



# The Prolactin Receptor Gene Expression Variance in Marshes and Riverine Buffalos in Iraq

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## Abstract

The study area eight stations in southern Iraqi marshes in Missan governorate, and six stations were in Al-Qadisiya and Al-Najaf governorates, the southern Iraqi marsh was proper environment to culture of the riverine buffalo breed, yet the water buffalo which preferring the middle area [1], because the molecular information of the local buffalo in Iraq is very rare [2], the present study selection is the improvement of yield of milk with the advances in molecular biology, the identification of the genes underlying by quantitative (rt-PCR) technique to possible efficient the prolactin (PRL-R) gene expression and to better understand the actions of mammary gland gene expression on milk production in two buffalo breeds in Iraq by determined levels of the PRL-R gene expression in somatic cells in the mammary gland by randomly milk samples of swamp and riverine buffalos during first and last lactation period, the results appeared showed high expression of PRL-R gene in last lactation period of riverine buffalo than swamp buffalo breed, this gene may have potential direct or indirect effect on milk production.

The transcription levels of *PRL-R gene* in milk of swamp buffalo were found to be significantly down-regulated in first stage of lactation period, but its where up-regulated in second stage of this period, and significantly decreased this regulation in last period, while very down regulation in first stage of lactation of riverine buffalo and curved to a highly induced regulation in second and late lactation period.

**Keywords:** Buffalos; Prolactin receptor; Gene expression

## Introduction

The Buffaloes is considered one of the animals that has wide spread in the marshes area in the south of Iraq [1,3] it's an important dairy animal, because of the opportunities of milk production despite feeding with low quality roughage [4-6]. The domestic buffalo belongs to the family *Bovidae*, sub family *Bovinae*, genus *bubalis* [7,8]. The buffalo population in Iraq exist as two main types, swamp and river buffalo [9,10].

In the present study, we evaluated the PRL-R gene expression in milk somatic cells of lactating period, by used the quantitative real-time PCR (qPCR) is considered the gold standard for gene expression analyses because of its high sensitivity, specificity, and reproducibility [11]. The prolactin hormone and other environmental factors which stimulates growth of the mammary gland [12,13], this Prolactin hormone binding to specific receptor (PRL-R) which responsible on this hormonal action [14-16]. In cattle, two isoforms of PRL-R have been found, resulting from alternative splicing: a long form, with a length of 557 amino acids, and a short one, with a length of 272 amino acids. The PRL-R gene is mapped on the bovine chromosome

20 and is originally described as having 10 exons [17-19], this polymorphic sites in the bovine PRL-R gene were discovered in

2006 [20], also the first polymorphism in the bovine prolactin receptor gene, identified in 2005, in the region involved in the alternative splicing of the transcript [21-23], this gene coding for bovine PRL-R was mapped to chromosome 20 in cattle and chromosome 19 in buffalo [24].

Most transcripts of the PRL-R gene are processed for the synthesis of PRL-R mRNA in the epithelial cells of mammary ducts and alveoli [25-27], and has nine exons that code for a polypeptide of 581 amino acids [22], yet the PRL-R belongs to type I transmembrane receptor family and structurally resembles the class I cytokine receptor super family [17,28].

The aim of this study was to examine the changes of mRNA expression using quantitative real-time polymerase chain reaction because its technique considered the gold standard for gene expression analyses because of its high sensitivity, specificity, and reproducibility [11], with (GAPDH) as a reference gene which have been found to vary with tissue type, developmental

stage, and environmental stimuli [29]. In this study we trying a compartment between of the prolactin receptor gene expression level in milk somatic cells or lactating mammary gland of cows during early, mid and last lactation period in both swamp and riverine buffalos breeds, due to milk collection is routinely performed and is less expensive and more easily accomplished than blood collection according to study of [30].

