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Potential utility of RSAD2 transcript and protein in early detection of pregnancy in buffaloes

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Research Article

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Abstract

This study investigates a novel early pregnancy marker in water buffaloes, focusing on RSAD2 mRNA expression, known to be upregulated by interferon-tau (IFNT) during pregnancy. While RSAD2 is primarily recognized for its antiviral effect, we hypothesized its role as a conceptus-induced component in regulating pregnancy in buffaloes. Given its differential expression compared to other IFNT-induced genes in cows, *RSAD2* may serve as a biomarker for early pregnancy detection in buffaloes. RNA, cDNA, and plasma samples were obtained from archived samples collected before insemination (d0) and at d20, d25 and d40 after insemination. Twelve RNA samples, having optimal optical density and concentration, from six pregnant and six non-pregnant buffaloes were selected. The cDNA was analyzed to measure the abundance of *RSAD2* mRNA using real-time quantitative PCR (RT-qPCR) and plasma for protein expression analysis using Western blot. The RT-qPCR analysis showed a transcript of *RSAD2* increased significantly by 7-fold and 6-fold on d20 and d25, compared to both d0 and d40 in the pregnant group only. At d20, the sensitivity of *RSAD2* was 100% and the specificity was 83.3%, and at d25-d both the sensitivity and specificity was 100%, indicating low incidences of misdiagnosing early pregnancy in buffaloes. In the non-pregnant group, *RSAD2* expression remained low and did not change after insemination. Western blot analysis revealed an immunoreactive RSAD2 protein band. Densitometry analysis of the RSAD2-specific protein band, based on gray mean value, showed significantly increased expression of RSAD2 at d25 compared to d0 in the pregnant group. In conclusion, these results indicated that RSAD2 expressions at both the mRNA and protein levels show promising potential for detecting pregnancy at d25 post-insemination.

Advances in animal reproductive technologies have expanded our understanding of buffalo reproduction, of which pregnancy diagnosis remains a crucial process. Addressing declining fertility in livestock is a significant challenge in improving reproductive efficiency within the livestock sector (Wiltbank *et al*., 2016), which underscores the importance of early pregnancy diagnosis. To sustain pregnancy recognition, interferon tau (IFNT) is produced by trophectodermal cells of blastocysts in ruminants IFNT influences the expression of interferon stimulated genes (ISGs) in various cells (Shirasuna *et al*., 2012; Toji *et al*., 2018).

About 10% of the genes in the genome are influenced by interferon (IFN) regulation (Schoggins, 2019). A successful approach for investigating early pregnancy detection in cows and buffaloes is gene expression analysis of several unique ISGs in blood cells. Relative mRNA expression of ISGs such as interferon-stimulated protein 15 kDa (*ISG15)*, myxovirus resistance-1 and -2 (*MX1*, *MX2*) and oligoadenylate synthetase-1 (*OAS1*) in peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs) have shown potential for use in diagnosing early pregnancy in cows (Panda *et al*., 2020) and buffaloes (Casano *et al*., 2022). Also in a recent study, the expression of certain ISG transcripts namely; dermokine (*DMKN*), interferon induced transmembrane protein 2 (*IFITM2*), galectin 3 binding protein (*LGALS3BP*), TNF superfamily 13b (*TNFSF13B*), C-type lectin domain family 3-member B (*CLEC3B*), interferon alpha protein 6 and 44 (*IFI6*; *IFI44*), 2′-5′-oligoadenylate synthetase 2 (*OAS2*) and radical *S*-adenosyl methionine domain containing 2 (*RSAD2*) was found upregulated at d18 to d20 of pregnancy in peripheral blood leukocytes (PBLs) of cows (Rocha *et al*., 2020).

RSAD2, also known as viperin, has long been associated with antiviral potency in bovine species, notably cows and buffaloes (Tirumurugaan *et al*., 2020). It is found to be highly up-regulated in cattle in response to peste des petits ruminants virus (PPRV) infection, contributing to host defense and decreased viral replication (Tirumurugaan *et al*., 2020). However a new hypothesis emerges: Could RSAD2 also be involved in controlling buffalo pregnancy, particularly in response to conceptus-induced factors? Although previous studies have suggested a potential link between RSAD2 expression and pregnancy status in cows (Rocha *et al*., 2020), further investigation is needed to determine if similar associations exist in buffaloes. To explore this hypothesis, we employed real-time quantitative polymerase chain reaction

(RT-qPCR) and Western blot analysis. We sought to decipher RSAD2 mRNA and protein expression patterns in early pregnant and non-pregnant buffaloes.

