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Original Article

Changes in peripheral blood mononuclear cells' mRNA expression of *TLRs* and *CD14* during puerperal metritis in dairy cattle

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Abstract

Background: Peripheral blood mononuclear cells (PBMCs), commonly referred to as lymphocytes and monocytes, representing cells of the innate and adaptive immune systems. **Aims:** To find out whether changes in PBMCs' mRNA expression of pattern recognition receptors (PRRs) are associated with puerperal metritis in Holstein cows. **Methods:** Peripheral blood mononuclear cells were collected from 20 cows with puerperal metritis and 20 cows without metritis at 10 days postpartum. Expression of toll-like receptors 2 and 4 (*TLR2* and *TLR4*), and cluster of differentiation 14 (*CD14*) genes were assessed in PBMCs using a quantitative real time-polymerase chain reaction (qRT-PCR) technique. The data was normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a reference gene, and $2^{-\Delta\Delta Ct}$ methodology was used for relative quantification. **Results:** The results of the present study demonstrated that the expression of *TLR4* (P=0.04) and *CD14* (P=0.008) was significantly greater in cows with puerperal metritis compared to the control group. However, the expression of *TLR2* (P=0.06) was not significantly different between cows with puerperal metritis and healthy cows. **Conclusion:** This study suggests that puerperal metritis significantly increases the expression of *TLR4* and *CD14* genes in the PBMCs which contributes to the proper stimulation of inflammation and uterine clearance of bacteria soon after calving.

Key words: Cattle, CD14, Metritis, Toll-like receptors

Introduction

Metritis is a deep inflammation of the uterine wall with symptoms of a systemic disease. It can occur up to 21 days postpartum but often strikes in less than the first 10 days (Sheldon *et al*., 2006). The disease can affect huge numbers of herds and cause production losses with a negative influence on reproduction and survival (Mahnani *et al*., 2015; Cunha *et al*., 2018; Ahmadi *et al*., 2019). In several studies, the prevalence rate of clinical and puerperal metritis have been reported to be 36-50% and 18-21%, respectively (Reppert, 2015). As uterine diseases can affect large numbers of herds and severe economic loss, studying the molecular mechanisms of the immune response of the uterus is essential for the management of postpartum uterine infections in dairy cows (Herath *et al*., 2006a).

Infection is known to incite inflammation when microbial pathogens are recognized by the host's native immune system (Takeuchi and Akira, 2010). Innate immune responses may be stimulated by various forms of pattern-recognition receptors (*PRRs*), the most common of which are toll-like receptors (*TLRs*). Toll-like receptors such as *TLR2* are the best types of PRRs, and play a crucial role in innate and adaptive immunity while acting as potential structures to identify the bacterial infections caused by gram bacteria (Yapan *et al*., 2014). An extraordinary feature of *TLR2* and *TLR4* is their ability to collaborate with cluster of differentiation 14 (*CD14*) on the host cell surface to identify lipopolysaccharides (LPS) Gram-negative bacterial infection (O'Neill *et al*., 2009). These immune agents identify Gram-negative bacteria through the LPS or lipid A of their cell walls and stimulate the inflammation process (Brubaker *et al*., 2015). Cytokines such as tumor necrosis factor-alpha (TNF-α), also lead to the migration of immune cells to the uterus and contribute to the cleaning of infections (Sheldon and Dobson, 2004).

Studies have indicated that endotoxins that are able to translocate from the uterus into the circulation increase pro-inflammatory cytokines and cause a systemic inflammatory response. Increases in the levels of LPS in the peripheral plasma causes inflammatory responses in the uterus and expression genes of pro-inflammatory cytokines (Bilal *et al*., 2016; Emily *et al*., 2016). Studies clearly revealed that several immune genes secreted into the uterine lumen enter the uterine vein and directly upregulate the expression of immune genes in both uterus and peripheral blood cells (van der Molen *et al*., 2014; Bilal *et al*., 2016; Emily *et al*., 2016). The

upregulation of the immune gene expression in peripheral blood is a potential target for original approaches of the early diagnosis of uterine infections. The objective of this study was to identify changes in some immune gene expressions in peripheral blood mononuclear cells (PBMCs) of metritis cows, in order to identify novel disease biomarkers, and to help clarify the functional role of immunity and the inflammatory process of this infection.

