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Expression dynamics of ISGs and chemokines in maternal whole blood as an indicator of healthy embryonic implantation in buffalo

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ABSTRACT

Successful pregnancy establishment has a major influence on reproductive efficiencies of dairy buffaloes. This success depends on coordinated communications between conceptus and maternally derived cells, and any disturbance in this communication network during this process leads to early embryonic losses. Since, early embryonic losses is one of the prime cause of pregnancy failure in dairy buffaloes, present investigation was aimed to understand the expression dynamics and correlation pattern of Interferon tau stimulated genes and chemokines during peri-implantation period in dairy buffaloes through transcriptional profiling followed by Principal Component Analysis. Our study revealed upregulation of all the candidate genes in maternal whole blood across the parity, emphasizing the involvement of immune responses as signatures for healthy pregnancy. Understanding these embryo-maternal communication signals offers potential to prevent incidences of early embryonic losses and can provide insights on possible targets for therapy too.

Keywords: Buffalo, Chemokines, Embryo maternal signaling, Early embryonic losses, ISGs

Pregnancy in dairy buffaloes is a highly celebrated event. It initiates from the day of conception and continues till parturition, and its success explicitly depends on the coordinated communications between the mother and foetus. Since the day of conception, the process of signalling starts for establishing crosstalk and collaboration between conceptus and maternally derived cells leading to maternal recognition of pregnancy (MRP). Any disturbance in this communication network, during this process, leads to early embryonic losses. In ruminants, the principal signal for MRP is identified to be interferon Tau, which is a functional connection between the maternal immune system and the developing embryo (Rocha *et al.* 2020), and is secreted by trophoectoderm of blastocyst for a limited period during early pregnancy. This interferon Tau exerts its antiluteolytic action by suppressing the normal pattern of pulsatile release of PGF2 in late estrous cycle, possibly by a mechanism that involves downregulation of estrogen receptor in uterine epithelium, which in turn prevents a rise in oxytocin receptor ((Bazer *et al.* 2013). In addition to this, the survivability and opportunity of successful development of an embryo is also influenced directly and indirectly by various paracrine and autocrine factors (steroid hormones, growth factors, and cytokines), controlling uterine microenvironment, which is affirmed from the recent investigations underscoring the implications of cytokines (CCL8 and CXCL10) in pregnancy establishment in bovines and goat (Imakawa *et al.* 2005, Sakumoto *et al.* 2018, Kumari *et al.* 2021). Both these chemokines belong to cytokine family and are classified into -CC- and –CXC- group respectively, based on the presence and position of conserved cysteine residue and act through specific G-protein coupled receptors.

Present study is an attempt to provide insights into the poorly understood embryo-maternal dialogue during peri-implantation period in pregnant and non-pregnant Murrah buffaloes in terms of variation and correlation in expression dynamics of ISGs (ISG 15, MX 1 and MX 2) and chemokine genes (CCL8 and CXCL10). The study is aimed to enhance our understanding regarding molecular interactions underlying embryonic implantation. Understanding these embryo-maternal communication signals has the potential to prevent incidences of early embryonic losses and can provide insights on possible targets for therapy too.

MATERIALS AND METHODS

Experimental animals and sample collection: Present study was conducted on Murrah breed of buffaloes maintained at Livestock Farm Unit, Division of Livestock and Fishery Management of ICAR-Research Complex for Eastern Region, Patna. Twenty healthy cyclic Murrah

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buffaloes (age 2.5 years to 8 years) were enrolled for study. All animals were kept in a dry, ventilated shed under proper hygienic conditions. Animals were observed for estrus detection and AI was done on day of estrus using frozen semen. The day of AI was designated as day 0. Blood samples were collected using EDTA as an anticoagulant by jugular vein puncture under sterile conditions. Sample was further collected from animals on 12th, 15th, 18th and 21st day post insemination and immediately processed for RNA extraction. Plasma was separated from blood samples and progesterone level was determined by Bovine Progesterone (PROG) ELISA kit. Pregnant and non-pregnant conditions in animals were confirmed by trans-rectal ultrasound and rectal palpation on days 35 and 60 post insemination respectively.

RNA extraction, assessment and cDNA synthesis: Blood samples collected (containing EDTA) were immediately processed for extracting total RNA as per the method followed by Batra *et al.* (2019). Quality and quantity of RNA was assessed by nanodrop. RNA sample with ratio of 2.0-2.2 was considered good and taken for further analysis. Integrity of total RNA was also checked using 1% agarose gel electrophoresis and visualization under UV light. Two intact bands of 28 S and 18 S with smearing indicated good quality and intactness of RNA. One microgram of total RNA from different samples was reverse transcribed to cDNA (with confirmed integrity) using Quantitect Reverse Transcription Kit (Qiagen) according to manufacturer's instruction.