Through a comprehensive examination of the utility of RSAD2 transcript and protein, this study seeks to bridge the gap between molecular biology and buffalo reproductive management. The results of this study would offer a potential breakthrough in reproductive health management and provide an optimal window for buffalo pregnancy diagnosis in the farm for improved reproductive efficiency.

Materials and methods

Experimental animals

The study was conducted on archived samples of RNA and cDNA stored at −80°C. Ethical permission to conduct research was previously approved by the IAEC GADVASU ethical committee (Reference No. GADVASU/2021/IAEC/61/05 dated 19/10/21). Briefly, in an earlier study, blood from 18 buffaloes (9 pregnant and 9 non-pregnant animals) maintained at the Directorate of Livestock Farm, Guru Angad Dev and Animal Sciences University, Ludhiana, Punjab, was obtained in November 2021 to April 2022. Twelve archived RNA/cDNA and plasma (6 pregnant and 6 non-pregnant buffaloes) were obtained to conduct this study. RNA isolation and cDNA synthesis procedures have been published earlier (Sharma *et al.*, 2023).

RT-qPCR analysis of RSAD2

Primer sequences were designed using Primer-BLAST and manufactured by Sahagene, TL, India, for this study. The target sequence size and amplification temperature are shown in online Supplementary Table S1. PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, MA, USA) was prepared. An equal amount of the master mix was dispensed in sterile 4-strip RT-qPCR tubes. Subsequently, 10-fold diluted templates were added to each tube separately. Once the reactions in each tube were ready, they were kept inside the real-time thermocycler (Roto-Gene-Q; Qiagen, Hilden, Germany), and the PCR setting was done using SYBR green. The reaction condition for the amplification of the target was 40 cycles of PCR at 50°C for 2 min (UDG activation), 95°C for 2 min (initial denaturation), 95°C for 3 s, 60°C for 30 s (annealing/extension) and melt cure (90 to 95°C at the rate of 1°C/min of cooling rate, which was optimized for RSAD2. The $2^{\Delta\Delta Ct}$ method of relative quantification was used to compare the fold change difference between the expression of genes between pregnant and non-pregnant buffaloes across the time points d0 (before A.I.) and 20, 25 and 40 d after insemination (Livak and Schmittgen, 2001).

Receiver operating characteristic (ROC) curves were constructed where day 30 pregnancy status based on ultrasound was designated as the true positive. Predicted cutoff values were chosen based on the optimal criterion, considering sensitivity, specificity and pregnancy prevalence. Differences were considered significant where $P \leq 0.05$.

Western blot of RSAD2

The plasma samples collected at different time points (d0, 20 and 25) were quantified using Nano-drop. 20 ng of total protein was digested and electrophoresed on SDS-PAGE gels. The protein separated on the gel was transferred to a nitrocellulose membrane, 0.22 µm and blocked (5% BSA) for 2 h at room temperature. The membrane was incubated with the primary antibody, target protein (Anti-RSAD2 rabbit monoclonal antibody, BioRad, CA, USA, 1:5000 in blocking buffer), and endogenous protein (Anti-ACTB mouse monoclonal, Thermo Scientific, catalog no. BSM-33036M, 1:5000 in blocking buffer) overnight at 4°C; both target and endogenous protein were tagged separately. Species-specific secondary antibody IgG-HRP (1: 20 000) was incubated for 1 h at room temperature. The blot was developed using SuperSignal®West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, MA, USA) reagents and visualized in G: BOX Chemi XRQ gel doc system (Syngene, MA, USA). The blot image was further analyzed using ImageJ (ver. 1.53k) (Schneider *et al.*, 2012).

Image analysis of RSAD2 protein band

Densitometry analysis of protein band from the captured images was analyzed using ImageJ software with image background subtraction method for accurate Western blot semi-quantification (Gallo-Oller *et al.*, 2018). Briefly, using the rectangular selection tool, the outline of the first gel was selected as the region of interest (ROI). Next, the rectangular selection tool was moved to the next well over the RSAD2 band, and the next ROI was selected by holding the shift key while dragging. The procedure was repeated until the last well of the gel image. After all the gel lanes had been selected, 'plots' were created by choosing 'plot lanes' from the drop-down 'Analyze' menu. A window appeared in each band, representing the peak of the specific protein band. Using the line drawing tool, the total area of the peak enclosed was captured. A magic wand tool was selected to measure each peak, and the measured area can be annotated automatically by selecting label peaks. The area of measurement was recorded in the tabular form displayed under the 'result' and was saved as an option under the 'file' menu for statistical analysis.

