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

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Abstract

The study area consisted of eight stations in southern Iraqi marshes in the Missan governorate, and six stations in the Al-Qadisiya and Al-Najaf governorates. The southern Iraqi marshes had the proper environment for the culture of the riverine buffalo breed, yet the water buffalo prefer 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 milk yield with the advances in molecular biology, the identification of the underlying genes by quantitative (rt-PCR) technique responsible for the efficiency of the prolactin (PRL-R)gene expression and to better understand the actions of mammary gland gene expression on milk production in the two buffalo breeds in Iraq, by determining levels of the PRL-R gene expression in somatic cells in the mammary gland by random milk samples of swamp and riverine buffalos during first medal and last lactation period. The results showed high expression of PRL-R gene in last lactation period of riverine buffalo than in swamp buffalo breed, and hence this gene may have potential direct or indirect effect on milk production. The transcription levels of PRL-R gene in the milk of swamp buffalo were found to be significantly down regulated in the first stage of lactation period, but were up-regulated in second stage of this period, and this regulation significantly decreased in the last period. In the riverine buffalo, the levels were very down regulated in the first stage of and curved to a highly induced regulation in second and late lactation period.

Keywords: Buffalos; Prolactin; Gene Expression

Abbreviations: PRL-R: Prolactin Regulation; GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase; CMT: California Mastitis Test; PBS: Phosphate Buffer Slain; OD: Optical Density

Introduction

Buffaloes are considered one of the animals that are wide spread in the marshes area in the south of Iraq [1,3]. It is an important dairy animal, because of the opportunities of milk production, despite feeding on 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 exists 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 during lactating period, by using the quantitative real-time PCR (qPCR), which 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 stimulate the growth of the mammary gland [12,13]. This Prolactin hormone binds to specific receptor (PRL-R) which is responsible for the hormonal action [14-16].

In cattle, two isoforms of PRL-R have been found, resulting from alternative splicing: long form, with a length of 557 amino acids, and a short one, with a length of 272 amino acids. The PRL-R gene, mapped on the bovine chromosome 20, is originally described as having 10 exons [17-19]. These polymorphic sites in the bovine PRL-Rgene were discovered in 2006 [20], while the first polymorphism in the bovine prolactin receptor gene was 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 have 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 [28,17].

The aim of this study is to examine the changes of mRNA expression using quantitative real-time polymerase chain reaction, because this technique is considered the gold standard

for gene expression analyses because of its high sensitivity, specificity, and reproducibility [11], with (GAPDH) as a reference gene which has been found to vary with tissue type, developmental stage, and environmental stimuli [29]. In this study, we attempt to examine 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 buffalo breeds. The milk collection is routinely performed and is less expensive and more easily accomplished than blood collection according to the study of Yang FL and Li XS [30].

Materials and Methods

Animals and milk samples

Twenty adult, lactating buffaloes 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). The volume of the samples collected from each animal was 50 ml during the three stages of lactation period, which are classified on physiochemical characteristics of milk samples according to stage of lactation period in swamp buffalo. Accordingly, first stage (S1) is 10-100 days, second stage (S2) is 101-180 days and third stage (S3) is 181 days. In riverine buffalo, the first stage (R1) was for 10-100 days, second stage (R2) for 101-180 days and third stage(R3) for 181 days [31], with minor modifications.

These samples were placed in ice box for 3-5hrs, and later subjected to Centrifugation at 12000 rpm for 10 min at 4^0C. The resulting supernatant containing the hard fat layer was aspirated and discarded, leaving a 5ml residual fluid at the bottom of the 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 4^0C. 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 5 min at 4^0C. The pellet obtained was washed with PBS three times, and stored at -70 to -80^0C in deep freeze system, until total RNA extraction, according to method of Sigl T et al. [32].

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)	5'-ACAGTCAAGGCAGAGAACGG-3'	501	2 min. 95^0C, 30 sec. 95 ^0C, 30 sec. 58^0C , 60 sec. 72^0C , 5 min. 72^0C
	5'-TGCGCTCACCTGACAATCTT-3'		
PRL-r (F)	5'-CTGCTGATCAGTGCCTGACA-3'	509	2 min. 95^0C, 30 sec. 95^0C, 30 sec. 58^0C, 60 sec. 72^0C, 5 min. 72^0C
	5'-ATGAGTCCCCATCCATCCCA-3'		

Primers

Two primer pairs were designed using the Primer Premier 5.0 software (http://www.premierbiosoft.com); the Housekeeping gene is GAPDH gene primer and the 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).

RNA extraction and purification

Table 2: The value of total RNA concentration by SP-3000 Nano (UV/V) 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

(*) significant differences

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, and 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 remove the contamination from genomic DNA, we used the DNase I (Ta- KaRa) Kit.

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 was performed according to manufacturer's protocol, by using PCR system (MultiGeneOptiMax Thermal Cycler TC9610 /TC9610-230), MIDSCI®, USA at the biotechnology college of Al-Qadissiya university. Finally, the samples were stored at -20^0C until quantitative real time PCR (rt- PCR) was performed.

Quantitative real-time PCR

The q (rt-PCR) was carried out as described by Priyanka S et al. [33], for the relative expression of target genes in somatic cells of swamp buffaloes, in comparison with riverine buffaloes breed. The ΔCT, using a reference gene method can be used by normalizing gene expression of target (PRL-R) genes with the gene expression of housekeeping (GAPDH) gene as a reference gene, by using the following formula:-

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

The mean of CT numbers for target genes was normalized with reference (GAPDH) gene expression and was determined by using the Microsoft excel according to Vandesompele J et al. [34], and evaluation of these results was done [35].