Materials and Methods

Animals

A total of 40 lactating Holstein cows were selected from industrial dairy cows at the Azarnegin farm in Tabriz, East Azerbaijan province, North West of Iran. The studied cows included two groups for sampling:

1) Cows with puerperal metritis

2) Clinically healthy cows that were free of any uterine infections

In this study, 20 registered multiparous Holstein cows with metritis and 20 healthy registered multiparous Holstein cows without any uterine infections were examined. The healthy cows were those that calved normally without peripartum diseases (dystocia, retained placenta, metritis, clinical hypocalcaemia, mastitis, ketosis, and displacement of abomasum) or other diseases. All cows were kept in an open shed system, milked three times a day. The first service was performed 55 days postpartum. The body condition scores of all animals were ≥2. All cows were kept on the same environmental and management conditions.

Evaluation of puerperal metritis

The cows' reproductive system was examined within 10 days postpartum to diagnose possible metritis. Uterine infection was determined as described by Sheldon *et al*. (2006). All animals that showed both defined local and systemic signs were identified as puerperal metritis. The infected cows exhibited systemic signs of illness including fever, loss of appetite, dullness and markedly reduced milk yield with a large volume of fetid, reddish fluid in their uterus within a few days of calving.

After clinical evaluation, samples were aseptically obtained from uterine contents, sent to the laboratory, and cultured for aerobic bacteria. Samples were collected by introducing sterile pipettes into the uterus. Sterile syringes were attached to the end of these pipettes. To isolate aerobic bacteria, the samples were then transferred to a tube containing Stuart medium (Koneman *et al*., 1971).

PBMCs separation

From all cows (n= 40), 4 ml blood was taken (at 10 days after calving) from the tail vein into tubes containing ethylenediaminetetraacetic acid (EDTA) anticoagulant. The samples were kept on ice and sent to the laboratory. Initially, the temperature of the solutions was set at 37°C in the incubator; the blood sample and phosphate buffered saline (PBS) were mixed with a ratio of 1:1 to a total volume of 4 ml and then layered on top of Lymphodex (Inno-train Diagnostic GMBH, Germany) as a density gradient for the isolation of PBMCs. The tube was centrifuged at room temperature at 300 g for 40 min. The PBMCs layer was then harvested and washed two times with PBS for 5 min at 300 g. After cell counting by hemocytometer, 5×10^6 PBMCs cells were mixed with 200 µL of Trizol solution and stored at -80°C for the next step, which was RNA extraction.

RNA extraction and cDNA synthesis

RNA extraction was performed using Accuzol solution from BIONEER (Bioneer Company, S. Korea) according to the company's recommendations. After extraction, RNA was treated with 1 unit of DNase (Yekta Tajhiz, Iran). The quality and quantity of the extracted RNA samples were then assessed using Agilent 2200 Tapestation system (Agilent Technologies, Santa Clara, CA, USA). For cDNA synthesis, 5 µL of the extracted RNA, 1 µL of Random Hexamer Primer and 13.4 µL of diethylpyrocarbonate (DEPC) water solution were mixed and centrifuged shortly (1613 g for 30 s). The mixture was placed in the incubator for 5 min, at 70°C and placed on ice later to be cooled. Four µL of 5X buffer, 1 µL of the first-strand buffer, 0.5 µL of deoxyribonucleotide triphosphate (dNTP), RNasein (40 µ/µL) and 1 µL moloney murine leukemia virus (MMLV) were added to the solution and centrifuged. The tubes were transferred into the incubator at 37°C for 60 min. In order to produce the second strand of cDNA, the product was transferred to an incubator at 70°C for 5 min. The production of cDNA was carried out using a commercial cDNA synthesis kit (YTA Company, Iran) according to the manufacturer's guidelines.

