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Differential cytokine expression profile in peripheral blood mononuclear cells of endometritic buffaloes

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ABSTRACT

The aim of present study was to elucidate the relative mRNA expression of certain cytokines, viz. interleukin-1ß (IL-1ß), IL-8, tumor necrosis factor alpha (TNFα) and IL-4 in peripheral blood mononuclear cells (PBMCs) of endometritic buffaloes. Out of 15 buffaloes, 9 buffaloes were diagnosed positive for endometritis whereas 6 buffaloes having no symptoms of endometritis were considered as healthy control. The blood samples were collected from each animal during diagnosis of endometritis and PBMCs were separated from collected blood samples by density gradient centrifugation. Total RNA was extracted from the cultured PBMCs isolates, reverse transcribed to synthesize the cDNA and amplified in real time PCR system. The relative fold of expression was estimated using $2^{-\Delta\Delta Ct}$ method. An up-regulation in all 3 pro-inflammatory cytokines (IL-1ß, IL-8 and TNFα) mRNA, ranging from 1.92 to 3.27 fold was observed in buffaloes with endometritis as compared to healthy. However, no alteration was detected in anti-inflammatory cytokine, IL-4 expression. Thus, it is concluded that certain cytokines expressed differentially in PBMCs in buffaloes with endometritis, that is first ever information in this species. Nevertheless, further research in respect to threshold level of each target cytokine in peripheral circulation involving more number of animals is required to establish these potential marker gene(s) for diagnosis of endometritis and for monitoring of new therapeutic approaches.

Key words: Buffalo, Cytokines, Endometritis, Peripheral blood mononuclear cells, Real-time PCR

Endometritis is one of the most commonly occurring uterine affections in field and farm conditions in dairy animals. The annual incidence of uterine infections in postpartum animals ranges from 20–75% in dairy buffaloes (Usmani *et al.* 2001). In India, the incidence of endometritis in buffaloes reportedly varied from 4.5 to 25% in an abattoir study and 2.4 to 20% in clinical surveys (Narasimha Rao and Sreemannarayana 1982). It prolongs the ovarian rebound, delays the rate of uterine involution, precludes fertilization, prevent successful implantation, and causes infertility at the time of infection but also results in sub-fertility even after successful clinical resolution of the disease (Semambo *et al.* 1991). The exact cause of this disorder is difficult to ascertain therefore, it is necessary to assess the risk factors for establishment of endometritis as early as possible and there must be a link to provide a method of early diagnosis and prophylaxis. Diagnosis of endometritis by rectal palpation and fortuitous observation of a vaginal discharge, if present in adequate amount is probably the basis for its treatment in the field (Lewis 1997).

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However, vaginoscopic examination (Barlund *et al.* 2008), endometritis clinical score (Williams *et al.* 2005), white side test (Sarkar *et al.* 2006), ultrasonography (Barlund *et al.* 2008), uterine cytology (Kashimanikam *et al.* 2005) and endometrial biopsy (Gilbert *et al.* 2005) have been practiced for pin-point diagnosis of endometritis in cattle under field and farm condition with a variable success. There is no cow side test available till date to diagnose the sub-clinical endometritis in buffaloes.

Uterine patho-physiology was studied using molecular techniques to elucidate certain pro-inflammatory cytokine gene (interleukin 1ß, IL-6, IL-8, TNF-α etc.) expression profile at endometrial tissues of cows and buffaloes positive for endometritis (Chapwanya *et al.* 2009, Fischer *et al.* 2010, Galvao *et al.* 2011, Loyi *et al.* 2013, Patra *et al.* 2014). The differential expression at endometrium level of certain cytokines mRNA was observed in animals positive for endometritis during different stages of postpartum. Thus, expression profiling of certain cytokine genes might have diagnostic importance for determining the severity of uterine inflammation and would serve as a prognostic indicator for future reproductive health. However, collecting tissue samples through uterine biopsy in live animals is challenging enough in field condition as repeated sampling at several sites of endometrium may impair fertility (Bonnett

et al. 1993). Therefore, exploration of alternative less invasive approaches, which could be well suited under field condition was utmost important.