## Materials and Methods

### Animals and milk Samples

Twenty adult lactating buffalo were selected from local breed swamp (n=10) from the middle (Diwaniya and Al-Najaf), and riverine buffalos (n=10) were collected from south of Iraq (Missan and ThiQar), the milk samples were determined to be free of mastitis defect according to routine testing by CMT (california mastitis test), they volume of samples which collected from each animal were(50 ml) during three stages of the lactation period which are classification on physiochemical characteristic of milk samples according to stage of lactation period in swamp buffalo where, first stage (S1) 10-100 days, second stage (S2) 101-180 days and third stage (S3) --> 181

days, while in riverine buffalo were first stage (R1) 10-100 days, second stage (R2) 101-180 days and third stage(R3) --> 181 days [31] with minor modification, this samples placed in ice box for 3- 5hrs, later Centrifugation 12000 rpm for 10 min at 4°C, and the supernatant containing the hard fat layer was aspirated and discarded, leaving a 5ml residual fluid at the bottom of tube PBS (phosphate buffer slain) (5ml) was added to the fluid and was re-suspended and centrifuged again at 1200 rpm for 5 min at 4°C. The supernatant was discarded and 1ml of the residual fluid at the bottom was transferred by the pipette into 1.5ml free RNase eppendorf tube and centrifuged at 1200 rpm for 5min at 4°C. The pellet obtained was washed with PBS three times, and stored at -70 to -80°C in deep freeze system until total RNA extraction, according to method of [32]

### Primers

Two primer pairs were designed using the Primer Premier 5.0 software (<http://www.premierbiosoft.com>); the Housekeeping genes are GAPDH gene primer and other primer used for PRL-R gene as a target gene (Table 1). The specificity of the primer sets designed was confirmed by sequencing analysis of amplicon (<http://dx.doi.org/10.3168/jds.2012-6437>).

**Table 1:** The PRL-R and GAPDH genes primers with their sequences, product size and PCR conditions.

Primer	Sequences	Product size (bp)	PCR conditions
GAPDH (F) GAPDH (R)	5'-ACAGTCAAGGCAGAGAACGG-3' 5'-TGGCGTCACCTGACAATCTT-3'	501	2 min. 95 °C ,30 sec. 95 °C , 30 sec. 58°C , 60 sec. 72 °C , 5 min. 72 °C
PRL-r (F) PRL-r (R)	5'-CTGCTGATCAGTGCCTGACA-3' 5'-ATGAGTCCCATCCATCCCA-3'	509	2 min. 95 °C ,30 sec. 95 °C , 30 sec. 58°C , 60 sec. 72 °C , 5 min. 72 °C

### RNA extraction and purification

The total RNA was extracted from somatic cells using Trizol® Reagent by, AMBION/RNA (LIFE TECHNOLOGIES Ltd.)CALIFORNIA, according to the manufacturer's protocol, the integrity of total RNA extracted was verified by agarose gel electrophoresis by, NANOPAC-300 (CLEAVER SCIENTIFIC

Ltd.) UK, the RNA was quantitated using SP-3000 Nano (UV/Vis Spectrophotometer, OPTIMA Tokyo, JAPAN), also the purity and quantity of the extracted total RNA were assessed, with the optical density (OD) ratio of OD260/OD280 being 1.8 to 2.0 for all samples (Table 2). To removal of the contamination from genomic DNA, we used the DNase I (Ta- KaRa) Kit.

**Table 2:** The value of total RNA concentration by SP-3000 Nano (UV/Vis Spectrophotometer).

Buffalo breeding samples	Mean ± Se. of total RNA concentration		
	First stage	Sec. stage	Third stage
Swamp breed	91.3±0.633	92.2±1.103*	90.6±1.275
Riverine breed	91.8±1.38	90.3±1.247	88.6±1.087

**cDNA Syntheses**

The First-strand cDNA was synthesized from 1500 ng of RNA using the Cloned AMV (*INVETROGEN®*) First-Strand cDNA Synthesis Kit (USA), The Reverse Transcriptase PCR is performed according to manufacturer’s protocol, under the following conditions, by using PCR system (Multi Gene Opti Max Thermal Cycler TC9610 /TC9610-230), MIDSCI®, USA (in biotechnology college of Al-Qadissiya univ.) finally the samples were stored at -20°C until performed q (rt- PCR).

