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Chronological profiling of early pregnancy transcripts in Murrah buffaloes

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Abstract

Effective reproductive management in cattle, such as cows and buffaloes, requires early and accurate pregnancy detection. Early identification of pregnancy enables farmers to promptly identify non-pregnant animals for treatment and/or rebreeding, thereby reducing the calving interval. This study aimed to standardize the expression of the *CCL8* and *CXCL10* genes as markers for early pregnancy detection in Murrah buffaloes. Blood samples were collected on the 16th day post-artificial insemination for gene expression analysis and on days zero, seven, 14, and 21 post-artificial insemination for progesterone concentration measurement. Buffaloes were categorized as pregnant (n = 6) or non-pregnant (n = 6) based on the resumption of estrus. Gene expression levels in peripheral blood leukocytes were analyzed using Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) with SYBR green dye. Amplicons of *CCL8*, *CXCL10*, and *GAPDH* genes were measured 108, 117, and 158 bp, respectively. Results showed that CCL8 mRNA expression in pregnant buffaloes was 5.13 and 12.21 times higher compared to non-pregnant buffaloes, while CXCL10 mRNA expression was 4.19 and 22.17 times higher. These findings indicated significantly elevated CCL8 and CXCL10 mRNA expression levels in peripheral blood leukocytes of pregnant buffaloes on the 16th day. Progesterone levels in the pregnant group were increased significantly from day zero to day 21, while no significant differences were observed between groups on days zero, seven and 14. Pregnancy was further confirmed via *per*-rectal examination on the 45th day post-artificial insemination Therefore, *CCL8* and *CXCL10* gene expression profiling on the 16th day could serve as reliable early pregnancy markers in Murrah buffaloes.

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Introduction

Indian buffaloes have long calving interval, late puberty and high incidence of anestrus. The lack of reliable methods for detecting early pregnancy in buffaloes exacerbates the situation.[1] In bovine species, several methods of pregnancy detection (cyesiognosis) are used, however, none is ideal due to inherent limitations in level of sensitivity, accuracy and reliability, specificity, speed and convenience of testing.[2] Due to the development of molecular techniques like proteomics and their applications in animal research, researchers are now looking for pregnancy biomarker molecules.[3,4]

In the past few decades, several methods of directly or indirectly diagnosing pregnancy have emerged such as rectal palpation,[5] ultrasound,[6] early pregnancy factor,[7] and pregnancy-associated glycoprotein (PAG) 1 is a pregnancy-specific protein B, within the broader family of PAGs found in ruminants.[8,9] In case of pregnant animals, progesterone level remains elevated throughout the pregnancy (until parturition) causing the absence of cyclic pattern. Progesterone is an example of non- pregnancy specific diagnosis method.[10]

Early and accurate pregnancy detection is a critical criterion for improving reproductive management in livestock such as cows and buffaloes. High reproductive efficiency is required to achieve high life-time production from dairy animals. Early pregnancy diagnosis is critical for shortening the calving interval by allowing the farmer to identify non pregnant animals as soon as possible and treat and/or rebreed them. A 60-day post-parturient barren interval is ideal for breeding in dairy animals.[11]

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Dairy farmers must identify non-pregnancy as soon as possible in order to rebreed the dam as soon as possible. It is estimated that the early embryonic time is 42 days after insemination.[12] Their pregnancy is safer and non-infectious embryonic losses are uncommon after implantation.