Quantitative real time PCR and relative quantification: Primers used in the study were selected based on the published literature. The sequences, optimised annealing temperature and expected PCR product length are presented in Table 1.

Quantitative real time PCR was performed (in triplicate) using Quantitect SYBR Green PCR kit (Qiagen) in Applied Biosystem 7500 real time PCR system. Each sample was run in triplicate in 20 µl reaction mixture which consisted 2 µl diluted cDNA, 10 µl of mixture composed of SYBR Green /ROX qPCR (2×) (Qiagen), 0.5 µl each of forward (0.5 mM) and reverse primers (0.5 mM) and 7 µl nuclease free water. The real-time PCR was run with initial denaturation at 95°C for 30 sec followed by 35 cycles of denaturation at 95°C for 5 sec, annealing for 12 sec and extension at 72°C for 10 sec. Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimers. Prior to relative quantification, real time PCR efficiencies for all target genes and endogenous control genes were determined by standard curve generation. Estimation of transcriptional abundance of target genes was performed using the ΔΔCt model. The Ct values for different genes obtained from day 0 samples were used as calibrator for obtaining relative mRNA expression. The Ct values of GAPDH were used as Ct of reference gene. The expression levels of target genes were normalized to reference gene and the relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Statistical analysis: The experimental data for real-time PCR are presented as the mean±SEM. The difference of mRNA expression in whole blood at different days post AI for pregnant and non-pregnant group was analyzed using one-way ANOVA for repeated measures with Dunnett's multiple comparison post hoc tests with the SPSS software package. The following general linear model was used for the analysis

$$Y_{ij} = \mu + D_i + e_{ij}$$

where Y_{ij}, jth observed value of the response variable for the ith day; µ, general mean effect; D_i, effect of ith day; e_{ij}, error term.

Principal Component Analysis (PCA) is a multivariate statistical analysis tool generally used to reduce the high dimensional correlated variables to low dimensional uncorrelated variables known as principal components. Principal components are the linear combinations of the original variables. PCA is popularly used in the fields of genetics, engineering, computer vision, etc. (Hubert *et al.* 2005). In this study, PCA biplot is used to visualise the expression of five selected maternal recognition of pregnancy (MRP) genes like ISG 15, MX1, MX2, CCL8 and CXCL10) at different days of post AI for both pregnant and non-pregnant buffalo. The studied data

Table 1. Primers used in the study

Target gene	Product size	Sequence (5′-3′)	Gene Accession No	Annealing temperature	Reference
MX1	111 bp	For: AATGGAGTGCTGTGGTTGAGAA Rev: CACAAACCCTGGCAACTCTCTG	AF047692.1	58°C	Thakur *et al.* (2017)
ISG 15	91 bp	For: CCTGACGGTGAAGATGC Rev: CTGGGCGATGAACTGCTT	NM_174366.1	58°C	Thakur *et al.* (2017)
MX2	77 bp	For: GTCCGCTGGTGCTGAAACTGA Rev: TGCTGCGTAATGTTGCGGTAGGT	NM_173941.2	60°C	Thakur *et al.* (2017)
CCL8	108 bp	For: AACATGAAGGTCTCCGCTGG Rev: GCAGCAGGTGATTGGGGTAG	NM_174007	60°C	Sakumoto *et al.* (2017)
CXCL10	117 bp	For: CTCGAACACGGAAAGAGGCA Rev: TCCACGGACAATTAGGGCTT	NM_001046551	60°C	Sakumoto *et al.* (2017)
GAPDH	82 bp	For:GCGATACTCACTCTTCTACTTTCGA Rev: CGTACCAGGAAATGAGCTTGAC	U85042.1	60°C	Thakur *et al.* (2017)

were standardized before the analysis. Data analysis was performed in R version 3.2.6 package (R Core Team, 2020).

RESULTS AND DISCUSSION

Transcriptional abundance of ISGs and chemokines: The differential expression levels of genes (MX1, MX2, ISG15, CCL8 and CXCL10) are presented in Table 2. In pregnant buffaloes, ISGs viz. ISG15 and MX2 (Supplementary Figs 1- 2) along with chemokines viz. CCL 8 and CXCL 10 (Supplementary Figs 4-5) were found to be upregulated, during days 0 to 21 post AI. Down regulation was observed in case of MX1 gene (Supplementary Fig. 3) post day 18 of AI. Fold change in expression level between day 12 and 15 post AI did not vary significantly for ISG 15 and MX2 gene. Chemokines (CXCL10 and CCL8) also showed consistent increase in expression levels during the period, but the difference in expression level was not significant between day 15 and 18 post AI for CXCL10 gene. Significant difference in fold change in expression level for CCL8 gene on all days post AI was observed. We also attempted to measure the concentration of proteins of candidate genes using commercial ELISA kit in plasma, but none of the proteins were detected. It may be due to low concentrations of these proteins, as also reported by Sakumoto *et al.* (2018).

