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Dr. Ashish Bhaladhare
Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Dr. Amit Kumar
Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Dr. Pushpendra Kumar
Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Dr. Vikrant Singh
Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Dr. Gyanendra Singh
Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Corresponding Author:
Dr. Ashish Bhaladhare
Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

Expression profiling of PARVB in pregnant water Buffaloes (*Bubalus bubalis*)

Dr. Ashish Bhaladhare, Dr. Amit Kumar, Dr. Pushpendra Kumar, Dr. Vikrant Singh and Dr. Gyanendra Singh

Abstract
The role of PARVB in the early pregnancy of buffalo was investigated using real-time PCR analysis of its expression in the endometrial tissue of pregnant and non-pregnant buffaloes. The results showed that PARVB expression was significantly higher in the endometrial tissue of pregnant buffaloes compared to non-pregnant buffaloes. PARVB is a cytoskeletal protein involved in the regulation of cell adhesion and migration, which are critical processes for successful implantation and early embryonic development. These findings suggest that PARVB may have a conserved role in the regulation of early pregnancy in mammals. Further studies are warranted to elucidate the specific mechanisms by which PARVB contributes to early pregnancy in buffalo and other mammals.

Keywords: PARVB, early pregnancy, buffalo, real-time PCR, endometrial tissue, cell adhesion, cell migration

Introduction
Buffalo is renowned as black gold in Indian farmer community. Mostly reared for milk throughout India but also used in farmers field for various work. Indian dairy market size was about 13.17 Lakh crore in 2021 (DAHD, 2022-23) [11]. Indian dairy market size growing with growth rate of 15% per annul during last 15 year, and expected to reach 30.84 lakh crore in 2027 as per report of International market analysis and consulting services Private Ltd. (IMARC) 2021. Milk production in the country in 2021-22 has been estimated at about 22 crore metric tonnes registering a growth of 6% per year during last 5 years. The per capita availability of milk has reached to 444 grams per day in 2021-22. The milk production is estimated to reach about 30 crore tonnes by 2030 as per NITI Aayog report. Buffalo contribute nearly 50% of total milk production of India. At present total 20 of breeds buffalo present in India. India having diversified genetic resources of buffalo in the world, which includes Murrah, Nili Ravi, Jaffarabadi and others.

Complexity of reproductive nature of Buffaloes and early embryonic mortality always a major hurdle in rearing and management. Early embryonic mortality in early ages between 0-40 days is high as compare to reports of 40 days and above pregnancy (Reese *et al*., 2018) [1]. Major problem associated with early embryonic mortality is delay in implantation of embryo and establishment of firm attachment in uterus during early embryonic life in buffaloes. The Parvin-Beta (Parvb) gene codes for the protein "beta-parvin," which is involved in cell adhesion, cytoskeletal organization, and cell migration. The Parvb gene in the domestic water buffalo consists of 15 exons and 14 introns, and it is located on chromosome 5. The gene has a length of 14,049 base pairs and is oriented on the negative strand (NCBI Data base). Beta-parvin interacts with other proteins such as integrin's, talin, and vinculin to regulate cell adhesion, migration, and signaling.

The process of early pregnancy in buffalo is a complex and multi factorial phenomenon that involves various molecular and cellular processes. In recent years, several studies have focused on identifying key molecules and genes that play a crucial role in the early stages of pregnancy. One such molecule that has gained attention in recent years is PARVB (beta-parvin), which is known to regulate cellular adhesion and migration. However, the role of PARVB in the early pregnancy of buffalo is not well understood.

Real-time PCR (polymerase chain reaction) is a highly sensitive and reliable technique for quantifying gene expression levels in biological samples. This method has been widely used in the field of reproductive biology to investigate the expression of various genes involved in

different stages of pregnancy. In this study, we aimed to investigate the role of PARVB in early pregnancy of buffalo using real-time PCR.

The findings of this study will provide valuable insights into the molecular mechanisms underlying early pregnancy in buffalo and could contribute to the development of new strategies for improving reproductive efficiency in buffalo. Understanding the role of PARVB in early pregnancy may also have implications for improving reproductive outcomes in other livestock species.

Materials and Methods

Sample collection

A total of 6 cotyledonary tissues samples from three different stages of buffaloes, belongs to 2 early, one mid and three after birth stages were collected. First two stages of samples were collected from local abattoir house while after birth samples were collected from Seoni District Madhya Pradesh after taking consent from Farmers.

In the present research, the age of the embryo was determined based on crown-rump vertebral length using the Soliman formula (1975) [4], which was quoted by Tomar *et al*. (2018) [5] and Gupta, *et al*. (2014) [3] in their research.

Y = 28.66 + 4.496 x (CVRL < 20 cm)

Y = 73.544 + 2.256 x (CVRL > 20 cm)

The curved crown-rump length (CVRL) is the length of the embryo from the anterior frontal bone to the rump at ischiatic tuberosity, measured with the help of a thick cotton thread (Edwards, 1965; Gupta *et al*., 2014) [3]. The fetuses were divided into two groups.