Cytokines play a controversial role in early pregnancies in mammals. These low molecular proteins are responsible for maintaining corpus luteum, fetal adhesion and invasion, implantation, fetal growth, placental differentiation and various immunomodulatory mechanisms.[13] In some cases, the availability of sufficient numbers on a local level is important for embryo survival.[14] Cytokines are small proteins secreted by cells that have a specific effect on cell interactions and communication.[15] It stimulates the expression of interferon-stimulated genes, such as interferon-stimulated protein 15.00 kDa, myxovirus-resistance proteins 1 and 2, and 2′-5′-oligoadenylate synthetase 1 in cow peripheral blood leukocytes (PBLs).[16-23] In early pregnancy, 2′-5′-oligoadenylate synthetase 1 protein expression in buffaloes is a reliable marker of conceptus implantation.[24] The determination of interferon -stimulated genes in circulating immune cells has previously been shown to be a potential pregnancy diagnostic test for beef,[22-25] dairy products,[19,26] cattle, there is still no method that has been shown to be both accurate and practical for detecting pregnancy before day 20.[18,25,27] After comparing these four methods as pregnancy predictors on the 20th day after timed AI in dairy cattle, the abundance of two genes (*interferon-stimulated protein 15 kDa* and *LGALS3BP*) was evaluated which was stimulated by the conceptus using samples collected from four cell types: Peripheral blood mononuclear cells , total blood leukocytes, cervical cells and total milk leukocytes.[28] They found that interferon-stimulated protein 15 kDa abundance using peripheral blood mononuclear cells is the best pregnancy predictor on day 20 post-TAI among the cell types evaluated. At the fetomaternal interface in the uteri of pregnant buffaloes collected from a abattoir, the pregnancy-associated proteins PAG-1 and PAG-2 were discovered as early markers for pregnancy and were found to be expressed from one to four months of pregnancy.[29] These results allow the diagnosis of pregnancy within 3 weeks of artificial insemination (AI), however, it have shown varying degrees of effectiveness after AI. The accuracy as a marker for early pregnancy in dairy cows was almost not true, although the accuracy was increased from 17 to 25 days.[16] In dairy cows, the level of myxovirus-resistance 2 mRNA in PBL of dairy heifers did not change significantly from 0 to 18 days after AI,[17] while the 2′-5′-oligoadenylate synthetase 1 mRNA level has been successfully used to assess the 18-day gestation of heifers, but it has not been used in dairy cows.[19] Also, the combination of interferon-stimulated gene expression in PBL and color Doppler ultrasound of the corpus luteum on 20 days after AI of cattle is a possible high-precision diagnosis of pregnancy.[22]

A study showed that the mRNA of Chemokine (C-C motif) ligand 8 (also known as MCP2) and (C-X-C motif) ligand 10 (also known as IP10) expression in bovine endometrium was higher on the 15th and 18th days of pregnancy than in the non-pregnant stage.[30] In addition, CCL8 and CXCL10 expression were increased after interferon tau stimulation in an *in vitro* endometrial culture system.[30] The mRNA expression of the *CCL8* and *CXCL10* genes was increased in PBLs from 14 to 18 days of pregnancy, whereas no significant changes were observed in cows with early embryonic mortality or late embryonic mortality,[19,31] because interferon tau stimulated the expression of CCL8 and CXCL10 in cultured PBLs and the rise in CCL8 and CXCL10 levels might be pregnancy-related. Furthermore, CCL16 and interferon tau stimulated the expression of CCL8 and CXCL10 in cultured PBLs, implying that chemokines such as CCL8, CXCL10, and CCL16 may play some role in maternal recognition.[31] The cytokine related gene expression in PBLs from early pregnant buffaloes has not been studied till date. Therefore, considering all the above points, we have studied the expression of CCL8 and CXCL10 in buffaloes PBLs on the 16th day after AI in pregnant and non-pregnant buffaloes.

Materials and Methods

Collection of buffalos' peripheral blood. Entire procedure was conducted according to the guidelines of IAEC committee registered as Registration No 2058/GO/Re/SL/19/CPCSEA dated 19.03.2019 meeting held at College of Veterinary and Animal Sciences, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut-250110, India. Murrah buffaloes from Livestock Research Farm Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut were used for the study. The animals were kept in a dry, clean and well-ventilated sheds and reared under strict management and proper hygienic conditions throughout the study. Prophylactic measures against cattle/bovine diseases were carried out as prescribed by the health calendar of the institute to ensure that the animals were in a healthy condition throughout the study. Blood samples were collected through sterile ethylenediaminetetraacetic acid coated vacutainers containing 0.50% (10.00 µL mL^{-1} of blood) anticoagulant from jugular vein of buffaloes on day zero and after post AI on the 16th day. Precautions were taken to minimize the effect of ribonuclease activity while transport to laboratory and subsequent processing. The blood samples were immediately processed for extraction of total RNA to prevent its degradation. The data were collected separately for the pregnant (n = 6) and non-pregnant (n = 6) Murrah buffaloes.