Performance of q (real time-PCR)

The SYBER Green I based two-step reaction of q(rt-¬PCR) was performed according to 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, at the veterinary hospital laboratory of Al-najaf, according to the method described by Chen HF [36]. Therefore two q (rt-PCR) master mixes were prepared, for the PRL, and GAPDH genes, as following: 20 μL of total volume cDNA template for target genes from cDNA template (10 μL), forward (2 μL) and reverse (2 μL) primers and DEPC water (6 μL). These master mixes were added into Power SYBR® Green PCR Master Mix kit q (rt-PCR) PreMix tube, and then Exicycler™ 96 Real-Time Quantitative thermal Block instrument was used for 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 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 SYBR green I based rt-PCR assay was divided into primer efficiency estimation and relative quantification of PRL-R gene expression level, which was normalized by housekeeping gene expression (GAPDH). The Ct values of GAPDH are 23.2676 in swamp breed and 23.4184 in riverine breed, obtained as 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 is 0.6145, second stage up-regulation is 1.653 and decreased regulation is 1.1272 in the last period, while in riverine buffalo, this expression has very low regulation (0.4045) in first stage of lactation and curved to a highly induced regulation in the second (2.1312) and late lactation period (4.4201) in mammary gland (Table 3) (Figure 1 & 2).

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	Mean ± Se. of CT values			ΔCT	Expression value(2^ΔCT)
	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   R1= Riverine buffalo in 10-100

S2= Swamp buffalo in 100-180   R2= Riverine buffalo in 100-180

S3= Swamp buffalo in > 180   R3= Riverine buffalo in > 180
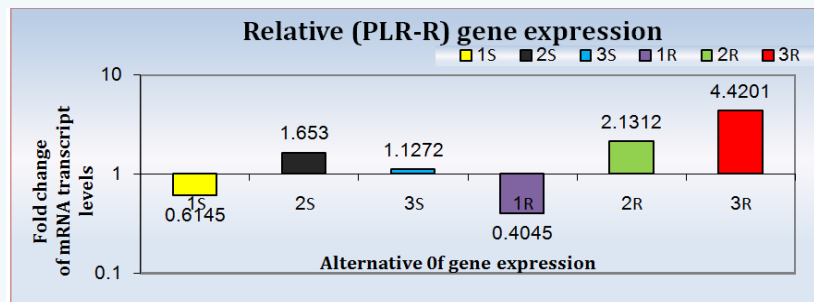
Figure 1: The mean of fold change of mRNA transcript levels to the PRL-R gene and GAPDH-R gene in swamp, and riverine buffalo breed.
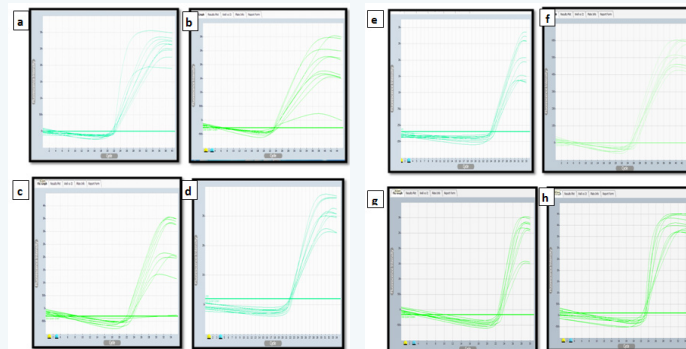

Figure 2: Real Time PCR Amplification plot for PRL-R gene-(S) swamp breed, (R) riverine breed:
[a] First period (S1),
[b] Second period (S2),
[c] Third period (S3),
[d] First period (R1)
[e] Second period (R2),
[f] Third period (R3),
[g] GAPDH gene in swamp breed and
[h] GAPDH gene in riverine breed by (Excecycler 96)® system.

Discussion

In mammals, the hormone prolactin (PRL) is best known for its role in the regulation of lactation [39-41], and it also has important functions like the development of mammary gland, affecting milk yield and composition [42-45]. Also according to Imagawa M et al. [13], this hormone is a stimulant for the growth of mammary ductal and alveolar cells by binding to PRL-R. Yet, the physiological functions of the PRL hormone to induce lactation are through the effect of PRI-R [46]. The prolactin receptor (PRL-R) gene was reportedly associated with milk protein and milk fat yields in the swamp buffalo [47]. The statistic significance of the study [48] confirmed the associations between PRL-R and milk production in livestock.

Therefore the results of the previous study confirmed the up-regulation of PRL-R gene expression in last lactation period of riverine buffalo (4.4201) more than in the swamp buffalo, which are down-regulated (1.1272) in this period. These results are in agreement with the previous studies [46,49-51] and also support the concept that the PRL-R's higher sensitivity to PRL during lactation period may be associated with an increase in subsequent milk yield in riverine buffalo more than the swamp buffalo. The previous studies [52] observed that the swamp buffalo produces relatively small quantities of milk, while [53] it was recorded that in the riverine breeds, milk yield is high, at about six to seven liters per day.

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 observation disagrees with the study of Auchtung TL et al. [40], that recorded that 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 produces 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, 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 [56].

Conclusion

The data normalization of the reference gene (GAPDH) was done with PRL-R gene expression in milk somatic cells of the two lactating local buffalo breeds. In the present study, we compared

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

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