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Differential expression of pro-inflammatory cytokines in endometrial tissue of buffaloes with clinical and sub-clinical endometritis ☆

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ABSTRACT

The objective of this study was to investigate the endometrial expression of pro-inflammatory cytokines (IL1β, IL6, IL8 and TNFα) along with TLR4 and CD14 in normal and endometritic buffaloes. The genitalia were collected in the abattoir and divided into three groups as normal (gr. I = 12), clinical endometritis (CE, gr. II = 12) based on positive color reaction to white side test of uterine discharge and sub-clinical endometritis (SCE, gr. III = 12) based on endometrial cytology (presence of ⩾5% PMNs) and histopathology. The equal numbers of genitalia were grouped into follicular and luteal stage in each group. Endometrial tissue scrapings were used for total RNA extraction and cDNA was transcribed and amplified by Real time PCR. The results showed several fold higher expression of all cytokine transcripts in CE (gr. II), whereas significant up-regulation of CD14 (1 to 2-fold), IL6 (15 to 36-fold), IL8 (8 to 14-fold) and TNFα (10 to 11-fold) mRNA was observed in SCE. This indicates that the evaluation of expression patterns of certain cytokines gene holds promise to diagnose the severity and degree of uterine inflammation.

The postpartum uterine contamination by a range of non-specific pathogen is usually eliminated in the process of involution, but approximately 30% cows are unable to resolve the inflammatory response to the pathogens, thereby developing endometritis (Herath et al., 2009) which may persist to cause subclinical endometritis (SCE) resulting in repeat breeding condition (Singh et al., 2008). Endometritis is also the most common uterine lesion observed in buffaloes slaughtered at abattoirs (Azawi et al., 2008). The infertility associated with endometritis is correlated to tissue damage and inflammation (Gilbert et al., 2005). Rectal examination (LeBlanc et al., 2002), vaginoscopic examination (Gilbert, 1992), endometritis clinical score (Williams et al., 2005), white side test (Sarkar et al., 2006) and uterine biopsy (Chapwanya et al., 2009) have been used for detection of clinical endometritis (CE) or SCE. Diagnosis of sub-clinical condition is based mainly on histological findings of endometrial tissue and number of polymorphonuclear cells (PMNs) in the uterine cytology. However, the percentage of PMN cells considered being diagnostic for SCE is variable (Kasimanickam et al., 2004). Further, evidence from uterine biopsy studies show that many of the cases of SCE are not described (Parkinson, 2009). Therefore, SCE is an emerging issue although less well characterized in dairy animals. These facts leave much to be explored in the area of diagnosis of sub-clinical uterine infection.

Since, lipopolysaccharide is known to be the most important virulent factors of coliform bacteria that is recognized by the complex TLR4–CD14 on cell membrane (Herath et al., 2006) and initiate signaling cascades for expression of several inflammatory mediators including cytokines (Dohmen et al., 2000) that mobilize and activate immune cells (Akira et al., 2006), which in the case of bovine uterine disease is particularly associated with the influx of PMNs and macrophages into the uterus (Zerbe et al., 2003). Therefore, cytokines are now recognized as principal component of the complex and balanced intracellular communication among cells in the uterus. Recently, the expression profile of inflammatory mediators (IL1β, IL6, IL8 and TNFα) in endometritic cows have been studied by few researchers in developed countries and they found its significant correlation with severity and persistence of uterine inflammation (Chapwanya et al., 2009; Fischer et al., 2010; Gabler et al., 2010; Galvão et al., 2011). Moreover, the information with respect to pro-inflammatory endometrial cytokines in buffalo is lacking. The aim of the present study was to combine conventional evaluation for CE and SCE with endometrial mRNA expression of

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pro-inflammatory cytokines, TLR-4 and CD14 to build an accurate picture for diagnosis of uterine inflammation in buffaloes.

Buffalo genitalia were obtained from the local abattoir, Bareilly, Uttar Pradesh in ice within half an hour of slaughtering the animal for further processing in the laboratory. The cyclical stage (follicular and luteal stages) of the genital organs was determined on the basis of gross ovarian morphology (Ireland et al., 1980). Six genitalia each of follicular and luteal phase were included in three different groups viz., normal (gr. I, $n = 12$), CE (gr. II, $n = 12$), and SCE (gr. III, $n = 12$). Genitalia were diagnosed for CE by presence of mucopurulent discharge followed by positive color reaction to white side test (Sarkar et al., 2006), SCE by endometrial cytology (Kasimanickam et al., 2004) and histopathology. Slight modification was made with respect to preparation for cytological slides for quantitative assessment of endometrial inflammation A simple sterilized brush was used to collect the sample directly from the dissected morbid genitalia and rolled on clean grease free glass slide and allowed to dry at room temperature followed by fixing in methanol for 10 min and then stained with Giemsa (Merck, India) for 45 min. A minimum of 200 cells were counted in 400× magnifications in each smear and identified as epithelial cells, PMNs and mononuclear cells. Samples with PMNs exceeding the 5% cut off were taken as SCE (Fischer et al., 2010). Further, a section of entire thickness of uterine wall was transferred to 10% formal saline for histological sectioning and staining. Formalin fixed uterine tissues were paraffin embedded, sectioned in 5–8 μm thickness and stained with haematoxylin and eosin by standard procedure. The stained slides were examined under 100× & 200× magnification of light microscope and graded as normal, CE and SCE tissue based on various microscopic lesions that describe endometritis (Azawi et al., 2008; Chapwanya et al., 2009).