**Quantitative real-time PCR**

The q (rt-PCR) was carried out as described by [33], for relative expression of target genes in somatic cells of swamp and buffaloes in comparison with riverine buffaloes breed, the  $\Delta CT$  USING A REFERENCE GENE METHOD can be used by normalizing gene expression of target (PRL-R) genes with gene expression of housekeeping (GAPDH) gene as a reference gene, by using a following formula:-

$$\text{Expression value (Fold yield)} = 2^{\Delta CT(\text{reference}) - \Delta CT(\text{target})}$$

The mean of Ct numbers for target genes were normalized with reference (GAPDH) gene expression were determined by using the Microsoft excel according to [34], and evaluation of this results according of recommended by [35].

**Performed of q (real time-PCR)**

The SYBER Green I based The two-step reaction of q(rt-PCR) which performed by manufacturer’s protocol of Power SYBR® Green PCR Master Mix kit (applied biosystem ) California USA and Exicycler™ 96 Real-Time Quantitative Thermal Block instrument(Bioneer, Korea), in veterinary hospital laboratory of Al-najaf, according to method described by Chen [36], therefore preparing two q(rt-PCR) master mixes, for the PRL, and GAPDH genes, as the following: (20 µL) of total volume cDNA template for target genesfrom cDNA template(10 µL), forward (2 µL) & revers

(2 µL) primers and DEPC water (6 µL), this master mixes were added into Power SYBR® Green PCR Master Mix kit q(rt-PCR) PreMix tube, then start Exicycler™ 96 Real-Time Quantitative, thermal Block instrument to relative quantification, according to kit instruction.

**Statistical analysis**

All the values are expressed as mean ± SE. data of results were analyzed using student t-test and appropriate p-values of less than 0.05 (P<0.05) were considered as statistically significant [37].

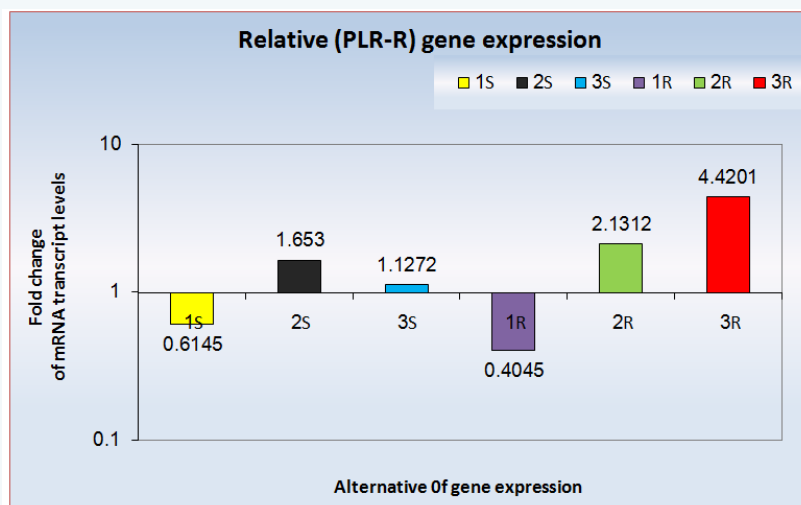
**Results**

In our study, the value of total RNA concentration (Table 2) was highly significant different (94.374 ±3.07 ng/µl) in somatic cells of the mammary gland, its total RNA samples were used in cDNA synthesis step by using First-Strand cDNA Synthesis Kit.

