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EXPRESSION OF SALIVARY TLR4 (Toll Like Receptor-4) GENE DURING ESTROUS CYCLE IN SURTI BUFFALOES

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Abstract: Buffalo is a shy breeder and does not manifest overt signs of estrus that make estrus detection difficult resulting in a poor conception rate. An attempt was made to identify the expression of TLR4 genes in saliva as an estrus biomarker in Surti buffaloes. Additionally, to monitor the reproductive status, the concentration of estrogen and progesterone in buffalo saliva during estrus and diestrus phases of estrus cycle were also estimated. A total twelve Surti buffalo animals maintained at Livestock Research Station, Kamdhenu University, Navsari were selected for the study. About 8 – 10 ml of saliva was collected from the animals during estrus and diestrus phases of estrus cycle. After centrifugation of saliva samples, RNA was extracted from 250 µL of cell free saliva by Trizol method. Average total RNA yield from all the 24 samples was 45.3±8.51 ng/µL. Previously reported bovine specific primers were used for amplification of TLR4 genes through Real time PCR. All the samples were run in duplicate in Real time PCR and GAPDH gene was used as an internal control gene to analyze the gene expression. GAPDH gene could be amplified consistently in the Real time PCR, however, the expression of TLR4 gene was inconsistent. Estrogen and progesterone concentration detection was carried out by Bovine estrogen and Bovine progesterone ELISA kit, respectively. Concentration of estrogen during estrus phase was 74.39±6.80 and 108.72±25.94 pg/ml and that during diestrus phase was 60.80±8.62 and 89.70±14.25 pg/ml in dam and daughter animals respectively. Similarly, 2.23±0.33 and 3.42±0.55 ng/ml progesterone level was found during estrus phase and 3.17±0.55 and 3.57±0.82 ng/ml level of that was found during diestrus phase in dam and daughters respectively.

Keywords: TLR4, Saliva, Surti, Estrogen, Progesterone

Introduction

India has rich in buffalo biodiversity with 20 distinct recognised buffalo breeds. Gujarat ranks third in terms of buffalo population, with 10.5 million heads. Surti buffalo is named after the town of Surat located in southern Gujarat; however, the breeding tract of this breed is around Kheda, Vadodara, Anand and Bharuch districts of the middle and southern part of Gujarat.

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Riverine buffaloes widely considered seasonally polyestrus animals express estrus behaviour during September to January, with a peak during October to November (Kumar *et al*., 2013)[1]. Buffaloes are mainly considered as peculiar shy breeders. The duration and intensity of estrus behaviour may be genetically influenced. Genomic studies can help us in understanding the different regulatory mechanisms that lead to expression of estrus. Boer *et al.* (2010)[2] mentioned that changes in systems that regulate estrus behaviour could be manifested by altered gene expression. Saliva is considered as the best source of biological material for biomarker discovery studies since it is non-invasive in comparison to other body sources (Li *et al*., 2004)[3]. Saliva contains specific biomolecules like mRNA, miRNA, DNA, or protein that reflect the individual's physiology or disease condition. (Ang *et al.,* 2011)[4]. The TLR4 gene in water buffalo consists of three exons and two introns, and is located on chromosome number 8 (Gulhane and Sangwan, 2012)[5]. TLR4 participate in ovulation, fertilization, gestation, parturition, placental function, trophoblast invasion, parturition, and protection of the reproductive tract from pathogen (Kannaki *et al*., 2011)[6].

Progesterone plays an essential role in various reproductive functions, including regulating the length of the estrous cycle, maintaining pregnancy (Rekawiecki *et al*., 2008)[7] and regulating embryonic growth and development. Monitoring P4 in plasma/serum (Lobago *et al*., 2007) [8] or milk (Domènech *et al*., 2011)[9] using an Enzyme Linked Immunosorbent Assay (ELISA) or Radioimmunoassay (RIA) has been used to assess the reproductive status of cattle and ensure successful breeding, or inform decisions to cull non-productive cows/heifers. Circulating concentrations of estrogen during the preovulatory period can influence the establishment and maintenance of pregnancy by altering the uterine environment (Miller *et al.,* 1977)[10]. Keeping this in mind a study was planned to explore the possible role of TLR4 gene during estrus and diestrus phases of estrous cycle in Surti buffaloes.