Real time-polymerase chain reaction (RT-PCR)

The level of *mRNA* gene expression of *TLR4*, *TLR2*, and *CD14* genes was determined by quantitative real time-polymerase chain reaction (qRT-PCR) test using the SYBR Green I in a Rotogen 6000 system (QIAGEN, Germany). Polymerase chain reaction were performed in 20 µL reaction mixture containing 1 µL cDNA, 10 µL Master mix 1x (SYBR Premix Ex Taq (2x) (YTA Company, Iran), 0.5 µL of PCR forward and reverse primers and 8 µL of dH_2O (sterile distilled water). The primers (listed in Table 1 below) were designed for the target genes using Allele ID software (version 7.5).

The PCR thermal cycling consisted of one cycle of initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing 56°C for 2 min and cyclic extension at 60°C for 1 min. All trials were analyzed in duplicate and the average values were used for quantification. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a reference gene, and the $2^{-\Delta\Delta Ct}$ methodology was used for relative quantification.

Statistical analysis

Data achieved in this study were analyzed using the statistical software SPSS-21 (SPSS Corporation, USA).

Table 1: Characteristics of primers used for RT-PCR

Gene	Gene name	Primer	Nucleotide sequence (5′ → 3′)	GeneBank Accession No.
CD14	Cluster of differentiation 14	Forward Reverse	AGCCCTCCAGCACCAAAATGA CAGAAGGCTGGTTGGTTGAG	NM_174008.1
TLR2	Toll-like receptors 2	Forward Reverse	CATGGGTCTGGGCTGTCATC TCACACACCTCTGCAGGTCTC	NM_174197.2
TLR4	Toll-like receptors 4	Forward Reverse	CAAAAAGTATGGCAGGGGCGA CCTGAGACAAGATGGTGAAGGT	NM_174198.6
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Forward Reverse	GCTTTTGTGGAAACCCTCCTG ATGGGTGGAATCATACTGGAAC	NM_001190390

RT-PCR: Real time-polymerase chain reaction

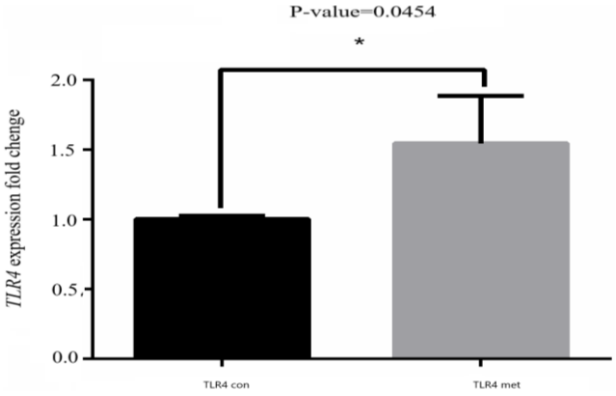

Fig. 1: Expression levels of toll-like receptors 4 (*TLR4*) (mean±SE) in cows with metritis (TLR4 met) and healthy cows (TLR4 con). * P<0.05

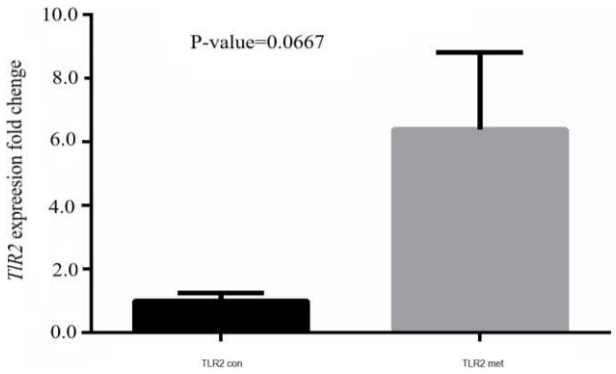

Fig. 2: Expression levels of toll-like receptors 2 (*TLR2*) (mean±SE) in cows with metritis (TLR2 met) and healthy cows (TLR2 con)