Statistical analysis

The data was presented as median fold change value. Friedman's non-parametric test similar to one-way ANOVA, was applied. A Dunn's multiple-comparison was applied to compare multiple means, taking the rank of each column with the mean rank of control (d0). The adjusted *P-value* for the statistical differences <0.05 was taken to declare the significance level.

Results

We investigated the differential expression profiling of genes associated with early conceptus in buffaloes. The RT-qPCR and Western blot analyses were done to investigate the differential expression of RSAD2 at various days after insemination and compared with the value before insemination.

The mRNA expression of RSAD2 in PBMCs

The RSAD2 primer amplification efficiency was 99%, with an R^2 value of 0.99510, indicating efficient amplification of PCR, and observance of the single melt peak depicts the formation of a single qPCR product. We verified a single PCR product of the

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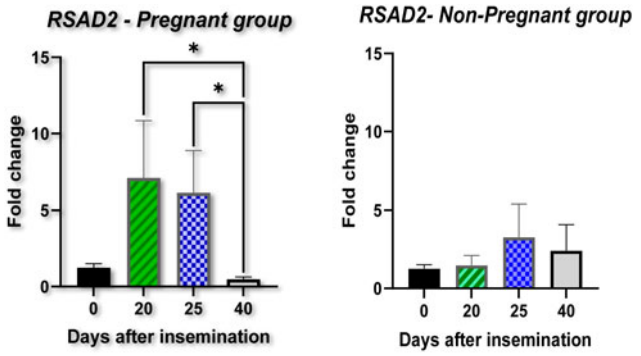

Figure 1. Fold change expression of *RSAD2* in PBMC of pregnant and non-pregnant buffaloes at d0, d20, d25 and d40 after artificial insemination.

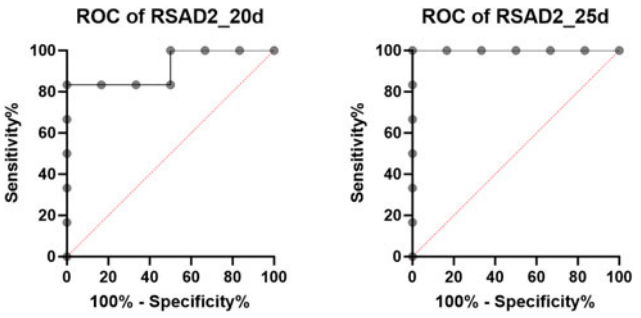

Figure 2. Receiver operating characteristics (ROC) curves for the detection of early pregnancy in buffaloes at d20 (panel A) and d25 (panel B) post-insemination.

intended size by running an amplicon in agarose gel electrophoresis.

In the pregnant group, the *RSAD2* gene expression was up-regulated at d20 and d25 (vs. d0) 7- and 6-fold (χ^2 (3) = 13.4, $P < 0.001$), respectively, and a significant difference ($P < 0.05$) was observed at d20 and d25 compared to d40. However, in non-pregnant animals, the mRNA expression remained at the basal level across all the time points. The data are shown in Fig. 1.

The ROC curves analysis of early pregnancy diagnosis in buffaloes was also established for *RSAD2* on d20 and d25. The area under the ROC curve (AUC) representing the diagnosis effectiveness of pregnancy using *RSAD2* expression showed a value of 91% at d20 and 100% at d25. The cutoff value was the threshold determined by the maximum value of the Youden index (YD). The sensitivity of *RSAD2* for diagnosing pregnancy was 83.3%, but the specificity was 100%. Interestingly, at d25, the diagnosing efficiency of *RSAD2* mRNA was 100% specific and sensitive (Fig. 2). The optimal cut-off values (fold change) for predicting pregnancy were >1.99 and >2.11 at d20 and d25 post-insemination, respectively (Table 1).

Protein expression of RSAD2 in plasma

Western blotting analyzes the protein presence and abundance in the biological sample. RSAD2 was subjected to Western blotting to check its suitability as a protein marker. The protein abundance was measured at d0, d20 and d25 of gestation. The expression level was significantly higher at d25 compared to d0 in the pregnant group of animals (Fig. 3A, B) whereas at d20, the expression level did not differ from d0. For the control run, beta-actin showed a similar intensity of protein band for all the groups of pregnant and non-pregnant samples (not shown).