Isolation of total RNA using Trizol reagent. Extraction of total RNA from the blood samples was carried out using Trizol reagent (ThermoFisher Scientific, Waltham, USA). Briefly, 600 µL of blood was mixed with 900 µL of Trizol reagent and vortexes uniformly to completely dissociate the blood cells. Then 180 µL of chloroform (0.20 volumes Trizol Reagent) was added. Tubes were shaken vigorously for 15 sec and incubated on ice for 15 min and further, tubes were centrifuged at 12,000 *g* for 15 min at 4.00 ˚C. After centrifugation, the mixture was separated into 2 phases *i.e.,* first phase is aqueous phase which was transparent in color and second in interphase. The upper aqueous layer was removed carefully into the labeled tube without disturbing the interphase and 500 µL of isopropanol was added to each of these tubes and incubated for overnight at – 20.00 ˚C. Tubes were centrifuged again at 12,000 *g* for 10 min at 4.00 ˚C. A pellet was visible at this stage after centrifuge. The supernatant was removed. The pellet was washed with 1.00 mL of 75.00% ethanol and mixed by vortexing. Tubes were again centrifuged at 12,000 *g* for 10 min at 4.00 ˚C. Supernatant was poured off to waste and tubes were inverted on clean tissue towel for 10 min to let the ethanol evaporate. Depending on the size, the pellet was resuspended in 15.00 - 200 µL of diethyl pyrocarbonate-treated water and stored at – 80.00 ˚C.

Quality and quantity check of RNA. The total RNA was quantified and purity was checked by using the nanodrop spectrophotometer reading (ThermoFisher Scientific). One µL total RNA was used and absorbance at 260 and 280 nm wavelengths were recorded against nuclease free water as blank. The RNA samples showing the optical density 260: optical density 280 value more than 1.80 were considered to contain no protein and ratio of 2.00 - 2.20 was taken for further analysis.

Reverse transcription and synthesis of complimentary DNAs (cDNA). The first stand cDNA was synthesized using Promega First Strand cDNA Synthesis Kit (Promega, Madison, USA) according to manufacturer's instruction from the isolated total RNA. Reverse transcription was carried out in 20.00 µL reaction mixtures. Calculation was done using the concentration of total RNA from NanoDrop reading (ng μL^{-1}) to take 5.00 µL of total RNA for each reaction and dissolved in nuclease free water by 6.50 µL and add 1.00 µL of Random primer to make final volume 12.50 µL, then incubated at 70.00 ˚C for 5 min and after that snap chilled on ice for 5 min and mixture was added. Reaction mixture was mixed to RNA-primer complex and spin, followed by incubation at 70.00 ˚C for 5 min. Reaction was stopped by incubating for 5 min at 70.00 ˚C and finally at 4.00 ˚C forever. The resulting (cDNAs were used in quantitative RT-PCR (qRT-PCR) reactions. The cDNA was stored at – 20.00 ˚C for less than 1 week and – 80.00 ˚C for long term use.

Confirmation of cDNA with GAPDH primers. The integrity of the cDNA was checked by PCR with GAPDH primers. The amplification of 158 bp *GAPDH* gene fragment from the cDNA indicated that the cDNA was made from the RNA extracted from blood was of good quality.

Optimization of end point PCR. End point PCR conditions were optimized to amplify buffaloes *CCL8* and *CXCL10* gene sequences in thermo cycler. Factor specific primers were used for the amplification of genes. Primers were synthesized using Fast PCR (version 6.2.73) primer designing software. Details of primers have been given in Table 1. The annealing temperature was standardized by cDNA prepared from mRNA of the blood by PCR. The reaction was carried out at different annealing temperatures, primer concentrations, magnesium chloride concentration, template RNA and Taq polymerase. The optimum temperature of 60.00 ˚C for CCL8 and CXCL10 were found to be most suitable for annealing for respective primers and was used in subsequent PCRs. The above reactants were added to a nuclease free thin walled 0.20 mL microcentrifuge tube pre-chilled on ice. The contents were gently vortexed and then spun down to collect at the bottom of tube by brief centrifugation. The reaction was carried out in a thermal cycler using the cycling parameters that have been found optimum for amplification of gene fragments given in Table 1.