Material and methods:

Selection of animals

Twelve Surti buffaloes (six dam-daughter pairs) maintained at Livestock Research Station, KU, Navsari were selected for the study. The Surti buffaloes were examined for the presence of dominant follicle (DF) or corpus luteum (CL) on the ovarian surface using per-rectal examination and trans-rectal ultrasonography (USG). The animals were regularly inspected during early morning and evening hours for estrus (heat) detection. The onset of estrus in buffaloes were detected initially by observation of estrus signs followed by detection through teaser bull. Some of the common behavioural signs exhibited by buffaloes during estrus phase

were frequent urination, bellowing, chin resting, sniffing of genitalia, cervical mucus discharge and restlessness. Vulvar edema was a prominent sign during estrus in buffaloes.

Collection and processing of samples

Saliva samples were collected in the morning before feeding, on the day of estrus (day 0) and diestrus (13th – 15th day of estrous cycle). About 8 - 10 ml saliva samples from each animal were collected in 15 ml DNAse/RNAse free sterile centrifuge tube using IV (Intra Venous) set tube. One cut end of the tube was intubated in mouth (lower jaw) of the animal and 20 ml syringe was attached with another end of the tube to aspirate the saliva. The aspirated saliva samples were transferred to sterile 15 ml centrifuge tube. The samples were immediately kept on ice and transferred to the laboratory. The samples were centrifuged at 3000 g for 10 min at 4˚C, the supernatant was collected and stored in 2 ml sterile microcentrifuge tube at -40 ˚C. About 1-2 ml of supernatant was stored and an equal amount of TRIzol reagent was added and further processed for RNA isolation and gene expression study.

Total RNA extraction

Total RNA was extracted using TRIzol as follows. About 250 µL of supernatant or cell free saliva samples were transferred to microcentrifuge tubes for RNA extraction. Microcentrifuge tube containing 250 µL of sample with 750 µL of TRIzol reagent were added. The mixture was vortexed and allowed to stand at Room Temperature (RT) for 10 min. After incubation, 200 µL of chloroform was added and the mixture was again vortexed vigorously for 15 sec. After 10 min incubation at RT, the mixture was centrifuged at 13000g for 10 min at 4°C. The aqueous supernatant containing total RNA was recovered and mixed with 600 µL isopropyl alcohol. The mixture was kept at -40 °C overnight. Next day the mixture was centrifuged at 13000g for 10 min at 4 °C to obtain the pellet. Supernatant was discarded and the pellet was washed with 1 ml of 75% ethanol followed by vortexing of 15 sec. The mixture was centrifuged at 13000g for 10 min at 4°C. The Supernatant was discarded and the pellet was air dried. Properly air-dried pellet was dissolved in 20 µL of RNase free water. Isolated RNA was stored at – 80 °C till further use. Concentration and purity (260/280) of total RNA extracted was measured in Nanodrop spectrophotometric (Thermo Scientific ND 2000_C). The samples with a 260/280 ratio between 1.8 and 2.0 were used for cDNA synthesis and downstream processing.

cDNA synthesis and optimization of PCR

An accurate amount of 300 ng RNA was taken and reverse transcription was carried out in total 32 µL reaction volume according to the manufacturer instructions (QuantiTect® Reverse Transcription Kit) in a PCR thermocycler (ABi) as follows. Template RNA was thawed on ice.

QuantiTect® Reverse Transcriptase, gDNA wipeout buffer, QuantiTect RT buffer, RT primer mix and RNase free water were also thawed at room temperature (15-25°C). gDNA elimination reaction was prepared on ice according to (Table 1). Contents were mixed and kept on ice for further use. Master mix of gDNA wipeout buffer and RNase free water was prepared in a volume 10 percent greater than that required for the total number of reactions to be performed. Master mix was appropriately distributed into individual tubes, followed by addition of each RNA sample.