MATERIALS AND METHODS

The present study was designed to explore the differential expression of certain cytokines (IL-1ß, IL-8, TNFα and IL-4) gene in peripheral blood mononuclear cells (PBMCs) of buffaloes with and without endometritis. Buffaloes (15) at second to fourth parities, from livestock farm of the Institute, were included of which, 9 buffaloes were diagnosed positive for endometritis based on presence of mucopurulent discharge on vaginal inspection (Williams *et al.* 2005), positive color reaction to white side test of cervico-vaginal mucus (Sarkar *et al.* 2006). Another 6 buffaloes having no symptoms of endometritis and negative reaction to white side test were considered as healthy control. Blood samples (10 ml) were collected from each animal immediately after diagnosis of endometritis.

The PBMCs were separated in collected blood samples by density gradient centrifugation using Lymphocyte Separation Media (LSM, density 1.077±0.001) and cultured in RPMI 1640 media in 6 well microplate at final cell concentration of 2×10^6 cells/ml. 1 µg *E. coli* LPS was added per ml of cell suspension and the cells were allowed to grow for 48 h at 37°C in CO_2 environment. After 48 h of incubation the cells were harvested by centrifugation in a DEPC treated micro-centrifuge tube at 3,000 rpm for 3 min.

Total RNA was extracted from the collected tissue samples using trizol reagent. The quantity and quality of total RNA was checked in a nanodrop spectrophotometer at 260 and 280 nm. The RNA samples with OD value ranging between 1.8 and 2.0 indicated presence of more than 90% total RNA with least protein and DNA contaminants and were considered for use in cDNA synthesis.

The extracted total RNA (2µg) was reverse-transcribed using RevertAid M-MuLV reverse transcriptase enzyme and oligo $(dT)_{18}$ primer to synthesize the complimentary DNA (cDNA) following manufacturers' instructions using a negative control. Both forward and reverse primers for target genes (IL-1ß, IL-8, TNFα and IL-4) including house-keeping gene (ß-actin) were designed using online primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) from the published complete or partial gene sequences available with the National Centre for Biotechnology information (Table 1). The primer sets of 20 base pairs were custom synthesized and procured from a commercial firm.

The PCR cyclic conditions of selected target genes and house-keeping gene (ß-actin) were standardized by using different concentration of magnesium chloride and annealing temperatures in a thermal cycler. The initial denaturation was done at 94°C for 5 min followed by 30 cycles of cyclic denaturation at 94°C for 30 sec, annealing at temperature specified as in Table 1, for 30 sec and extension at 72°C for 30 sec followed by final extension at 72°C for 5 min. The amplified PCR product was verified by 1.5% (w/v) agarose gel electrophoresis using known 100 bp ready to use DNA ladder (Fig. 1).

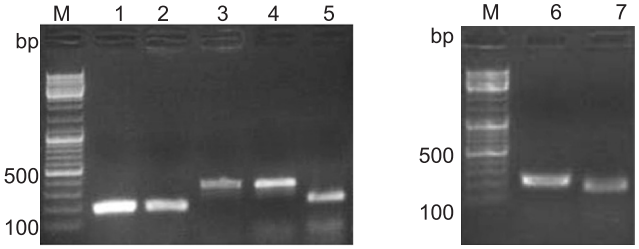

Fig. 1. Agarose gel (1.5 % w/v) electrophoresis of amplified PCR products in peripheral blood mononuclear cells of buffaloes. Lane M: 100 bp DNA ladder; lane 1–2: IL-8 (201 bp); Lane 3–4: TNFα (351 bp); lane 5: IL-1ß (205 bp); lane 6: ß-actin (227 bp); lane 7: IL-4 (197 bp).

The target genes (IL-1ß, IL-8, and TNF-α) and house-keeping gene (ß-actin) were amplified in real-time PCR system. The PCR master mix (2×) provided with the kit was used in real-time PCR. The reaction mixture was prepared as per the manufacturer specification and the reaction was run following standard cyclic condition used in real-time PCR. The threshold cycle value for each target and house-keeping genes was recorded for evaluation of

Table 1. List of primers used for amplification of cytokine genes in peripheral blood mononuclear cells of buffaloes with and without endometritis

Target gene	Primer sequence	ACC No.	Product size	Annealing temperature
ß-actin	F -5'-CGC ACC ACT GGC ATT GTC AT-3' R- 5'-TCC AAG GCG ACG TAG CAG AG-3'	K00662	227 bp	57°C
IL-1ß	F- 5'- ACC AGC TCT ACA ACA AAA GC- 3' R- 5'-TTG CAC TTT ACT GAC TGC AC- 3'	AY514903	205 bp	53°C
TNF-α	F- 5'-CAA GTA ACA AGC CGG TAG CC-3' R- 5'-TGG AAG ACC CCT CCC TGG TA-3'	EF424255.1	351 bp	61°C
IL-8	F 5'-CTG CAG TTC TGT CAA GGA TG -3' R 5'-CAA CCT TCT GCA CCC ACT TT -3'	AY952930	201 bp	57°C
IL-4	F- 5'-GTA CCA GTC ACT TCG TCC AT-3' R-5'-GCT CCT GTA GAT ACG CCT AA-3'	AY293620.1	197 bp	52°C

fold of expression. The dissociation curve for each amplified product of the target genes was analyzed to verify the specificity of the product and ruled out any false amplification due to primer-dimer.