Discussion

Pregnancy diagnosis is important in sound reproductive management and profitability of a good buffalo farm. A number of tests are available for cattle, including estimation of progesterone pregnancy-specific protein B (e.g. BioRRYN: Abdulkareem *et al.*, 2011), estrone sulfate (Aba *et al.*, 1998), rectal palpation, ELISA-based assays (Silva *et al.*, 2007), urine protein pregnancy markers (Rawat *et al.*, 2016), urinary metabolites (Sarangi *et al.*, 2022), real-time PCR-based assays (Casano *et al.*, 2022), blood tests for pregnancy-specific glycoproteins (PAGs: Pohler *et al.*, 2016; Van Hanh *et al.*, 2020; Tadeo *et al.*, 2021) and ultrasonography. Among these, progesterone assay is cost-effective and rectal palpation at day 50 is the field-based test. Ultrasonography is performed after day 35 for a confirmed diagnosis of pregnancy by visualizing conceptus, but it requires expertise and may not be practical in field conditions. In buffaloes, the sensitivity of monitoring early pregnancy using transrectal ultrasonography reported was less than 50% between 19–24 d (Barile *et al.*, 2021).

Determination of successful pregnancy at less than 30 d after insemination is considered early detection. In cattle, during early pregnancy, the elongating conceptus induces a change in maternal immune cells (Pugliesi *et al.*, 2014; Rocha *et al.*, 2020). In buffaloes, it could be hypothesized that the pregnancy is associated with paracrine signaling induced by conceptus-released IFNT (Casano *et al.*, 2022) that affects the endometrium, and hence immune responses. Analogous to the study performed on cows, we previously explored the differential expression of ISGs in pregnant buffalo immune cells, reporting *ISG15* and galectin-3 binding protein (*LGALS3BP*) expression in early pregnant buffaloes (Sharma *et al.*, 2023). In a continuation of this study, we also explored differential expression of pregnancy-specific proteins in blood and milk of early pregnant and non-pregnant buffaloes, and found differences in the expression of an ~73 kDa protein (Singh *et al.*, 2024). Based on an earlier published study (Rocha *et al.*, 2020) found differential expression of genes *IFI44, OAS2, TNFSF13B, CLEC3B* and *RSAD2* in cows and the current research now explores the utility of RSAD2 at gene and protein levels in detecting early pregnancy in buffalo.

Radical *S*-adenosyl methionine domain-containing 2 (RSAD2) is an antiviral protein, also known as viperin, which is produced

Table 1. Sensitivity and specificity of *RSAD2* mRNA in diagnosing early pregnancy in buffaloes at d20 and d25 after insemination

Day	Cutoff value of *RSAD2* (fold change)	Sensitivity %	CI 95%	Specificity %	95% CI
20	> 1.99	83.33	43.65–99.15%	100.0	60.97%–100.0%
25	>2.11	100	61–100%	100.0	60.97–100.0%

The calibrator was d0.

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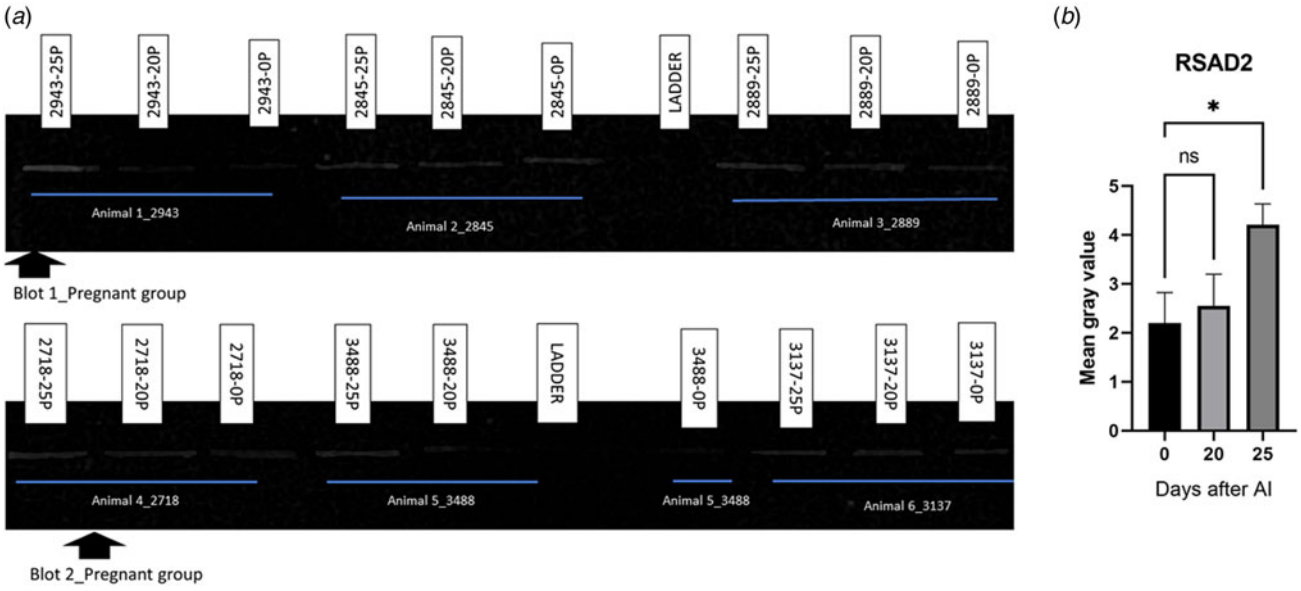

