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).

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

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 1β, 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 2x10⁶ 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 CO₂ 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 (2 µg) 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 (β-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 (β-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).

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence</th>
<th>ACC No.</th>
<th>Product size</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>F-5’-CGC ACC ACT GGC ATT GTC AT-3’</td>
<td>K00662</td>
<td>227 bp</td>
<td>57°C</td>
</tr>
<tr>
<td></td>
<td>R-5’-TCC AAG GCG AGC TAG CAG AG-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1ß</td>
<td>F-5’-ACC AGC TCT ACA ACA AAA GC-3’</td>
<td>AY514903</td>
<td>205 bp</td>
<td>53°C</td>
</tr>
<tr>
<td></td>
<td>R-5’-TTG CAC TTT ACT GAC TGC AC-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>F-5’-CAA GTA ACA AGC CGG TAG CC-3’</td>
<td>EF424255.1</td>
<td>351 bp</td>
<td>61°C</td>
</tr>
<tr>
<td></td>
<td>R-5’-TGG AAG ACC CCT CCC TGG TA-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>F-5’-CTG CAG TCT TGT CAA GGA TG-3’</td>
<td>AY952930</td>
<td>201 bp</td>
<td>57°C</td>
</tr>
<tr>
<td></td>
<td>R 5’-CAA CCT TCT GCA CCC ACT TT-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>F-5’-GTA CCA GTC ACT TCG TCC AT-3’</td>
<td>AY293620.1</td>
<td>197 bp</td>
<td>52°C</td>
</tr>
<tr>
<td></td>
<td>R-5’-GCT CCT GTC GAT ACG CCT AA-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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^-∆∆Ct) described by Livak and Schmittgen (2001). The difference in threshold cycle value (ΔCt) of target genes and house-keeping gene (β-actin) for each sample was considered for calculation of fold expression and expressed as ΔCt. The ΔCt 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^-Δ∆Ct.

Data obtained from this experiment were analyzed using SPSS-16.0. The Ct values of house-keeping gene (β-actin) were analyzed by Shapiro-Wilk test for normal distribution. Independent t-test was used to test the significance of mean Ct values of β-actin gene between healthy control and endometritis. The data related to fold expression (2^-∆Ct) of cytokines genes were not normally distributed, the statistical analysis was performed on the ΔCt values using independent t-test and then converted to 2^-ΔCt 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 β-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-1β 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).

A significant up-regulation of IL-1β, 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-1β, IL-6 and TNF-α in monocytes culture in postpartum cows that developed metritis. During endometritis IL-1β 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-1β 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-1β 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-1β 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.

### 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)

<table>
<thead>
<tr>
<th>Cytokine gene</th>
<th>Fold change (2^-∆Ct)</th>
<th>±S.E.M</th>
<th>P value</th>
<th>Change in expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>2.15†</td>
<td>0.44</td>
<td>0.03</td>
<td>Increase</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.92†</td>
<td>0.35</td>
<td>0.03</td>
<td>Increase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>3.27†</td>
<td>1.04</td>
<td>0.06</td>
<td>Increase</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.77</td>
<td>0.18</td>
<td>0.24</td>
<td>Nonsignificant</td>
</tr>
</tbody>
</table>

SEM, Standard error of mean; † indicates significant difference (P<0.05); †indicates tendency towards statistical difference (P<0.10).
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 why the anti-inflammatory cytokine IL-4, which also potentiates 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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