The mean fold change (n-fold) for each cytokine gene was determined using the relative quantification method ($2^{-\Delta\Delta Ct}$) described by Livak and Schmittgen (2001). The difference in threshold cycle value (Ct) of target genes and house-keeping gene (ß-actin) for each sample was considered for calculation of fold expression and expressed as ΔCt. The ΔΔCt of target gene in endometritic buffaloes was calculated by deducting the average ΔCt of target gene of healthy animal (normalized calibrator) from the ΔCt of target gene of endometritic buffaloes. The fold of expression of target gene in endometritic buffaloes was finally estimated as $2^{-\Delta\Delta Ct}$.

Data obtained from this experiment were analyzed using SPSS-16.0. The Ct values of house-keeping gene (ß-actin) were analyzed by Shapiro-Wilk test for normal distribution. Independent t-test was used to test the significance of mean Ct values of ß-actin gene between healthy control and endometritis. The data related to fold expression ($2^{-\Delta\Delta Ct}$) of cytokines genes were not normally distributed, the statistical analysis was performed on the ΔΔCt values using independent t-test and then converted to $2^{-\Delta\Delta Ct}$ for data presentation. The difference of mean values for all data analyzed with $P<0.05$ was considered as significant, whereas $0.05<P<0.10$ was considered as tendency.

RESULTS AND DISCUSSION

The result revealed an average Ct value of ß-actin as 22.06±0.68 and 21.98±0.91, respectively, in endometritic and non-endometritic buffaloes. There was no significant ($P=0.945$) difference in expression that confirms its suitability as reference gene. All 3 target pro-inflammatory cytokine transcripts were expressed differentially in PBMCs of endometritic buffaloes and the fold changes are shown in Table 2. The expression level of IL-1ß was increased 2.15 fold ($P<0.05$) in endometritic buffaloes as compared to healthy control. Similarly, IL-8 expression was also higher (1.92 fold, $P<0.05$) in endometritis as compared to the normal (non endometritis) buffaloes. Interestingly, the expression of TNF-α was the highest (3.27 fold) in buffaloes with endometritis compared to other pro-inflammatory cytokines and the fold change was tended to be significant ($P<0.10$).

Table 2. Cytokine gene expression profiles in the peripheral blood mononuclear cells (PBMCs) of buffaloes with endometritis (n=9) relative to non-endometritis (n=6)

Cytokine gene	Fold change ($2^{-\Delta\Delta Ct}$)	±S.E.M	P value	Change in expression
IL-1ß	2.15*	0.44	0.03	Increase
IL-8	1.92*	0.35	0.03	Increase
TNF-α	3.27†	1.04	0.06	Increase
IL-4	0.77	0.18	0.24	Nonsignificant

SEM, Standard error of mean; * indicates significant difference ($P<0.05$); †indicates tendency towards statistical difference ($P<0.10$).

A significant up-regulation of IL-1ß, IL-8 and TNF-α mRNA in PBMC culture in buffaloes with endometritis relative to the healthy ones is well supported by the results of Galvao *et al.* (2012) who also found differential level of expression in IL-1ß, IL-6 and TNF-α in monocytes culture in postpartum cows that developed metritis. During endometritis IL-1ß is mainly produced by mononuclear phagocytes, T and B lymphocytes cells that are infiltrated into the endometrium and elicit the release of histamine and PGE2 that trigger vasodilatation and increase permeability (Roach *et al.* 2002). Interleukin-1ß further stimulates the production of IL-8 responsible for chemo-attraction of neutrophils and monocytes to clear the pathogen and enhances synthesis of several other cytokines and inducible nitric oxide synthase to potentiate the immune response. The IL-8 is produced by mononuclear phagocytes, antigen – activated T cells, endothelial and epithelial cells, and even neutrophils (Miller and Krangel 1992). The main inflammatory impact of IL-8 lies in its chemotactic effects on neutrophils and its ability to stimulate granulocyte activity by up-regulating cell surface adhesion molecule expressions, thereby mediates recruitment and activation of neutrophils in inflamed tissue (Warren 1990). The other major pro-inflammatory cytokine, TNF-α is produced by activated macrophages, monocytes, mast cells and some T and natural killer cells and exerts secondary inflammatory effects by stimulating IL-6 synthesis in several cells including mononuclear phagocytes, T cells and fibroblasts. Interleukin -6 then mediates its own effects and those of TNFα and IL-1ß in inducing acute phase response, and thereby perpetuating inflammatory response through a cascade of cytokines with overlapping properties (Warren 1990).