Group I (Early Stage): Foetuses with CVRL between 0 and 2.7 cm

Group II (middle stage): Foetuses of CVRL between >3.0 and 28 cm

Group III (post-partition stage): Group three includes samples from post-parturition, when normally calved female buffaloes have completed their full-term gestation periods.

All samples were collected in RNA later and stored in LN2 for 12 hour than transferred to -80 ℃ till further use.

3.8.3 cDNA preparation

cDNA was prepared by RNA extracted in previous step with the Thermo scientific Revert Aid First Strand cDNA Synthesis Kit # K1621 20 rxns, following kit protocols in a 20ul reaction. Whole reaction divided in two parts 1st - we need to prepare 12 microliter of reaction with RNA template, Oligo (dT) 18 primer and NFW (Table 3.1)

Table 1: cDNA synthesis step 1

Reagents	Volume
Total RNA (2ug)	2 ul
Oligo (dT)18	1 ul
NFW	9 ul
Total Volume	12 ul

2nd 8 ul of reaction volume was prepared by added following mentioned reagents

Table 2: Final Reaction for cDNA synthesis

Reagents	Volume
5X Reaction Buffer	4 ul
Ribo Lock R Nase Inhibitor (20 U/ul)	1 ul
10 mM dNTP Mix	2 ul
Revert Aid M-MuLV RT (200 U/ul)	12 ul

These 8 ul mix. Was added in each sample tube to make total reaction volume 20 ul.

After gentle tapping each tube placed in Agilent Supercycler 8800 at 42 degree for 60 min. Termination of reaction required 5 min. heating at 70 degrees.

Real-time PCR

Real-time qPCR was performed using the Power up SYBR Green Master Mix (Quanti Fast SYBR® Green RT-PCR Kit) and the Step One plus Real-Time PCR System (Applied Biosystems, USA). Primers were designed using Primer-BLAST (NCBI) based on the buffalo PARVB gene sequence (Accession No. XM_006056225.2). The reference gene GAPDH (thermo cDNA Kit) was used for normalization. The primer sequences used in the study are given below:

Gene Name	Accession No		Primer Sequence	Final Product	Annealing temperature
PARVB	XM_025282715.3	F	GAGATGATGATGGGGCGCTT	139	61.0 ℃
		R	CCGTCACCTCCAGATTCAGC		

3.8.5 Primer reconstitution and standardization

All of the primers were purchased from BarCode ltd in lyophilized form. Each primer tube was first centrifuged at 3000 rpm for 3 min then NFW was added to make 100 um

stock solution as per manufacturer instruction. Each tube tapped gently for proper mixing and then kept at 4 degrees for 12 hours. Stock solution then diluted in 1:10 ratio to prepare a working solution.The primer were standardized by gradient PCR, using Promega PCR master mix for 30 cycles.

- **Denaturation:** 94 ℃ for 30 Second
- **Annealing (Gradient Step):** From 56 ℃ to 62 ℃ for 30 second
- Extention at 72 ℃ for 1 min

The PCR product of each primer was visualized by running on 2% agarose gel in 1x TBE buffur at 80 Volt.

Table 3: Reaction mixture for primer standardization for PCR

Reagents	Volume µL
Promega PCR master mix	12.5
Primer Forward	0.5
Primer Reverse	0.5
cDNA	1
NFW	10.5
Total	25

RT-qPCR was done by using Quantifast SYBR Green PCR Kit. Each sample was run in triplicate with GAPDH and NTC with following protocol in Himedia RT-qPCR in Physiology division:

PCR conditions were optimized for each primer set and included an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30s and annealing/extension at 60°C for 1 min. The specificity of the PCR products was confirmed by melting curve analysis. The relative expression levels of PARVB were calculated using the 2^-ΔΔCt method (Livok & Schmittgen; 2001) [2].

Results

The relative expression levels of PARVB in the cotyledonary tissue of pregnant buffaloes were analyzed by real-time PCR. The results showed that PARVB fold change expression was high in the cotyledonary tissue of pregnant buffaloes in early as well well as after birth stage as compare to middle stage.

The melting curve analysis confirmed the specificity of the PCR products. The amplification efficiency of the primers was found to be within the acceptable range (90-110%) and the correlation coefficients (R^2) of the standard curves were greater than 0.99.

These results suggest that PARVB may play a role in the early pregnancy of buffalo. The higher expression of PARVB in the endometrial tissue of pregnant buffaloes could be attributed to its involvement in the regulation of cellular adhesion and migration, which are crucial processes for successful implantation and early embryonic development.