Table 1: Genomic DNA elimination reaction components

Sr. No.	Components	Volume/ Reaction	Final Concentration
1.	gDNA wipeout buffer, 7x	3.4 µL	1 x
2.	Template RNA	Variable	300 ng
3.	RNase free water	Variable	**--**
Total reaction volume		24 µL	**--**

Reaction components were incubated for 2 min at 42°C in PCR thermocycler and subsequently kept on ice. Reverse transcription master mix was prepared on ice according to Table 2. The reverse transcription master mix contained all components required for first strand cDNA synthesis except template RNA. Master mix was prepared 10 percent greater than that required for total number of reactions to be performed. Template RNA was added from as per table 1 (24 µL) to each tube containing reverse transcription master mix. Components were mixed and then immediately transferred to PCR thermocycler. The individual tubes containing reaction mixture were incubated for 15 min at 42°C in PCR thermocycler.

Table 2: **Reverse-transcription reaction components**

S. No.	Components	Volume/Reaction
Reverse-transcription master mix		
1.	Quantitect Reverse Transcriptase	0.8 µL
2.	Quantitect RT buffer, 5x	6.4 µL
3.	RT Primer Mix	0.8 µL
Template RNA		
4.	Entire genomic DNA elimination reaction (As per table 1)	24 µL
Total reaction volume		32 µL

Further incubation was carried out for 3 min at 95°C to inactivate Quantitect Reverse Transcriptase. Reverse transcription products were stored at -20°C.

Real -Time PCR

Template cDNA, QuantiNova SYBR Green PCR master mix, primers, ROX dye and RNase-free water were thawed. Individual solutions were vortexed and a master mix was prepared as given in Table 3. Each well was loaded with 10 µL of reaction components. Samples were loaded in duplicate for each sample and each gene. The optical strip was kept on the wells and the 96-well plate was briefly rotated in an Eppendorf refrigerated centrifuge at 280 g for 1 min. The 96-well plate was placed in the qPCR machine in the correct orientation and optical lids were cleaned with the lens tissue to remove any dust before closing the qPCR machine lid. Gene name and sample number were assigned to each well in software program as per the instrument instruction manual. The PCR protocol was started with amplification protocol involving denaturation step (94°C, 15 sec), annealing - extension (60°C, 30 sec), cycling program (40 times) followed by melt curve (dissociation curve) analysis. Fluorescence signals were measured once in each cycle at the end of the amplification protocol. For each gene of interest, negative controls were used in which, instead of cDNA, RNase-free water was added. For each sample, a dissociation curve was generated after completion of amplification and analyzed in comparison to negative controls, to determine the specificity of PCR reaction. For improved visualization of melting temperature, melting peaks were derived by plotting the negative derivative of fluorescence over temperature versus temperature.

Table 3: Reaction components for Real Time PCR

Component	Volume/ reaction	Final concentration
SYBR Green PCR master mix (2X)	5 µL	1X
Primer A	0.5 µL	5 µmole
Primer B	0.5 µL	5 µmole
cDNA template	1.5 µL	Approx. 30 ng
ROX dye	1 µL	-
RNase-free water	1.5 µL	-
Total volume	**10 µL**	-

Table 4: Primer pairs and expected product size for various genes under study Gene

Gene	Sequence (5'-3')		Product size (bp)	References
TLR4	F	5'CTTGCGTACAGGTTGTTCCTAA3'	153	Lakshmi *et al.*, 2022[11]
	R	5'CTGGGAAGCTGGAGAAGTTATG3'		
GAPDH	F	5'GGTCATCATCTCTGCGCCTT3'	185	Singha *et al.*, 2022[22]
	R	5'CGTGGACGGTGGTCATAAGT3'		

Bovine Estrogen and Progesterone ELISA:

Estimation of estrogen and progesterone concentration in the collected saliva samples was carried out by Bovine Estrogen ELISA kit (KRISHGEN Biosystems) and Bovine Progesterone ELISA kit (KRISHGEN Biosystems) respectively as per manufacturer's protocol.