In our study the level of fold change of pro-inflammatory cytokines was relatively lower in PBMC culture than the level of expression observed in endometrium of buffaloes positive for endometritis (Patra *et al.* 2014). Sordillo *et al.* (1995) also suggested that PBMCs culture produces significantly lower TNFα than mammary lymphnode mononuclear cells in similar monocytes concentrations during the periparturient period. This indicated that the differential expression pattern may be due to variation in monocyte activation state with respect to peripheral circulation and tissue location (Elias *et al.* 1985).

Similarly, several other researchers also found a significant up-regulation of TNF-α level in plasma of cows that subsequently developed postpartum endometritis (Kim *et al.* 2005), and higher concentration of IL-1ß and TNF-α in lochia of women with postpartum endometritis than in healthy postpartum women (Sukhikh *et al.* 2005) and elevated IL-8 level in peritoneal fluid of women with endometriosis (Arici *et al.* 1998). Thus, the increased concentration of pro-inflammatory cytokines may have diagnostic importance and it reflects the severity and complication of uterine inflammation.

However, no significant change was observed in the IL-4 expression between endometritic and non-endometritic buffaloes (P>0.05, Table 2). It is difficult to explain that why the anti-inflammatory cytokine IL-4, which also potentiate humoral immune response was expressed similarly both in PBMC of buffaloes with and without endometritis. One probable reason might be that IL-4 is the cytokine, which is involved in chronic inflammation (Feghali and Wright 1997) and the animals used in our study may not have that kind of uterine infection/inflammation. Therefore, studies on a larger number of animals with endometritis need to be performed to confirm these results and to determine the threshold value of each cytokine gene expressed in PBMC that can be indicated to detect uterine infections as established for endometrial tissue.

It could be concluded from the results that the expression of IL-1ß, IL-8 and TNF-α transcripts increases in peripheral blood mononuclear cells of endometritic buffaloes and this is the first ever information in this species. Further, no change in IL-4 (anti-inflammatory cytokine) transcript expression was found in PBMC of buffaloes with or without endometritis; needs further investigation on a large number of animals.

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REFERENCES

Arici A, Seli E, Zeyneloglu H B, Senturk L M, Oral E. and Olive D L. 1998. Interleukin-8 induces proliferation of endometrial stromal cells: a potential autocrine growth factor. *Journal of Clinical Endocrinology and Metabolism* **83**: 1201–05.

Barlund C S, Carruthers T D, Waldner C L and Palmer C W. 2008. A comparison of diagnostic techniques for postpartum endometritis in dairy cattle. *Theriogenology* **69**: 714–23.

Bonnett B N, Martin S W and Meek A H. 1993. Associations of clinical findings, bacteriological and histological results of endometrial biopsy with reproductive performance of postpartum dairy cows. *Preventive Veterinary Medicine* **15**: 205–20.

Chapwanya A, Meade K G, Doherty M L, Callanan J J, Mee J F and O'Farrelly C. 2009. Histopathological and molecular evaluation of Holstein-Friesian cows postpartum: Toward an improved understanding of uterine innate immunity. *Theriogenology* **71**: 1396–407.

Elias J A, Schoursieiber A D, Gustilo K, Chen P, Rossman M D, Lammie P J and Daniele R P. 1985. Differential interleukin – 1 elaboration by unfractionated and denity fractionated human alveolar macrophages and blood monocytes relationship to cell maturity. *Journal of Immunology* **135**: 3198–202.

Feghali C A and Wright T M. 1997. Cytokines in acute and chronic inflammation. *Frontier in Bioscience* **2**: 12–26.

Fischer C, Drillich M, Odau S, Heuwieser W, Einspanier R and Gabler C. 2010. Selected proinflammatory factor transcripts in bovine endometrial epithelial cells are regulated during the oestrous cycle and elevated in case of sub-clinical or clinical endometritis. *Reproduction Fertility and Development* **22**: 818–29.