Real-time PCR. Quantitative real-time PCR was carried out using a stepOne™ real-time PCR from Applied Biosystems (Waltham, USA). The reaction was set up in a separate area from the nucleic acid preparation or PCR product analysis. Light exposure to the qPCR master mix was kept to a minimum. Pipetting was done with care to avoid creating bubbles, which would interfere with the instrument reading of fluorescence. For gene quantification, no template control was used to check for contamination in reaction components other than the cDNA. The absence of a detectable signal indicated that the samples were DNA-free. Only the real-time master mix and primers were used in the negative control. To set up the reaction,

Table 1. Gene transcripts, primer sequences and resulting fragment sizes (Quantitative reverse transcription polymerase chain reaction)

Targets	Sequence of nucleotides	Fragment size (bp)	Annealing temperature	Reference
CCL8	F: AACATGAAGGTCTCCGCTGG R: GCAGCAGGTGATTGGGGTAG	108	60.00 ˚C	BA01272031 BA01272032
CXCL10	F: CTCGAACACGGAAAGAGGCA R: TCCACGGACAATTAGGGCTT	117	60.00 ˚C	BA01272033 BA01272034
GAPDH	F: ACCCAGAAGACTGTGGATGG R: CAACAGACACGTTGGGAGTG	158	60.00 ˚C	BA01272039 BA01272040

optically clear caps were used. Three µL of cDNA was extracted. Following master mix (20.00 µL) was prepared by adding 10.00 µL Eva Green Mix, 0.50 µL each forward and reverse primer, 6.00 µL Nuclease Free Water and 3.00 µL template. Without gloves, no contact with the optical surface of the caps was made. Before beginning the cycling programme, the strips were centrifuged to spin down the solution to the bottom of the tubes and remove any possible bubbles. The GAPDH was chosen as a housekeeping gene. Three segmented qPCR amplification programme was used. The average cycle threshold (C_T) value obtained for *CCL8*, *CXCL10* and *GAPDH* gene was normalized so that the comparison could be made. The data obtained were subjected to comparative C_T method ($\Delta\Delta C_T$ method) for the analysis of the expression level of target (CCL8, CXCL10) and an endogenous control. The levels of these amplicons were compared to the calibrator sample. The $\Delta\Delta C_T$ and $n\Delta\Delta C_T$ value were calculated as follows:

$$\Delta\Delta C_T = C_{T\,Target} - C_{T\,Control}$$

$$n\Delta\Delta C_T = \Delta C_{T\,Test\,sample} - \Delta C_{T\,Calibrator\,sample}$$

Incorporation of $\Delta\Delta C_T$ value into the fold difference. The fold difference of the target gene transcript, relative to the calibrator was calculated by the $2^{\Delta\Delta Ct}$ value.[32]

Progesterone assay in early pregnant and non-pregnant buffalos. The blood collection was conducted between the months of February to March when the mean environmental temperature and relative humidity (17.00 - 23.00 ˚C and 78.00 - 91.00%, respectively) were ambient for buffaloes breeding at livestock farm, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, India. Twelve healthy buffaloes in breeding age were classified into two groups: Group 1 (n = 6): Animals in anestrus (non-pregnant) comprising the control group and animals inseminated as *per* heat detection and insemination schedule followed (pregnant) comprising the experimental group and group 2 (n = 6). Blood samples were collected from experimental group on days 14, 16, 18 and 20 post- AI and from the same days from control group using sterile clot activator vacutainers from jugular vein of buffaloes. Then serum was separated by centrifuging the clotted blood at 3,000 rpm for 15 min. Serum was separated and stored at – 20.00 ˚C in plastic bullets. Progesterone kits were used to estimate serum progesterone concentrations using the enzyme-linked immunosorbent assay technique (Xema Co., Moscow, Russia). The Xema Progesterone EIA is a solid phase immunoassay in 96 well plates that employs the competitive enzyme immunoassay principle. A standard curve was drawn using the optical density (y-axis) and calibrator concentration (x-axis), and the concentration of test samples was calculated using that by extrapolation curve. The concentrations of test samples were converted from nmol to ng using a conversion factor (1.00 nmol = 0.318 ng mL^{-1}). Progesterone levels of 2.50 or greater were used to determine pregnancy.