Peri-implantation period is the period during which blastocyst physically and physiologically comes into intimate contact with the endometrium and is followed by placentation. This process of implantation begins on day 16-18 in bovines (Pederson *et al.* 2017, Panigrahi *et al.* 2020) and occurs under strictly synchronized embryonic maternal dialogue (Salilew-Wondim *et al.* 2012). In recent decade, this period has remained area of keen interest for researchers and was extensively investigated with the aim for identification of early pregnancy biomarker, but understanding this complex and coordinated signaling underlying has always remained a fascinating area of research. And therefore, present investigation was aimed to provide further insight into the expression dynamics of ISGs (ISG 15, MX1, and MX2) along with chemokines (CCL8 and CXCL10) and correlation among them during this critically important period in whole blood of pregnant and non-pregnant buffaloes. ISG 15, MX1 and MX2 are interferon stimulated genes (ISGs) induced by Interferon-tau (IFNτ) (Toji *et al.* 2017) and have been previously reported for high abundance of their transcripts in peripheral blood mononuclear cells (PBMCs) of pregnant sheep (Yankey *et al.* 2001) and cows (Matsuyama *et al.* 2012). Painkra *et al.* (2019) studied differential neutrophil gene expression in blood and milk during pre-implantation pregnancy in cows and reported that though MX1 and OAS 1 were found to be differentially expressed during different days post AI, no significant difference in the fold change was observed between pregnant and non-pregnant animals. Chemokine CCL8 transcript was also identified in the bovine endometrium and increased in cows during an early stage of pregnancy (Mansouri *et al.* 2012, Sakumoto *et al.* 2017). Zlotkowska *et al.* (2019) reported chemokine CCL8 as an important participant in the establishment of appropriate environment for embryo implantation. The level of CXCL10 expression in the endometrium was also found to be higher during pregnancy than in the non-pregnant stage in goats (Imakawa *et al.* 2005) and cows (Sakumoto *et al.* 2017, Sakumoto *et al.* 2018). Similar to these species, we also observed upregulation of candidate genes in whole blood of pregnant buffaloes. Our findings substantiates the findings of Thakur *et al.* (2017), which also revealed downregulation of MX1 gene post day 18 of AI, in peripheral blood mononuclear cells of pregnant buffaloes.

Table 2. Differential gene expression at days post artificial insemination in pregnant and non-pregnant buffalo

Candidate gene	Days post AI	Pregnant	Non-pregnant
MX1	12	0.2 ± 0.045^{a}	0.21 ± 0.20^{a}
	15	0.62 ± 0.09^{b}	0.28 ± 0.16^{a}
	18	0.79 ± 0.09^{c}	0.32 ± 0.12^{a}
	21	0.59 ± 0.04^{b}	0.38 ± 0.11^{a}
MX2	12	0.08 ± 0.03^{a}	0.06 ± 0.032^{a}
	15	0.1 ± 0.01^{a}	0.08 ± 0.032^{a}
	18	0.24 ± 0.04^{b}	0.082 ± 0.026^{a}
	21	0.22 ± 0.02^{b}	0.091 ± 0.03^{a}
ISG 15	12	1.2 ± 0.50^{a}	1.1 ± 0.90^{a}
	15	1.6 ± 0.05^{a}	1.15 ± 0.85^{a}
	18	2.5 ± 0.50^{b}	1.2 ± 0.60^{a}
	21	3.8 ± 0.17^{c}	1.18 ± 0.62^{a}
CCL8	12	0.014 ± 0.001^{a}	0.011 ± 0.002^{a}
	15	0.02 ± 0.004^{b}	0.009 ± 0.002^{a}
	18	0.042 ± 0.014^{c}	0.012 ± 0.004^{a}
	21	0.062 ± 0.002^{d}	0.013 ± 0.006^{a}
CXCL10	12	0.04 ± 0.001^{a}	0.039 ± 0.02^{a}
	15	0.067 ± 0.025^{b}	0.045 ± 0.012^{a}
	18	0.094 ± 0.027^{bc}	0.055 ± 0.004^{a}
	21	0.12 ± 0.02^{c}	0.052 ± 0.008^{a}

Values with different superscript denotes significant difference ($P<0.05$).