Galvão K N, Santos N R, Galvão J S and Gilbert R O. 2011. Association between endometritis and endometrial cytokine expression in postpartum Holstein cows. *Theriogenology* **76**: 290–99.

Galvão K N, Feilippe M J B, Brittin S B, Sper R, Fraga M, Galvao J S, Caixeta L, Guard C L, Ricci A and Gilbert R O. 2012. Evaluation of cytokine expression by blood monocytes of lactating Holstein cows with or without postpartum uterine disease. *Theriogenology* **77**: 356–72.

Gilbert R O, Shin Sang T, Guard C L, Hollis E N and Frajblat M. 2005. Prevalence of endometritis and its effects on reproductive performance of dairy cows. *Theriogenology* **64**: 1879–88.

Kasimanickam R, Duffield T F, Foster R A, Gartley C J, Leslie K E, Walton J S and Johnson W H. 2005. A comparison of the cytobrush and uterine lavage techniques to evaluate endometrial cytology in clinically normal postpartum dairy cows. *Canadian Veterinary Journal* **46**: 255–59.

Kim I H, Na K J and Yang M P. 2005. Immune response during the postpartum period in dairy cows with postpartum endometritis. *Journal of Reproduction and Development* **51**: 757–64.

Lewis G S. 1997. Uterine health and disorders. *Journal Dairy Science* **80**: 984–94.

Livak K J and Schmittgen T D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**:402–08.

Loyi T, Kumar H, Nandi S, Mathapati B S, Patra M K and Pattnaik B. 2013. Differential expression of pro-inflammatory cytokines in endometrial tissue of buffaloes with clinical and sub-clinical endometritis. *Research in Veterinary Science* **94**: 336–40.

Miller M D and Krangel M S. 1992. Biology and biochemistry of the chemokines, a family of chemotactic and inflammatory cytokines. *Critical Review on Immunology* **12**: 17–46.

Narasimha Rao A V and Sreemannarayana O. 1982. Clinical analysis of reproductive failure among female buffaloes under village management in Andhra Pradesh. *Theriogenology* **18**: 403.

Patra M K, Kumar H, Nandi S, Loyi T and Islam R. 2014. Upregulation of TLR-4 and proinflammatory cytokine transcripts as diagnostic indicator of endometritis in buffaloes. *Journal of Applied Animal Research* **42**: 256–62.

Roach D R, Bean A G, Demangel C, France M P, Briscoe H and Britton W J. 2002. TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. *Journal of Immunology* **168**: 4620–27.

Sarkar P, Kumar H, Rawat M, Varshney V P, Goswami T K, Yadav M C and Srivastava S K. 2006. Effect of administration of garlic extract and PGF2α on hormonal changes and recovery in endometritis cows. *Asian-Australasian Journal of Animal Science* **19**: 964–69.

Semambo D K, Ayliffe T R, Boyd J S and Taylor D J. 1991. Early abortion in cattle induced by experimental intrauterine infection with pure cultures of *Actinomyces pyogenes*. *Veterinary Record* **129**: 12–16.

Sordillo L M, Pighetti G M and Davis M R. 1995. Enhanced production of bovine tumor necrosis factor-alpha during the periparturient period. *Veterinary Immunology and Immunopathology* **49**: 263–70.

Sukhikh G T, Kasabulatov N M, Van'ko L V, Ordzhonikidze N V, Veryasov V N and Danelyan S Zh. 2005. Ratio between the

number of Th1 and Th2 lymphocytes in the peripheral blood and concentration of pro-inflammatory cytokines in lochia of women with postpartum endometritis. *Bulletin of Experimental Biology and Medicine* **140**: 622–24.

Usmani R H, Ahmad N, Shafiq P and Mirza M A. 2001. Effect of sub-clinical uterine infections on cervical and uterine involution, estrous activity and fertility in postpartum buffaloes. *Theriogenology* **55**: 563–71.

Warren J S. 1990. Interleukins and tumor necrosis factor in inflammation. *Critical Review of Clinical Laboratory Science* **28**: 37–59.

Williams E J, Fischer D P, England G C W, Dobson H, Pfeiffer D U and Sheldon I M. 2005. Clinical evaluation of postpartum vaginal mucus reflects uterine bacterial infection and the inflammatory response to endometritis in cattle. *Theriogenology* **63**: 102–17.