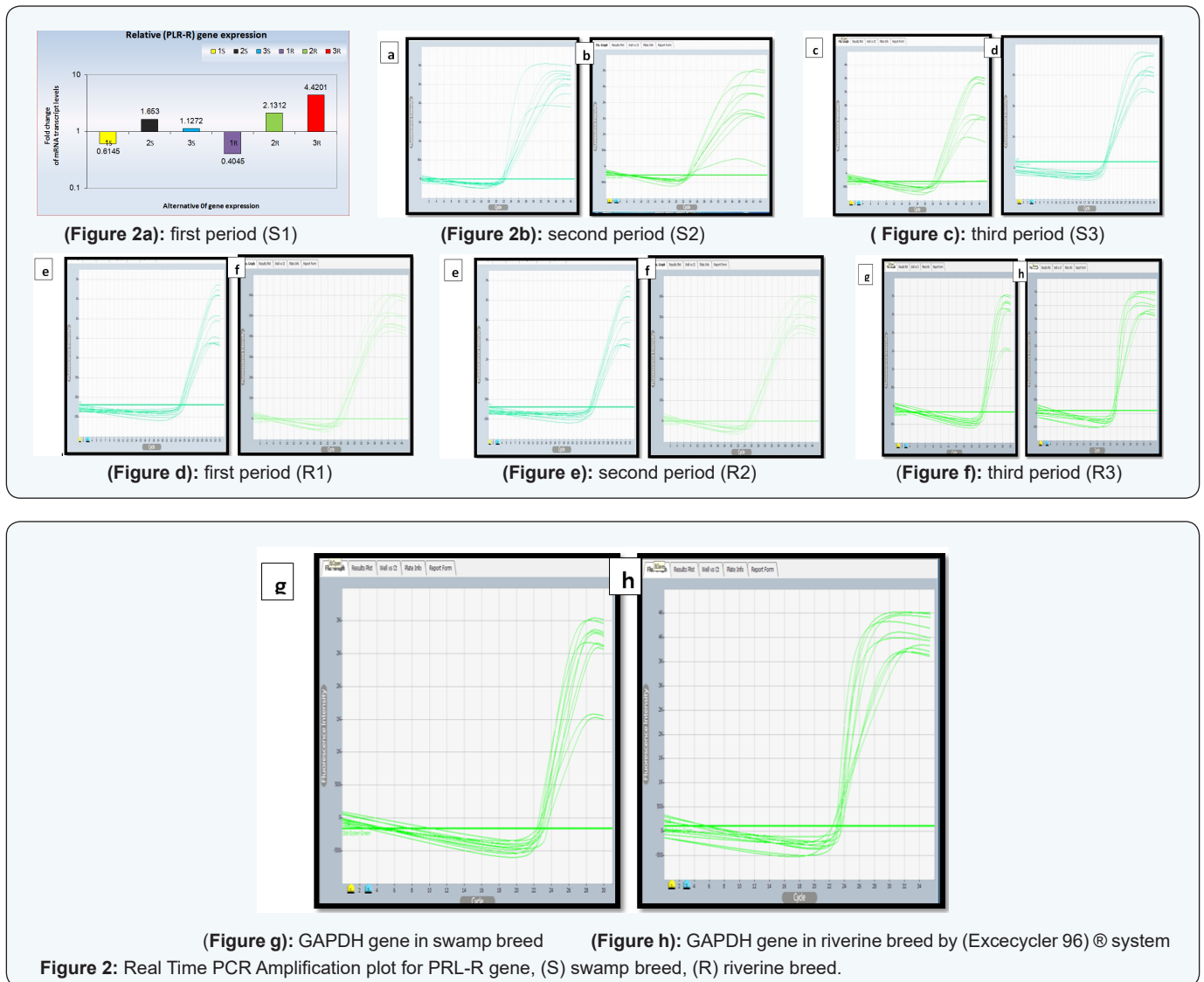
**The relative quantitative real time-pcr**

Data analysis of SYBRgreen I based rt-PCR assay were divided into primer efficiency estimation and relative quantification of PRL-R gene expression level which normalized by housekeeping gene expression (GAPDH).The Ct value of GAPDH are 23.2676 in swamp breed, and 23.4184 in riverine breed, there for the result of normalizing the PRL-R gene expression by The delta CT method Using a Reference Gene [38].

In swamp buffalo the expression value of PRL-R gene during lactation period which declines in first stage (0.6145), second stage up-regulation (1.653) and decreased this regulation (1.1272) in last period, while in riverine buffalo this expression are very down-regulation (0.4045) in first stage of lactation and curved to a highly induced regulation in second (2.1312) and late lactation period (4.4201) in mammary gland. (Table 3), (Figure 1,2).



