Expression profiling of chemokines CCL8 and CXCL10 during peri-implantation period in Murrah buffaloes as a possible signature of embryonic implantation

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ABSTRACT

The present investigation was aimed to evaluate novel implication of chemokine genes CXCL10 (C-X-C motif chemokine 10) and CCL 8 (C-C motif chemokine 8) genes for use as ideal pregnancy biomarker in dairy buffaloes. We studied expression profile of both these chemokine genes in whole blood of Murrah buffaloes on day 12, 15, 18 and 21 post artificial insemination (AI) using SYBR green chemistry based quantitative real time reverse transcription PCR. Our investigations revealed a consistent increase in transcriptional abundance of CCL8 and CXCL10 genes during this period, although the difference in expression level was not significant between day 15 and 18 post AI for CXCL10 gene. We also studied the effect of parity on the expression profile of these two genes and found that expression level of both these genes is independent of parity status of the animal. Based on the results, it can be concluded that these chemokine genes can be used as early pregnancy biomarker on any day between days 12 to 21 post artificial insemination in buffaloes irrespective of their parity status.

Keywords: Buffalo, Chemokine genes, Parity status, Pregnancy biomarker, Whole blood sample

Buffalo is an important animal in Indian dairy industry and profitability of this industry heavily depends on the reproductive management of these dairy animals. But, the incidence of reproductive challenges like silent heat and short duration heat, reduces the reproductive potential of this species. The phenomenon of silent heat is generally misdiagnosed as pregnancy by farmers (Batra *et al*. 2018), leading to long calving interval and low milk production which is the prime underlying reason for low milk productivity of buffaloes in the Indian subcontinent. This issue can be solved with the use of early pregnancy detection tools in dairy buffaloes, which will allow prompt re-insemination of animals failing to conceive at first service, in turn leading to increased reproductive efficiency and productivity. Extensive research has been conducted to develop ideal pregnancy detecting biomarker in dairy animals, based on quantitative real-time PCR. Findings of Green *et al*. (2005) provided the impetus in this field of research by reporting the possibility of pregnancy diagnosis post 18 days of artificial insemination (AI) in bovines, based on transcriptional abundance of interferon tau (IFNT) stimulated genes (ISGs) in circulatory leucocytes. Further research in this area revealed that use of ISGs as a marker

of early pregnancy diagnosis had variable effectiveness (Han *et al*. 2006, Stevenson *et al*. 2007), for example, OAS1 mRNA levels were successfully used to assess pregnancy on 18 d in heifers but not in cows (Green *et al*. 2010). Few investigations (Hannan and Salamonsen 2007, Sakumoto *et al*. 2017) have indicated role of the chemokines as important players in the process of embryonic implantation. Since, successful embryonic implantation is a well coordinated process involving maternal-embryonic communication by chemokine signaling and extensive endometrial modeling (Hannan and Salamonsen 2007, Kiefer and Siekmann 2011), functional involvement of chemokines cannot be denied. One study has reported increased expression of these chemokines in peripheral blood leucocytes (PBLs) of cows during peri-implantation period (Sakumoto *et al*. 2018). But, no study has explored the potential of chemokines as pregnancy biomarker in economically significant dairy buffalo of Indian subcontinent.

In view of above background, present investigation was carried out to determine expression profile of chemokine biomarker (CCL8 and CXCL10) during peri-implantation period in whole blood of artificially inseminated dairy buffaloes. We also investigated effect of parity on expression profiling of these genes.