Statistical analysis. All the normalized data regarding was statistically analyzed using SPSS Software (version 22.0; IBM Corp., Armonk, USA) according to standard method of Snedecor and Cochran.[33] All the data records after the experiment was compiled in Microsoft Excel and analyzed by using descriptive statistics. Data were analyzed using an ANOVA model with repeated measures to determine the significance difference within the group and Student *t*-test to determine the significance difference between the groups. Significant difference was considered if $p < 0.05$.

Results

Twelve buffaloes had been taken in this study and serum progesterone was estimated on day zero, seven, 14 and 21. Based on progesterone estimation, six buffaloes having progesterone concentration ≥ 2.50 ng mL^{-1} were considered as pregnant and remaining six having ≤ 2.50 ng mL^{-1} were considered as non- pregnant. On rectal examination on day 45, the six buffaloes that were predicted pregnant found pregnant and other six buffaloes that were predicted non-pregnant on the basis of progesterone assay found non-pregnant.

The mean serum progesterone concentration in pregnant buffaloes on days zero, seven, 14, 21 after AI were 0.44 ± 0.06, 1.18 ± 0.37, 3.00 ± 0.46, 4.17 ± 1.22 ng mL^{-1}, respectively. However, the mean progesterone concentration in non-pregnant buffaloes on day zero, seven, 14, 21 after AI were 0.45 ± 0.05, 1.06 ± 0.20, 1.77 ± 0.53, 0.64 ± 0.25 ng mL^{-1}, respectively. The mean progesterone concentrations were significantly different between pregnant and non-pregnant groups on day 21 (Table 2). It was observed that there was no significant difference in progesterone concentration between days zero (0.44 ± 0.06) and seven (1.18 ± 0.37), between days seven (1.18 ± 0.37) and 14 (3.00 ± 0.46) in pregnant Murrah buffaloes. Significant difference ($p < 0.05$) in progesterone concentration was observed among days zero (0.44 ± 0.06) and 14 (4.17 ± 1.22) and 21 (4.17 ± 1.22). Significant difference ($p < 0.05$) was also observed in progesterone concentration (ng mL^{-1}) at day 21 post AI between pregnant and non-pregnant buffaloes.

The mean serum progesterone concentration of pregnant group was significantly increased from day zero to day seven (0.44 ± 0.06 *vs*. 1.18 ± 0.37) but they showed that there was no significant difference ($p > 0.05$). However, the mean serum progesterone concentration of pregnant group was significantly increased from day 14 to day 21 (3.00 ± 0.46 *vs*. 4.17 ± 1.22) within the pregnant Murrah buffaloes.

Table 2. Quantitative reverse transcription polymerase chain reaction (ng mL^{-1}; mean ± SE) among pregnant and non-pregnant buffaloes on day zero, seven, 14 and 21 post-inseminations.

Groups	Day 0	Day 7	Day 14	Day 21
Pregnant	0.44 ± 0.06^{A}	1.18 ± 0.37^{AB}	3.00 ± 0.46^{BC}	4.17 ± 1.22^{Cb}
Non-pregnant	0.45 ± 0.05	1.06 ± 0.20	1.77 ± 0.53	0.64 ± 0.25^{a}

ABC and ab Means bearing different superscripts differ significantly within the groups and between the groups, respectively ($p < 0.05$).

Amplification of CCL8 and CXCL10 and GAPDH transcript cDNA. The *CCL8* and *CXCL10* gene expression in pregnant and non- pregnant Murrah buffaloes was observed by amplifying cDNA with CCL8, CXCL10 specific primer and GAPDH primer. The amplicons with CCL8, CXCL10 and GAPDH, were present respectively. The PCR products were loaded in Agarose Gel Electrophoresis (2.50% gel) to confirm the size. The band in agarose gel indicated the *CCL8* and *CXCL10* gene expression in pregnant animals on the 16th day whereas corresponding band was absent in non-pregnant Murrah buffaloes. However, PCR product specific to *GAPDH* gene showed similar amplicon in both pregnant and non-pregnant Murrah buffaloes (Fig. 1).