**Figure 1:** The mean of Fold change of mRNA transcript levels to the PRL-R gene and GAPDH-R gene in swamp, and riverine buffalo breed.



**Figure 2:** Real Time PCR Amplification plot for PRL-R gene, (S) swamp breed, (R) riverine breed.

**Table 3:** The mean of Ct values and expression value of the PRL-R gene in the somatic cells of swamp breed and riverine breed buffalo.

Buffalo breeding	Buffalo breeding			$\Delta$ CT	Expression value( $2^{-\Delta$ CT)
	Lactation period	PLR-R (Target) gene	GAPDH-R (Reference) gene		
Swamp breed	S1-group	22.5652	23.2676	-0.7024	0.6145
	S2-group	23.9927	23.2676	0.7251	1.653*
	S3-group	23.4404	23.2676	0.1728	1.1272
Riverine breed	R1-group	22.1127	23.4184	-1.3057	0.4045
	R2-group	24.5101	23.4184	1.0917	2.1312*
	R3-group	25.5625	23.4184	2.1441	4.4201**

(\*) significant differences. (\*\*) significant differences.

Experimental groups (n=10) according to days of lactation period

S1: Swamp buffalo in 10-100; S2: Swamp buffalo in 100-180; S3: Swamp buffalo in > 180; R1: Riverine buffalo in 10-100; R2: Riverine buffalo in 100-180; R3: Riverine buffalo in > 180

## Discussion

In mammals, the hormone prolactin (PRL) is best known for its role in the regulation of lactation [39-41], also it has important functions like the development of mammary gland affecting milk yield and composition [42-45]. Also according to [13] this is hormone stimulant for growth of mammary ductal and alveolar cells by binding to PRL-R. yet the physiological functions of the PRL hormone to induce lactation by this acting through the PRL-R [46]. The prolactin receptor (PRL-R) gene was reportedly associated with milk protein and milk fat yields in the swamp buffalo [47] also the statistically significant of study the [48] were confirmed the associations between PRL-R milk production in livestock, therefore the results of the previous study were conferment that up-regulation of PRL-R gene expression in last lactation period of riverine buffalo (4.4201) in more than swamp buffalo which are down-regulated (1.1272) in this period, this a results agreement with Previous studies like [46,49].

[50,51] as well as these data support the concept that the PRL-R were higher sensitivity to PRL during lactation period may be associated with an increase in subsequent milk yield in riverine buffalo more than swamp buffalo, consequently the previous studies like [52] were observed the swamp buffalo produce relatively small quantities of milk, yet [53] recorded that the high milk yield is about six to seven liters per day in riverine breeds Indian buffalo.

In the mammary gland of lactating mice, the PRL-R is highly expressed both at the end of pregnancy and during lactation [54].

PRL-R1 mRNA expression is highly induced in the mammary gland during late pregnancy and abruptly declines on the first day of lactation for the HT rats [55], but this observe disagreement with study of [40], that were recorded a short day photoperiod was associated with reduced PRL, whereas milk yield and expression of PRL-R mRNA in lymphocytes and mammary tissue were increased. The swamp buffalo produce low amount of milk (1.0 - 1.5) litres per day, so they are not heavily used in milk production [52], but the milk yield of Indian riverine breeds, there is about (6 - 7) liters per day [53]. The PRL-R numbers begin to decrease in early pregnancy and are maintained at a low level until late pregnancy [55].

## Conclusion

The data normalization of the reference gene (GAPDH) with PRL-R gene expression in milk somatic cells of lactating two local buffalo breeds. In the present study, we compared the transcription levels of PRL-R gene in milk of swamp buffalo were found to be significantly down regulated in first stage of lactation period, but its where up regulated in second stage of this period, and significantly decreased this regulation in last period, while in riverine buffalo this expression are very down regulation in first stage of lactation and curved to a highly induced regulation in second and late lactation period.

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