Result and Discussion:

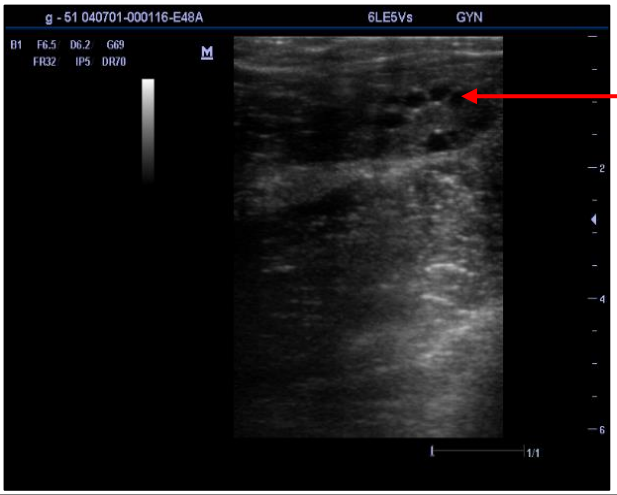

Plate 1: Presence of ovarian follicles during estrus phase

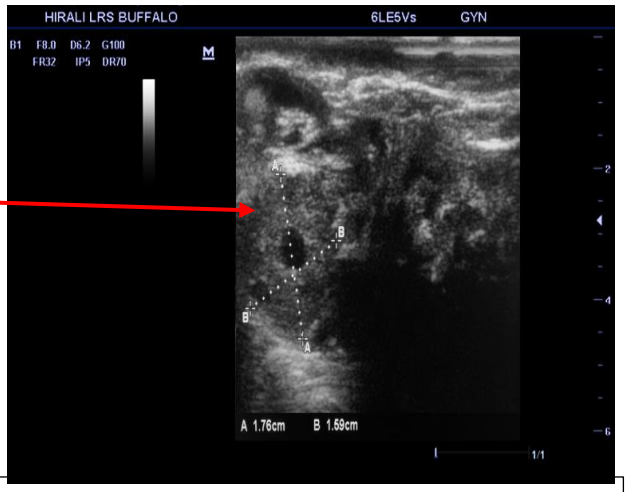

Plate 2: Presence of corpus luteum during diestrus phase

The estrus phase and diestrus phase were confirmed by the state of ovarian follicle and the state of corpus luteum respectively through Trans-rectal ultrasonography in the representative animal (Plate – 1 & 2). In the present study 15 – 150 ng/µL concentration of total RNA in 20 µL volume was obtained from 250 µL of saliva samples with average total RNA yield of 45.3±8.51 ng/µL. On an average total RNA yield was 905±170 ng from 250 µL of cell free saliva. Integrity of RNA was verified by 1 percent agarose gel electrophoresis wherein single distinct bands of 28s RNA with almost twice the intensity compared to single compact band of 18s. Total cDNA synthesized was in the range of 1800 to 2200 ng/µL with OD 260/280 ratio for all the samples were in the range of 1.8 – 1.9, indicating good quantity and quality of cDNA. GAPDH was used as an endogenous control and all 24 samples were

run in duplicate. The C_T value of each sample was recorded and average C_T value was calculated.

GAPDH gene was amplified successfully in all the 24 samples as evident by agarose gel analysis (Figure-1), gene amplification plot (Figure-2) and melting curve (Figure-3) of the representative samples. The expression profile of TLR4 was studied using previously reported primers (Lakshmi *et al.,* 2022)[11] through Real time PCR Fast SYBR green assay. The TLR4 gene inconsistently amplified at estrus and diestrus phases of estrous cycle in Surti buffaloes by Real Time PCR in the present study. This may be due to the lower expression of TLR4 gene in the saliva of buffalo during estrus and diestrus phases of estrous cycle which may not be detectable by Real Time PCR.

Onteru *et al*. (2016)[12] reported the higher expression of TLR4 gene during estrus as compared to diestrus phase from direct buffalo saliva samples. Muthukumar *et al*. (2014)[13] carried out salivary proteome analysis and reported higher expression of TLR4 during estrus phase as compared to other phases of estrous cycle suggesting TLR4 as a biomarker for estrus detection in buffaloes. Vahanan *et al*. (2008)[14] and Tirumurugaan *et al.* (2010)[15] reported the expression of TLR4 gene in tissues mainly ovary and uterus in buffalo and goat respectively. Ajevar *et al*. (2014)[16] studied the transcriptional profile of TLR4 from buffalo endometrium during the follicular, early, mid, and late luteal phases of estrous cycle and reported that TLR4 was upregulated during mid-luteal phase of the estrous cycle. As the estrogen triggers the expression of TLR4, (Rettew *et al*., 2009)[17], the gene should be more expressed during estrus phase. However, there are mixed reports of higher expression of TLR4 during estrus and diestrus phase in various tissues and species including saliva in buffaloes and reproductive tissues in goat and female dogs.