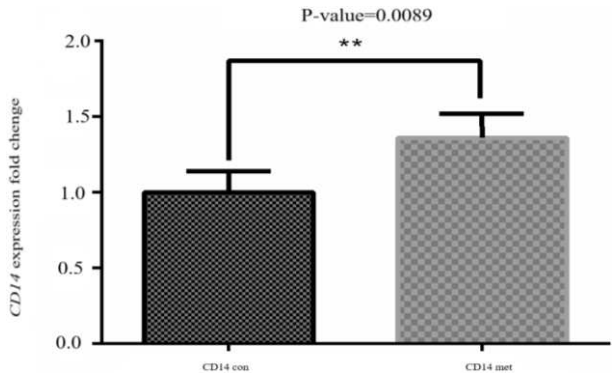

Fig. 3: Expression levels of cluster of differentiation 14 (*CD14*) (mean±SE) in cows with metritis (CD14 met) and healthy cows (CD14 con). ** P<0.01

An independent t-test was used to assess the difference of the mean cycle threshold (Ct) values of the *GAPDH* gene between healthy control, and puerperal metritis groups. The data obtained from RT-PCR for all target genes is presented as their relative fold change (n-fold).

Results

Bacterial isolation

Arcanobacter pyogenes, *Escherichia coli*, and non-differentiated *streptococci*, *staphylococci*, and *bacilli* were the most commonly isolated bacteria in both groups. However, *A. pyogenes* and *E. coli* bacteria were mostly isolated from the dairy cattle with puerperal metritis. The prevalence of pathogens associated with acute puerperal metritis is shown in Table 2.

Table 2: Prevalence of pathogens associated with acute puerperal metritis

Bacteria identified	Isolated from cows with metritis (n=20 samples)	Isolated from cows without metritis (n=20 samples)
Streptococcus sp.	4 (20%)	3 (15%)
Bacillus	1 (5%)	1 (5%)
Escherichia coli	6 (30%)	3 (15%)
Staphylococcus aureus	4 (20%)	2 (10%)
Arcanobacterium pyogenes	5 (25%)	2 (5%)
No growth	0 (0%)	9 (45%)

Gene expression

The results of our study revealed that *TLR4* gene expression was significantly greater in cows with puerperal metritis compared to the healthy control cows (P=0.04) (Fig. 1). *TLR2* gene expression for cows with metritis was higher than healthy control cows in relative terms, but this increase was not significant (P>0.05) (Fig. 2). *CD14* gene expression in cows with puerperal metritis was significantly higher than that of the healthy cows, (P=0.008) (Fig. 3).

Discussion

Neutrophil function is reduced close to the time of parturition in high-producing dairy cattle (Lange *et al*., 2016), especially those with uterine diseases (Cai *et al*., 1994; KIM *et al*., 2005). Recent studies show the role of uterine endometrium in inflammatory response and its modulation in cattle uterine infection (Fischer *et al*., 2010). Previous studies have also indicated that

endotoxins are able to translocate from the uterine into the systemic circulation. As a result, systemic immune responses might begin against endotoxins, which are the product of pro-inflammatory cytokines, and cause the expression of the genes associated with inflammation in the PBMCs (Bilal *et al*., 2016; Emily *et al*., 2016). In the present study, to determine the differences between the inflammatory processes of puerperal metritis and healthy cows, PBMCs gene expression was compared between infected and control cows. Highlighting the relation between uterine innate immune statues and postpartum uterine infections is vital for a good understanding of the susceptibility to or resistance against post-partum uterine infections.

Toll-like receptor 2 is critical for the recognition of Gram-positive bacteria, including anaerobes which are clinically relevant in cases of uterine inflammation (Takeuchi *et al*., 2000; Yoshimura *et al*., 2000). Toll-like receptor 2 ligands recognize pathogen-associated molecular patterns (PAMPs) from Gram-positive bacteria, as well as lipoteichoic acid, lipopeptides, and peptidoglycans (Zähringer *et al*., 2008). Surprisingly, we did not observe any differences in the *TLR2* gene expression following uterine infection with LPS and other bacteria. These findings are in agreement with (Martins *et al*., 2011), who stated that the levels of *TLR2* transcription did not differ between uterine biopsies of cows with uterine infection and healthy cows. Schöniger *et al*. (2017) demonstrated that there was no relationship between mare endometritis and the expression of *TLR2*. The reason for this inconsistency may rely on the fact that *TLR2* is important for identifying Gram-positive bacteria, while Gram-negative bacteria are involved in the establishment of metritis. Therefore, lower *TLR2* by 10 days postpartum could further compromise the leukocyte reply to bacteria attacking the uterus and lead to deficiency of recognition, hence the clearance of the bacteria.