MATERIALS AND METHODS

Experimental animals and sample collection: Present

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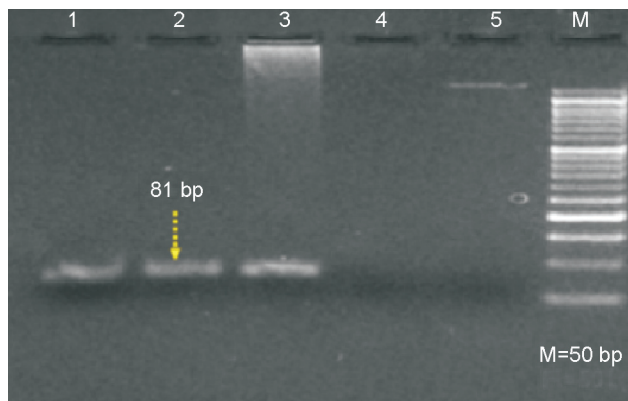
Fig. 1. Amplification of GAPDH gene in cDNA samples (Lanes 1–3), RT control (Lane 4) and non template control (Lane 5).

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. Fourteen healthy cyclic Murrah buffaloes (age 2.5 years to 8 years) were classified into primiparous (n=7) and multiparous (n=7) based on their parity status. 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. Animals which returned to heat after 21 days were considered as non-pregnant animals. Pregnant and non-pregnant conditions in animals were confirmed by trans-rectal ultrasound and rectal palpation on days 35 and 60 post insemination respectively. Pregnant animals were observed till successful calving after full gestation period for reliability of study.

RNA extraction, assessment and cDNA synthesis: Blood samples collected (containing EDTA) were immediately processed for extracting total RNA as per Batra *et al*. (2018). Quality and quantity of RNA was assessed by spectrophotometer. RNA sample with ratio of 2.0–2.2 was considered good and taken for further analysis. Integrity of total RNA was 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. The quality of synthesized cDNA was confirmed by semi-quantitative PCR using GAPDH primers (Fig. 1).

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 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 dimmers. Relative quantification 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 geometric mean of Ct values of GAPDH and β-actin 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 $2^{-\Delta\Delta C_T}$ 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 20.0 software package.

Table 1. Primers used in the study

| Target gene	Product size	Sequence (5′–3′)	Gene accession no	Annealing temperature	Reference
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)
ß-Actin	179 bp	For: GGCACCCAGCACAATGAAGATC Rev: ACAGTCCGCCTAGAAGCATTTGC	NM_173979.3	60°C	Sheikh *et al*. (2018)
GAPDH	81 bp	For: GCGATACTCACTCTTCTACTTTCGA Rev: CGTACCAGGAAATGAGCTTGAC	U85042.1	60°C	Thakur *et al*. (2017)
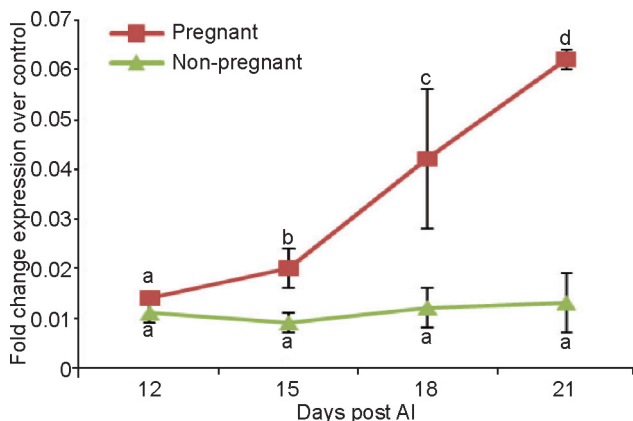
Fig. 2. Transcriptional abundance of CCL8 gene in pregnant and non-pregnant samples on different days post AI. Minimum level of significance was set at 95%. Each point represents mean±SEM. Points with different superscript denotes significant difference (P<0.05).

RESULTS AND DISCUSSION

Animals were classified into pregnant (n=8) and non pregnant animals (n=6) primarily based on the plasma progesterone levels followed by ultrasonographic examination and per rectal palpation findings.

Expression profile of chemokines: A consistent increase in expression levels of CCL8 and CXCL10 genes was observed during peri-implantation period, although the difference in expression level was not significant between day 15 and 18 post AI for CXCL10 gene. The transcriptional abundance of CCL8 in pregnant as well as non-pregnant samples is presented in Fig. 2.