Figure 3. Protein expression of RSAD2 in the blood plasma of early pregnant buffalos. (A) Protein intensity of RSAD2 across d0, d20 and d25 of the same animals showing varying intensity. (B) Gray mean value of RSAD2 protein bands at d0 *vs.* d20 and d25.

by type I IFNs and can prevent the human cytomegalovirus from infecting cells (Chin and Cresswell, 2001). The production of RSAD2 and interferon-induced with helicase C domain 1 (IFIHI) is seen during viral infection as a result of IFNs, which influence the adaptive immune responses (Katze *et al.*, 2002; Helbig *et al.*, 2005). RSAD2 is engaged in sustaining uterine receptivity by inducing an antiviral state and modulating immune cells (Song *et al.*, 2007) and contributes to the production and degradation of antibiotics, herbicides, vitamins, DNA precursors and cofactors (Sofia *et al.*, 2001). These compounds could hold significance for endometrial cells in aiding conceptus growth and implantation during the peri-implantation phase.

We showed the expression of RSAD2 at gene and protein levels in immune cells and its utility in determining early pregnant buffaloes. RSAD2 significantly increased d6 post-insemination in cow endometrial tissue (Forde *et al.*, 2011; Mamo *et al.*, 2012). Likewise, its mRNA expression in peripheral blood leukocytes at d21 was 2.3 times higher than at d0, again in cows (Kizaki *et al.*, 2013; Yoshino *et al.*, 2018). However, the expression level remained relatively stable at d14. In primiparous cows, a difference in RSAD2 expression in peripheral blood leukocytes between d15 and d18 was observed (Green *et al.*, 2010) and in a related study *RSAD2* expression increased exponentially from d18 to d21, then declined (Cheng *et al.*, 2019). Our results indicated a 6–7-fold increase in transcripts of *RSAD2* in PBMCs of d20 and d25 pregnant buffaloes. This concurs with the results where *RSAD2* mRNA expression in pregnant cows was significantly upregulated at d18 and d20 (Rocha *et al.*, 2020). Genetics of animals (comparing *Bos taurus* with *Bos indicus*, e.g.: Rocha *et al.*, 2023), breed, age, parity, and species may influence the expression of ISGs, including *RSAD2*. Also, these findings suggest that *RSAD2* expression might be indirectly affected by genetic composition, leading to associations with progesterone concentrations, where an inverse relation was observed. Further analysis using ROC curves showed the sensitivity was 100% at both d20 and d25, implying that the risk of misdiagnosing pregnant buffaloes as non-pregnant will be low. The study reports *RSAD2* as a good biomarker for early pregnancy diagnosis in buffaloes at both transcript and protein levels.

This research method of early pregnancy diagnosis in buffaloes is based on measuring the transcript of *RSAD2* in PBMCs. Therefore, factors like existing viral infection in the animals and early embryonic mortality could possibly affect the expression of RSAD2 and, hence, pregnancy detection. As indicated above, *RSAD2* is one of the ISGs that increases host defense and decreases viral replication (Helbig *et al.*, 2005; Tirumurugaan *et al.*, 2020). Likewise, in repeat breeding in the event of early embryonic mortality, increased levels of ISGs from previous pregnancy could produce false positive results. In addition, buffaloes are seasonal breeders, meaning reproductive efficiency is affected by seasonal variation. During the day length lengthening period (spring-summer), embryonic losses in buffaloes are as high as 20–40% as compared to 7% during the decreasing daylight length (winter) (Campanile *et al.*, 2016; Barbato *et al.*, 2022).

PAGs have been used to detect pregnancy in buffaloes. However, the specificity of PAG-based radio immune assay (RIA) at d19 to d24 after insemination in buffaloes was extremely low (11%) (Karen *et al.*, 2007). Later, the specific RIA system detected pregnant buffaloes at d28d with 98% specificity (Barbato *et al.*, 2017). An alternative protein biomarker like RSAD2 is required to develop protein-based assays. Estimation of RSAD2 protein in buffalo plasma for pregnancy diagnosis has not been reported earlier.

In conclusion, we have shown that the interferon-stimulating gene *RSAD2* shows promise for diagnosing early pregnancy in buffalo at transcript and protein levels.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029924000360

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