Pregnancy diagnosis. Positive pregnancy status of inseminated animals in experimental group was confirmed at around 45 days post AI by performing *per*-rectal examination of the animals at Livestock Research Centre, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, India. Six out of 12 inseminated animals in experimental group were confirmed to be pregnant, by feeling amniotic membrane slip and fetal bumps. Other six animals were confirmed to be non-pregnant on account of return to estrous in next cycle (*i.e.*, within next 21 days). The selection of animals in the control group was done on the basis of anestrous. The animals which had not come in estrous since many cycles and were also confirmed to be non-pregnant, were assumed to be in anestrous and chosen as control group. Thus, real- time expression study was done only for six pregnant animals in the experimental group and six animals from the control group.

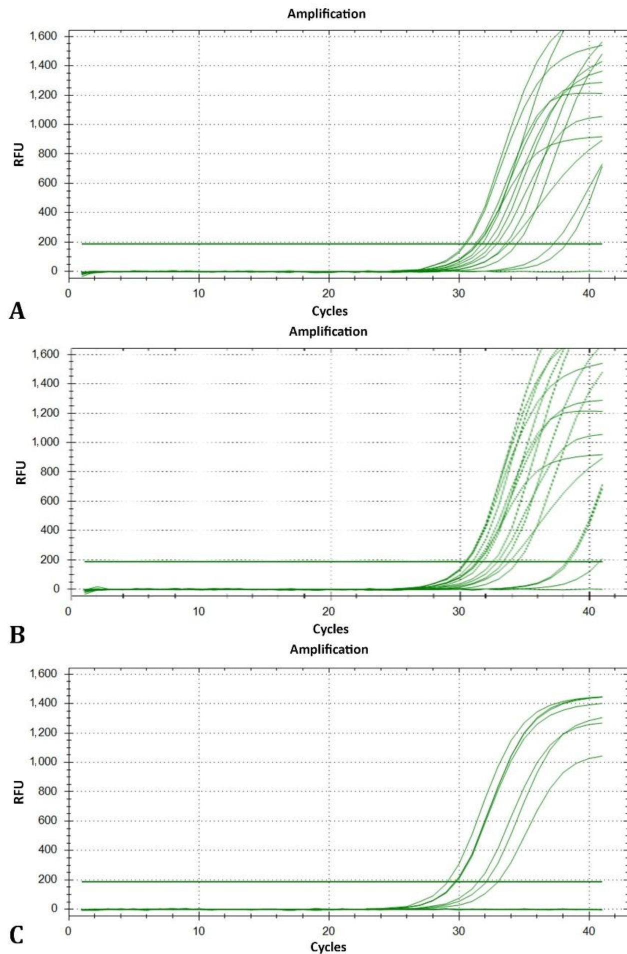

Fig. 1. Amplification plot of **A)** *GAPDH*, **B)** *CCL8* and **C)** *CXCL10* genes transcript in RT-PCR.