The genitalia were washed with phosphate buffered saline and endometrial tissue scraping was collected from all the organs classified, at horn-body junction of the uterus in 500 μl RNA later (Fermentas Life Sciences, India) solution. Phosphate buffered saline (500 μl) was added to 50 mg of endometrial scraping in a microcentrifuge tube and centrifuged at 5000 rpm for 2 min. The supernatant was decanted and the endometrial cell pellet was used for RNA extraction. Total RNA was extracted by using TRIzol reagent. The total RNA extracted was quantified using a software ND 1000 V3.6.0 in a Nano-Drop 1000 spectrophotometer. The purity of extracted RNA was deduced from the ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}) measured by Nano-Drop, which was observed ranging in between 1.8 and 2.0.

A total quantity of 2 μg total RNA was treated with 2 μl of DNase I (Fermentas Life Sciences, India) prior to reverse transcription to remove any DNA contamination in extracted RNA. The reverse transcription was performed using M-MuLV Reverse Transcriptase (Promega, New Delhi, India). Briefly, 2 μg of total RNA was mixed with 2 μl of oligo$(dT)_{18}$ primer (Fermentas Life Sciences, India) in a 0.5 ml PCR tube and incubated at 72 °C for 5 min to remove the secondary structures within the template. Then the tubes were snap chilled on ice to prevent formation of secondary structures. This was followed by adding of 5 μl 5X RT-Buffer, 2 μl of dNTP (10 mM each), 25 Units of Ribonuclease inhibitor, 200 Units of RT enzyme and final volume was made to 25 μl with nuclease free water. The contents were mixed properly and incubated at 42 °C for 1 h in a water bath for reverse transcription to occur. The reaction was terminated by heating at 90 °C for 2 min to inactivate M-MuLV RT enzyme. The cDNA prepared was stored at −80 °C in small aliquots for further use. The primers (Table 1) for different gene expression were designed using sequence analysis Primer3 software freely available online at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, based on the sequence data available for cytokine gene in the NCBI nucleotide database. Primers were procured from Biolink, M/s Eurofins Genomics India Pvt. Ltd., Bangalore, India. The PCR cyclic condition was optimized for each target primer sets in conventional PCR using 10 pmol of each primer. The amplification of specific PCR products were confirmed on 2% agarose gel by electrophoresis (Fig. 1).

For a valid ΔΔCt calculation, the efficiency of the target gene and reference gene amplification must be approximately equal, such that the slope (m) value should be <0.1. Therefore, a 2-fold serial dilution of RNA was prepared according to guidelines of Applied Biosystems for Real-Time Quantitative PCR and seven dilutions were subjected to amplification in the ABI 7500 real time system. The ΔCt values vs. log input amount of RNA were plotted to draw a linear regression line: $y = mx + b$, where, $y = \Delta Ct$, m = slope, x = log10 input RNA and b = y intercept.

Real-Time PCR was performed with Quantitect SYBR Green PCR kit (Qiagen, India) in Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems). In short, 1 μl of cDNA template for each known sample was added to MicroAmp optical 96 well reaction plate in duplicates along with no template controls (NTC) to all wells containing 12.5 μl qPCR master mix (2X), 0.5 μl (10 pmol) of forward and reverse primer. The reaction plate was given a quick spin in a Hermle plate centrifuge. The plate was then placed in the Miniioptican apparatus on ABI PRISM 7500 SDS platform and subjected to the following thermal cycle conditions: Initial activation at 95 °C for 15 min (1 cycle) followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at temperature as shown in Table 1 for 30 s, extension at 72 °C for 30 s and for melt curve analysis: 1 cycle each of 95 °C for 15 s, 50 °C for 1 min, 95 °C for 30 s and 50 °C for 15 s. Data were acquired during the annealing step. The specific products obtained for each primer set was purified using Mini

Table 1
Primers used for real time-PCR amplification of endometrial cytokines in buffalo.

Name of primer	Sequence (5′-3′)	Annealing temp. (°C)	Product size (bp)
CD14	FP 5′-GCAGCCTGGAACAGTTTCTC-3′ RP 5′-CGGTTACTTCCAGGTCCTCA-3′	53	192
IL1β	FP 5′-ACCAGCTCTACAACAAAAGA-3′ RP 5′-TTGCACTTTACTGACTGCAC-3′	55	205
IL6	FP 5′-AGGCAGACTACTTCTGACCA-3′ RP 5′-TACTCCAGAAGACCAGCAGT-3′	52	232
IL8	FP 5′-CTGCAGTTCTGTCAAGGATG-3′ RP 5′-CAACCTTCTGCACCCACTTT-3′	57	201
TLR4	FP 5′-AGACGACACATTTCAGGGCC-3′ RP 5′-CCAGGTTGGGCAGGTTAGAA-3′	53	251
TNFα	FP 5′-CAGTCTCCTACCAGACCAAG-3′ RP 5′-CAGCATAGTCCAGGTAGTCC-3′	53	186
β-actin	FP 5′-GACATCAAGGAGAAGCTCTG-3′ RP 5′-TGGAATTGAAGGTAGTTTCG-3′	53	209