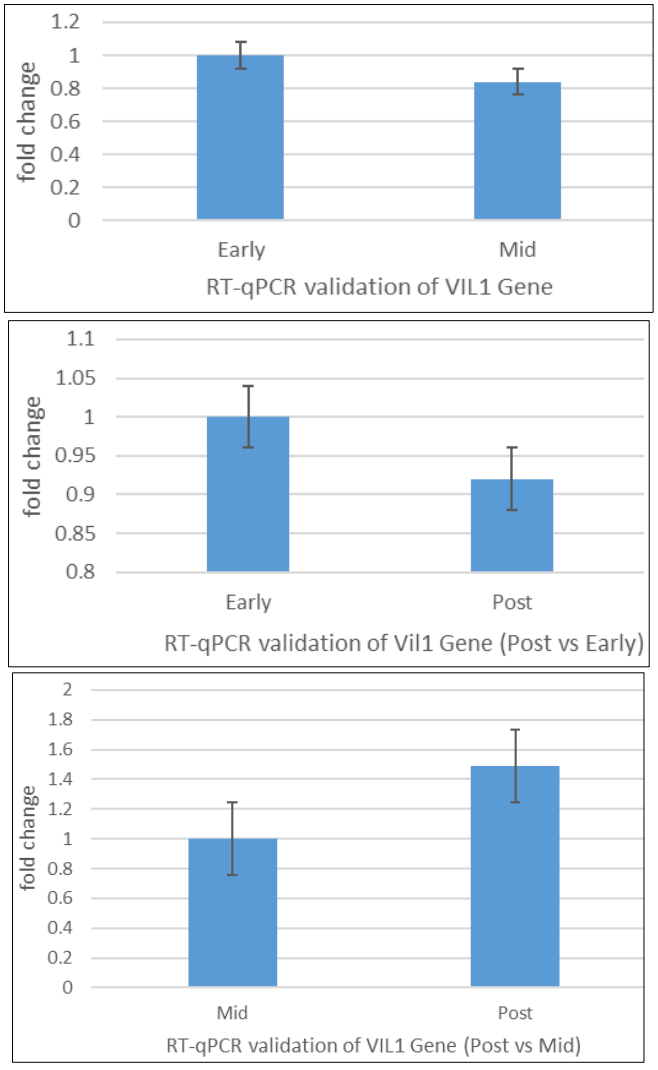

Fig 1: Relative expression levels of PARVB in the endometrial tissue of pregnant and non-pregnant buffaloes.

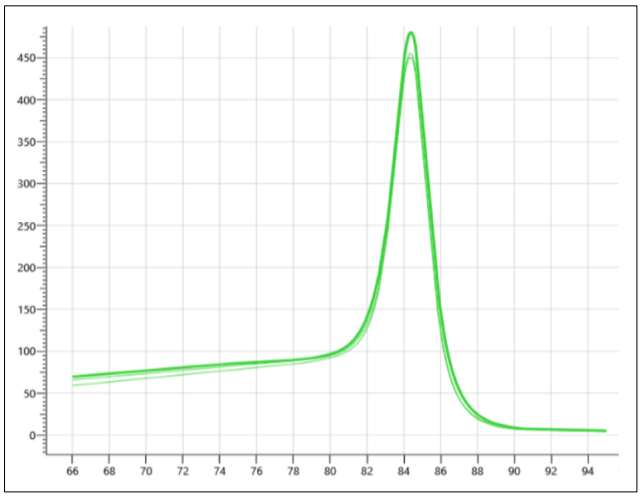

Fig 2: Melt curve of PARVB gene

Discussion

The present study investigated the role of PARVB in the early pregnancy of buffalo by analyzing its expression in the cotyledonary tissue of pregnant buffaloes in early, mid and after birth stages using real-time PCR. The results showed that PARVB expression in terms of fold change was higher in early and after birth stages as compare to middle stage.

PARVB is a member of the parvin family of cytoskeletal proteins that are involved in the regulation of cell adhesion, migration, and survival (Eslami *et al*., 2015) [6]. PARVB has been reported to interact with integrin-linked kinase (ILK) and promote the formation of focal adhesions, which are critical for cell-substrate adhesion and migration (Sakai, T., *et al*., 2003) [8]. In addition, PARVB has been shown to modulate the activity of Rho GTPases, which are key regulators of cell migration and cytoskeletal dynamics (Lawson & Burridge, 2014) [7].

In the context of early pregnancy, the endometrium undergoes significant changes to facilitate embryo implantation and establishment of pregnancy. The expression and activity of integrins and other adhesion molecules are critical for the adhesion and migration of the blastocyst to the endometrial surface (Sepulveda & Wu., 2006) [12]. The formation of focal adhesions and actin cytoskeleton remodeling are also essential for the successful attachment and invasion of the embryo. Therefore, the higher expression of PARVB in the cotyledonary tissue of pregnant buffaloes could indicate its involvement in the regulation of these processes.

Interestingly, previous studies have reported the differential expression of PARVB in the endometrium during the estrus cycle and early pregnancy of cattle and humans. In cows, PARVB expression was found to be higher in the early pregnant endometrium compared to the estrus and non-pregnant endometrium (Dickson *et al*., 2022) [9]. In humans, PARVB expression was up-regulated in the endometrium during the window of implantation (Chi *et al*., 2022). These findings suggest that PARVB may have a conserved role in the regulation of early pregnancy in mammals.

In conclusion, our study provides evidence for the involvement of PARVB in the early pregnancy of buffalo. The higher expression of PARVB in the endometrial tissue of pregnant buffaloes suggests its role in the regulation of cell adhesion and migration, which are crucial processes for successful implantation and early embryonic development. Further studies are warranted to elucidate the specific mechanisms by which PARVB contributes to early pregnancy in buffalo and other mammals.

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