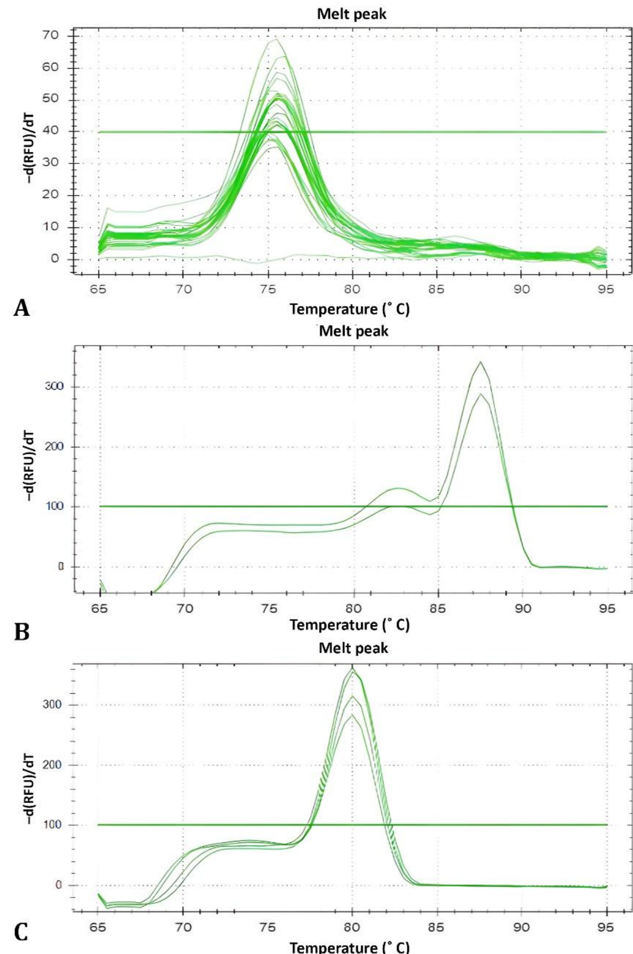

Fig. 2. Dissociation curve of **A)** *GAPDH*, **B)** *CCL8* and **C)** *CXCL10* gene transcript in RT-PCR.

Relative gene expression analysis of *CCL8* and *CXCL10* by real-time PCR. The $\Delta\Delta C_T$ method was a suitable method of real-time PCR to analyze the relative changes in gene expression of *CCL8* and *CXCL10* transcript in both pregnant and non-pregnant Murrah buffaloes. The *GAPDH* gene was used as an endogenous control (reference gene) during relative quantification. The amplification plots of all the three genes *i.e.*, (*CCL8*, *CXCL10* and *GAPDH*) were identically similar, confirming the equality in kinetics of real-time PCR of all the three genes involved (Fig. 1). The dissociation curves of the amplified products of target gene *CCL8*, *CXCL10* as well as endogenous control gene *GAPDH* showed the single peak confirming the specificity of the amplification (Fig. 2). The fold changes between both pregnant and non-pregnant Murrah buffaloes on day 16th were summarized and the expression level of *CCL8* and *CXCL10* gene in both pregnant and non-pregnant Murrah buffaloes in term of fold change is given in Figure 3.

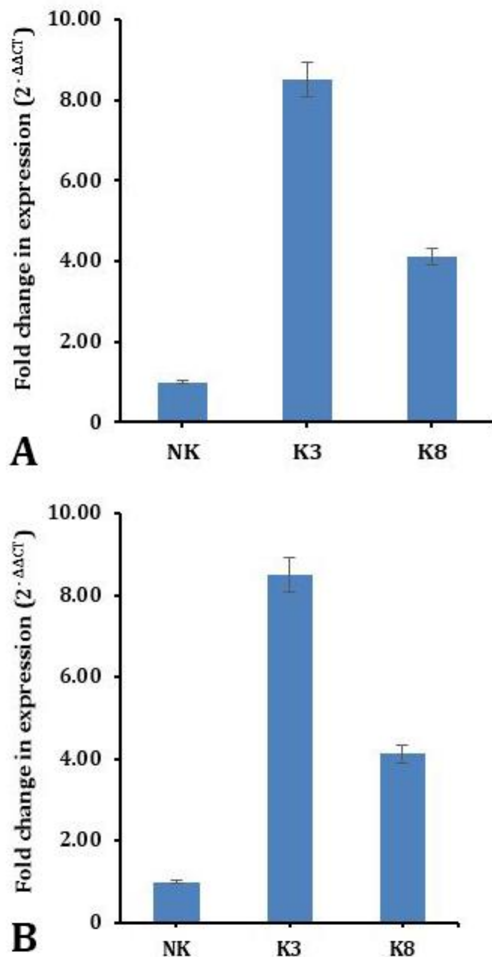

Fig. 3. Fold change in mRNA expression of **A)** *CCL8* and **B)** *CXCL10* genes on the 16th day. NK: Non-pregnant; K3 and K8: Pregnant buffaloes. Data are expressed as means ± SEM of six buffaloes and are expressed as relative ratios of the mRNAs to GAPDH at significance level of $p < 0.05$.