RT-PCR failed to show bubaline TLR4 in some of the salivary samples which might be due to the following reasons due to random variation or limited sensitivity to amplify low copy numbers in saliva.

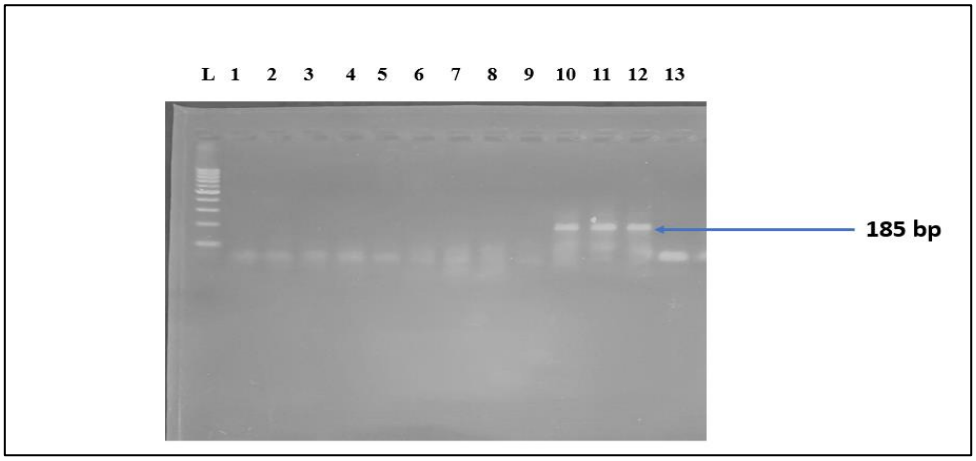

Figure-1 Agarose gel electrophoresis of Real Time PCR products

L = Ladder (100bp), Lane 1, 2, 3, 4 CYP19A1 gene transcript, 5 Negative Template Control of CYP19A1. Lane 6, 7, 8 TLR4 gene transcript, 9 Negative Template Control of TLR4. Lane 10, 11, 12 GAPDH gene transcripts, 13 Negative Template Control of GAPDH.