Toll-like receptor 4 recognizes Gram-negative bacteria (Herath *et al*., 2006b; Herath *et al*., 2009; Sheldon *et al*., 2010), such as *E. coli*, which is one of the most-commonly found bacteria in the uterus environment during the first weeks postpartum (Sheldon *et al*., 2002; Williams *et al*., 2005). Considering the fact that Gram-negative bacteria are often associated with acute puerperal metritis in cows, (Sheldon *et al*., 2010) this positive correlation can support the idea that escalated levels of *TLR4* mRNA can be related to an over responsiveness to uterine bacterial colonization at early stages of the postpartum period. In the current study, the *TLR4* expression for the cows with puerperal metritis increased in comparison with healthy cows. The results of this study are in agreement with Herath *et al*. (2009), demonstrating that persistent infections of the uterus are associated with elevated *TLR4*, *CD14*, and myeloid differentiation factor-2 (*MD-2)* expressions at time points during the first-week postpartum. *In vitro* research showed that bovine endometrial cells exposed to LPS, escalate in the expression of *TLR4* and *CD14* as well as the induction of the expression of chemokines and cytokines approximately 24 h after the revelation (Swangchan *et al*., 2012). In another study, Patra *et al*. (2014) claimed that *TLR4* expression was higher in buffaloes with endometritis compared to healthy buffaloes. They also demonstrated that the *TLR4* expression gene could be a distinguishing factor for the diagnosis of endometritis in buffaloes. Moreover (Davies *et al*., 2008), showed that *TLR4/MD2/CD14* complex (which is due to the presence of LPS of Gram-negative bacteria) plays a key role in the secretion of prostaglandin E2 (PGE2) from epithelial cells and increases the days open of cows with endometritis. Additionally, the co-receptors *CD14* and *MD-2* are needed for the recognition of LPS by *TLR4*. The interaction of LPS and *TLR4*/*CD14*/*MD-2* induces the expression of cytokines, antimicrobial peptides and chemokines and causes an inflammatory response. The lack of *CD14* results in a feeble interaction between LPS and the *TLR4–MD-2* complex (Triantafilou and Triantafilou, 2002; Miyake, 2006; Fu *et al*., 2013). Thus, once an infection in the uterus is proven, expression levels of *TLRs* in PBMCs could reflect a response rather than a prejudicing factor to the infection.

In the current study, the expression of *CD14* increased in PBMCs cows with puerperal metritis compared to the control cows. This report is in agreement with Herath *et al*. (2009) who reported that uterine infections are related to escalated expression levels of *CD14* and *MD-2* at the first week after calving. In another study, Loyi *et al*. (2015) reported that the expression of *CD14* increased significantly in subclinical endometritis cows compared with healthy cows. This consistency is likely due to the fact that *CD14* acts as a receptor for the *MD-2* complex which is activated in the presence of LPS during metritis (Takeuchi *et al*., 2000; Yoshimura *et al*., 2000). Nonetheless, the increased expression of genes linked to innate immunity or inflammation, when infection/inflammation is already established, does not provide evidence for the cause/effect relationships of these genes and the development of the infection/inflammation, because the up-regulation of these genes under the mentioned circumstances is probably a response to the infection rather than a predisposing factor.

Consequently, our findings of higher transcription levels of *TLR4* and *CD14* at 10 days postpartum, mostly in dairy cows with puerperal metritis, may be related to the pathogenesis of puerperal metritis even if a clear cause and effect relationship remains to be established. Our study suggests that the increased expression levels of *TLR4* and *CD14* genes in the PBMCs may contribute to a proper activation of the inflammation process and consequently, clear the bacteria from the uterus soon after calving.

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Conflict of interest

The authors state that they do not have any conflicts of interest.

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