Significant increase in transcriptional abundance of CCL8 gene was observed throughout the peri-implantation period in pregnant buffaloes with the C_t value of 0.014±0.001 at day 12 post AI to 0.062±0.002 at day 21 respectively. The difference in the expression level on all days was found significant (P<0.05). Expression level of CCL8 gene remained unchanged (P>0.05) throughout the
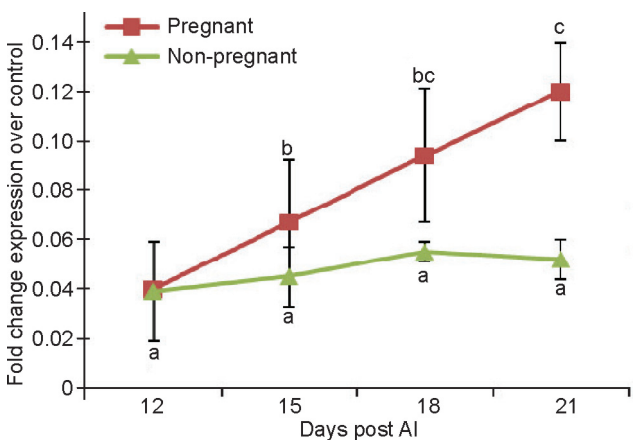

Fig. 3. Transcriptional abundance of CXCL10 gene in pregnant and non-pregnant samples. Minimum level of significance was set at 95%. Each point represents mean±SEM. Points with different superscript denotes significant difference (P<0.05).

peri-implantation period in case of non-pregnant animals. The transcriptional abundance of CXCL10 in pregnant as well as non-pregnant samples is presented in Fig. 3.

Expression of CXCL10 in pregnant buffaloes also showed significantly greater expression (P<0.05) on day 12 post AI to day 21 than control (non-pregnant) group. The expression showed significant increase (P<0.05) post day 12 to day 21 of AI with the values of 0.040±0.001 and 0.12±0.02, respectively. Difference in the expression level at day 15 and 18 was nonsignificant on (P>0.05) with the values of 0.067±0.025 and 0.094±0.027, respectively. The expression level of CXCL10 gene remained unchanged (P>0.05) throughout the peri-implantation period in case of non-pregnant animals. The accuracy of amplification of both these genes was ensured by the presence of a unique peak during the dissociation step at the end of the qPCR reaction (melt curve). Agarose gel electrophoresis (3%) of real time PCR amplified products reconfirmed accuracy of melt curve analysis.

Our findings are in concordance with the findings of earlier investigations (Sakumoto *et al.* 2017), where, increased expression of CCL8 and CXCL10 in endometrium and PBLs of pregnant Japanese Black cows was reported. The reason for increase in CCL8 and CXCL10 is supported by the findings of Zlotkowska *et al.* (2019), who suggested role of CCL8 as one of the signals produced by the trophoblast to signify its presence in the uterine lumen and significance of CXCL10 in establishment of an immunotolerant environment for the embryo during embryonic implantation.

Effect of parity on expression profile of chemokine genes: Parity has been reported to play important role in reproductive performance of buffaloes. Parity has also been reported to affect expression profile of ISGs (Green *et al.* 2010). Since, effectiveness of a biomarker based assay relies on its utility across animals, irrespective of its parity status, therefore, effect of parity status on expression profile of chemokine CCL8 and CXCL10 genes in whole blood of pregnant animals was studied. Investigations in primiparous pregnant (n=5) and multiparous pregnant (n=3) revealed that parity does not exert any significant effect on the transcriptional abundance of CCL8 and CXCL10 gene (P>0.05). Therefore, we suggest that increased response of chemokines CCL8 and CXCL10 is a pregnancy dependent event and is independent of parity status of the animal.

Based on our findings, we concluded that expression profiling of chemokines CCL8 and CXCL10 can act as signature for embryonic implantation in dairy buffaloes between day 12 – 21 post AI irrespective of their parity status.

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