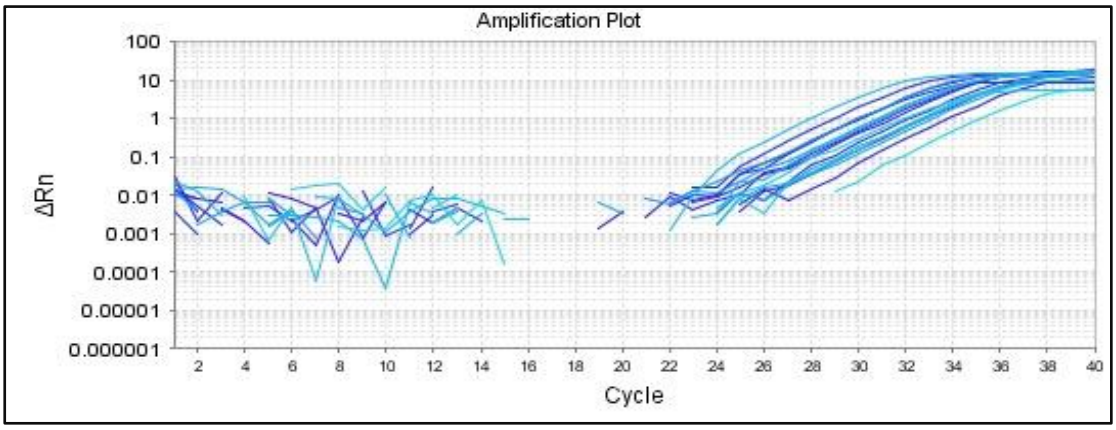

Figure-2 Amplification plot of GAPDH gene transcript

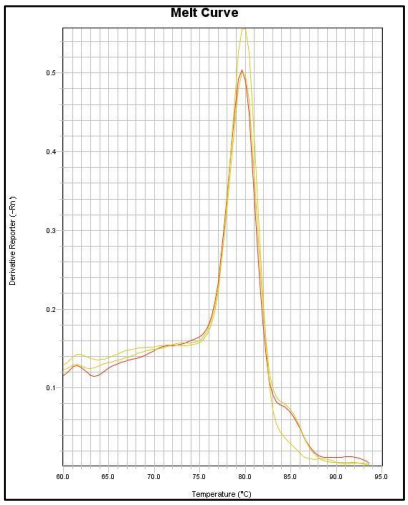

Figure-3 Melt curve of GAPDH gene

Bovine Estrogen and Progesterone ELISA

The concentration of estrogen during estrus phase was 74.39±6.80 and 108.72±25.94 pg/ml and that during diestrus phase was 60.80±8.62 and 89.70±14.25 pg/ml in dam and daughter animals respectively. Similarly, 2.23±0.33 and 3.42±0.55 ng/ml progesterone level was found during estrus phase and 3.17±0.55 and 3.57±0.82 ng/ml level of that was found during diestrus phase in dam and daughters respectively. No stage wise and animal class wise difference was observed in the salivary concentration of estrogen and progesterone. The results are depicted in table 5.

Table 5: Concentration of estrogen and progesterone in buffalo saliva during various phases of estrous cycle

	Estrogen (pg/ml)			Progesterone (ng/ml)		
	Estrus (6)	Diestrus (6)	p value	Estrus (6)	Diestrus (6)	p value
Dam (6)	74.39±6.80	60.80±8.62	0.282	2.23±0.33	3.17±0.55	0.29
Daughter (6)	108.72±25.94	89.70±14.25	0.305	3.42±0.55	3.57±0.82	0.834
p value	0.250	0.113		0.094	0.694	

In contradiction to the present result, Qureshi *et al*. (1999)[18] reported higher salivary estradiol concentration during proestrus and estrus phases as compared to other phases of estrous cycle in buffaloes. Ravinder *et al*. (2016)[19] also reported higher ($p < 0.05$) salivary estradiol levels and E2/P4 ratio at the estrus stage than the diestrus stage and salivary progesterone levels higher ($p < 0.05$) at the diestrus stage than the estrus stage in buffalo. Mekonnin *et al*. (2017)[20] found that salivary progesterone was significantly lower in saliva than in serum and milk during

diestrus phase of estrous cycle and pregnant cattle. They further reported higher estradiol in saliva during estrus than at other reproductive stages in cattle. Gali *et al*. (2017)[21] found higher concentration of salivary progesterone in pregnant animals as compared to non - pregnant sows.

The findings of the present study are not in line to the previously reported findings from buffaloes or other species. Moreover, as there are no reports on levels of estrogen and progesterone during various phases of estrous cycle from buffalo saliva, comparison of the same could not be carried out. More number of the studies from saliva during various physiological stages in different species may throw light on this issue.

Conclusions:

The major objectives of study were isolation of RNA from saliva, relative expression TLR4 genes and estimation of concentration of estrogen and progesterone from saliva during estrus and diestrus phases of estrous cycle in Surti buffalo. On an average total RNA yield was 905±170 ng from 250 µL of cell free saliva. TLR4 genes could not be amplified through Real Time PCR however GAPDH gene was successfully amplified. Concentration of estrogen during estrus phase was 74.39±6.80 and 108.72±25.94 pg/ml and that during diestrus phase was 60.80±8.62 and 89.70±14.25 pg/ml in dam and daughter animals respectively. Similarly, 2.23±0.33 and 3.42±0.55 ng/ml progesterone level was found during estrus phase and 3.17±0.55 and 3.57±0.82 ng/ml level of that was found during diestrus phase in dam and daughters respectively. No significant difference was observed between the concentration of estrogen and progesterone in saliva during estrus and diestrus phases of estrous cycle in dam daughter pairs of buffaloes.

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