Discussion

The necessary procedures used to improve the reproductive effectiveness in cattle like cows and buffaloes include an early and accurate diagnosis. Pregnancy detection tests that are affordable, precisely up or down-regulated, least impacted by non-animal variables, present in readily accessible body fluids produced over a long period of time, and leave no remnant after pregnancy are the most important requirements.

The mean progesterone concentration was significant between pregnant and non-pregnant group on day 21. It was observed that there was no significant difference in progesterone concentration between days zero and seven, between days seven and 14 in pregnant Murrah buffaloes. However, significant difference ($p < 0.05$) in progesterone concentration was observed among days zero and 14 and 21. Significant difference ($p < 0.05$) was also observed in progesterone concentration at day 21 post AI between pregnant and non-pregnant buffaloes. Six buffaloes who were expected to be pregnant were discovered to be pregnant during rectal examination on day 45, whereas, the remaining six buffaloes that were anticipated to be non-pregnant based on progesterone assay results were found to be non-pregnant.

Present study was aimed for the identification of early pregnancy markers. This study was the first in buffaloes although similar studies have been done in cattle. Earlier study showed that the level of CCL8, CXCL10 transcript (mRNA) increased gradually from 14 to 18 day in pregnant cows compared to non-pregnant cows' post AI.[31] By considering the above views, the present study was conducted on *CCL8* and *CXCL10* gene expression for pregnancy diagnosis in Murrah buffaloes at day 16th of post AI. It was hypothesized that that CCL8 and CXCL10 mRNA level in blood was up-regulated in blood from pregnant Murrah buffaloes compared to non-pregnant Murrah buffaloes.

The product size of amplicons of *CCL8* and *CXCL10* gene on Agrose gel electrophoresis having 107 and 117 bp was obtained in pregnant Murrah buffaloes (K3, K8) on day 16th of post AI (Fig. 1). However, the amplification was absent in the non-pregnant murrah buffaloes (NK). Thus, the current study showed correlation between *CCL8*, *CXCL10* gene expression in pregnant Murrah buffaloes. Further, the increased level of mRNA gene expression was correlated with serum progesterone concentration in pregnant Murrah buffaloes. Previous study showed that the expression of *CCL8* and *CXCL10* genes was increased in PBLs from day 14 to day 18 during early pregnancy in cows.[31] However, the expression of both CCL8 and CXCL10 in PBLs was stimulated and might play a role in regulating maternal recognition of pregnancy in cows.

The present study demonstrated several folds increase in expression of *CCL8* and *CXCL10* gene in pregnant Murrah

buffaloes compared to non-pregnant Murrah buffaloes (NK) on day 16 post AI (Figs 3A and B). In pregnant Murrah buffaloes (K3, K8), the mRNA gene expression of CCL8 was found as 12.21 and 5.13-fold higher compared to non-pregnant Murrah buffaloes (Fig. 3A), whereas, the mRNA expression for K3 and K8 of CXCL10 was found as 22.17 and 4.19-fold higher, respectively, compared to non-pregnant Murrah buffaloes (Fig. 3B). Thus, the average mRNA expression level of *CCL8* and *CXCL10* gene was higher in pregnant Murrah buffaloes compared to non-pregnant Murrah buffaloes.

However, the present study exhibited several increases in *CCL8* gene expression and *CXCL10* showed many fold increase in *CXCL10* gene expression on day 16 post AI in Murrah buffaloes. Previous studies also revealed that semi-quantitative RT-PCR and qPCR showed higher level of expression of CCL8 and CXCL10 transcript during 18th days post AI in blood of pregnant cows.[31,34.]

On the basis of relative expression analysis, it could be concluded that the expression of *CCL8* and *CXCL10* gene was significantly higher in pregnant Murrah buffaloes 16 days after A.I. compared to non-pregnant buffaloes. Therefore, *CCL8* and *CXCL10* gene transcripts on the 16th day might be considered as early pregnancy marker in Murrah buffaloes.

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Conflict of interest

The authors declare no conflicts of interest.

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