Analysis of abundance variation and correlation amongst candidate genes: Principal Component Analysis between five selected candidate genes (ISG 15, MX1, MX2, CCL8 and CXCL10) at different days post AI for both pregnant and non-pregnant buffalo presented in biplot results (Figs 1, 2) revealed that ISG 15, CCL8, CXCL10 and MX 2 dominated with principal component 1 (Dimension 1) that explained maximum variation (85.2%). Gene MX1 alone dominated on principal component 2 that explained minimum variation (12.2%) of the data sets. ISG 15, CCL8 expression was observed in latter days i.e. 21 days (D21) of pregnancy and both ISG 15, CCL8 were found to be highly correlated too. Same way, peak MX2 and CXCL10 expression was observed in latter days i.e. 21 days (D21) of pregnancy and they were also highly correlated. Peak of MX1 expression appeared in 18 days (D18) after pregnancy.

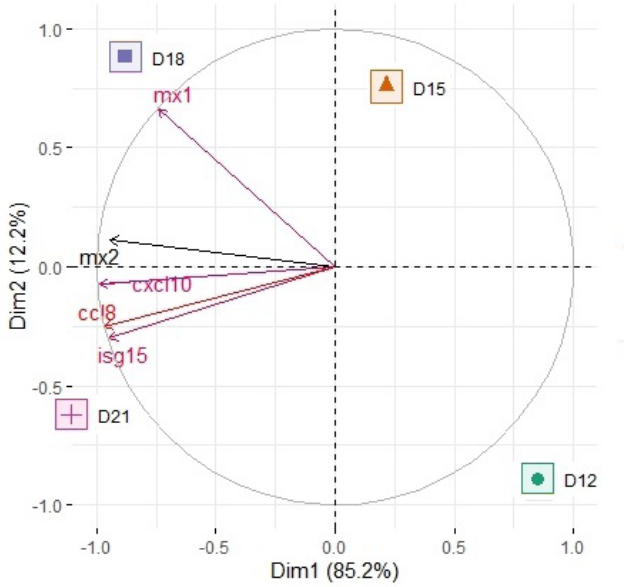

Fig. 1. PCA biplot between candidate maternal recognition of pregnancy genes (ISG 15, MX1, MX2, CCL8 and CXCL10) for their transcriptional abundance at different days post AI in pregnant buffalo.

MX 1 did not show correlation with any of the studied gene. This abundance and correlation pattern was exhibited by all the pregnant animals which underwent successful calving suggesting their role in the establishment of proper environment for embryonic implantation.

PCA biplot between five five candidate genes at different days of interval for non-pregnant buffalo showed that ISG 15, MX 2, CXCL10 and MX1 are dominated with principal component 1 (Dimension 1) with maximum variation (80.2%). CCL8 alone is dominated on principal component 2 that explained minimum variation (14.1%) of the data sets.

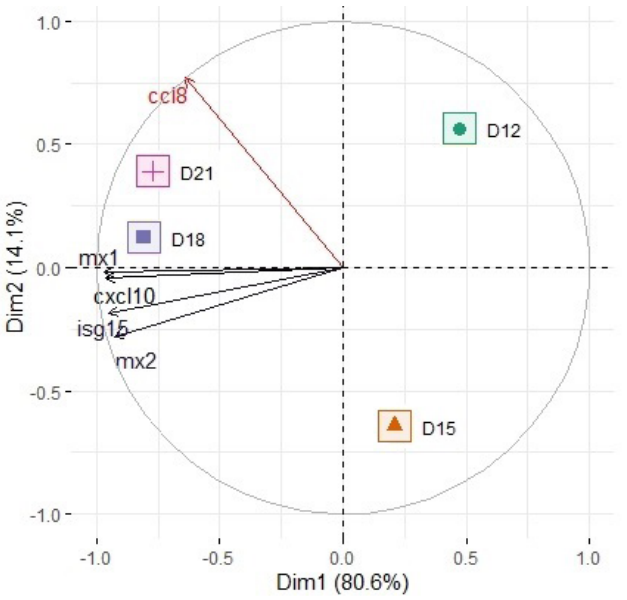

Fig. 2. PCA biplot between candidate maternal recognition of pregnancy genes (ISG 15, MX1, MX2, CCL8 and CXCL10) for their transcriptional abundance at different days post AI in non- pregnant buffalo.

CXCL10 and MX1 expression was observed on 18th day (D18) of pregnancy and both CXCL10 and MX1 are also highly correlated. CCL8 expression appeared in 21 days (D21) after pregnancy (Fig. 2).

From our findings, it can be stated that these genes influence embryonic implantation by participating as mediators of embryo-maternal communication and endorse the role of immune responses during establishment of pregnancy (Laura *et al.* 2018). These interactions can be used as a sign of healthy pregnancy in buffaloes during early 21 days post AI as these comprise few of maternal immune system adaptations, required for maintaining tolerance towards the allogeneic fetus.

ISGs and chemokines are involved in embryo-maternal communication signaling and can be used as signature of healthy embryonic implantation in buffaloes.

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