RESEARCH ARTICLE
Open Access

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2023.54.2.450-456

Expression study of pro-inflammatory cytokines and acute phase proteins in post-partum crossbred dairy cows with subclinical endometritis#

Sowmya Murali[1], Leeba Chacko[2], Renjith Sebastian[5], K. Promod,[3] and C. P. Abdul Azeez[4]
Department of Animal Reproduction, Gynaecology and Obstetrics
College of Veterinary and Animal Sciences, Pookode, Wayanad -673576,
Kerala Veterinary and Animal Sciences University,
Kerala, India

Citation: Murali, S., Chacko, L., Sebastian, R., Promod, K. and Azeez, C. P. A. 2023. Expression study of pro-inflammatory cytokines and acute phase proteins in post-partum crossbred dairy cows with subclinical endometritis. *J. Vet. Anim. Sci.* **54**(2):450-456
DOI: https://doi.org/10.51966/jvas.2023.54.2.450-456

Received: 11.11.2022 *Accepted: 2.02.2023* *Published: 30.06.2023*

Abstract

Subclinical endometritis (SCE) is one of the causes of poor reproductive performance in dairy cows. The objective of the study was to evaluate the expression of pro-inflammatory cytokines [PICs; Interleukin -1 beta (IL-1β), Interleukin -6 (IL-6), and Tumor necrosis factor- alpha (TNF-α)] and Acute phase proteins [APPs; Serum Amyloid- A (SAA) and Haptoglobin (HP)] in blood and uterine flushings of postpartum dairy cows with subclinical endometritis (SCE). Animals (n=49) were screened for SCE at 60 days postpartum (dpp) using the endometrial cytobrush cytology, and those with PMN cell percentage ≥ 18% were identified as SCE cows. The blood samples and uterine flushings from SCE (n=15) and the control group (n=15) were assessed for mRNA expressions of PICs and APPs using the Real-Time PCR method (RT-PCR). The PICs and APPs except TNF-α were non-significant (p>0.05) in blood samples whereas a significant difference (p<0.05) was found in uterine flushings. The PICs and APPs were significantly up-regulated in the uterine endometrium indicating that collection of uterine flushings at 60 dpp could be used as a diagnostic method for evaluating gene expressions of inflammatory mediators in dairy cows with SCE.

Keywords: *Endometrial cytobrush, pro-inflammatory cytokines, acute phase proteins*

#Part of MVSc thesis submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala

1. *MVSc Scholar*
2. *Assistant Professor*
3. *Professor and Head*
4. *Associate Professor*
5. *Assistant Professor, Department of Veterinary Biochemistry*

**Corresponding author: leeba@kvasu.ac.in, Ph. 9447640189*

Post-partum uterine infection is one of the most frequent causes of infertility in high-producing dairy cattle, which lowers the viability and sustainability of dairy farming. According to reports, 80–100 per cent of cows in dairy herds under intensive management have postpartum bacterial contamination of the uterine environment. Even while the immune system normally eradicates the majority of harmful microorganisms, 15 to 40 per cent of postpartum cows are reported to experience chronic illness (Sheldon *et al*., 2008). Subclinical endometritis (SCE) is an endometrial inflammatory disease with an increasing number of polymorphonuclear cells in the uterus. It has also been hypothesized that the subsequent onset of SCE may be influenced by an imbalance of pro-inflammatory/anti-inflammatory cytokines in the first week post-partum (Sheldon *et al*., 2006).

Toll-like receptors (TLRs), which are most frequently found in endometrial cells and macrophages, identify the pathogen through pathogen-associated molecular patterns (PAMPs) and release PICs such as IL-1β, IL-6, and TNF-α (Herath *et al*., 2009). The synthesis of APPs from the liver or other tissues is stimulated by the release of PIC (mostly IL-1β, TNFα-and IL-6) at the site of injury. Acute phase proteins are non-specific innate immune components, which maintain biological process equilibrium and regulate microbial proliferation (Chan *et al*., 2010). In the present study, the expression of PICs (IL-1 β, IL-6, and TNF- α) and APPs (SAA and HP) in dairy cows with SCE was compared to control animals at 60 dpp.

Material and methods

Experimental animals

The present study was conducted at the Instructional Livestock Farm Complex (ILFC), College of Veterinary and Animal Sciences, Pookode and Livestock Research Station (LRS), Thiruvazhamkunnu. Crossbred dairy cows between 3 to 10 years of age were evaluated for the percentage of PMN cells to diagnose SCE at 60 dpp.

Diagnosis of subclinical endometritis

A total of 49 crossbred dairy cows without any post-partum complications were screened for the detection of SCE based on endometrial cytology. The cytobrush technique (Kasimanickam *et al*., 2005; Gayathri *et al*., 2020) was used for collecting endometrial samples from all postpartum dairy cows and a smear was prepared by rolling cytobrush over a clean glass slide. These slides were stained using modified Wright Giemsa and observed under 400 ×× magnification of a light microscope and 200 nucleated cells were counted to estimate the percent of PMN cells. A total of 49 animals were screened, 15 post-partum dairy cows with more than 18 percent PMN cells were selected as positive for SCE (Group I) and 34 animals with less than 18 percent PMN cells were selected as control (Group II).

Sample collection

Blood (2 mL) was collected through jugular venepuncture into EDTA vials (Ultimate, Thrissur), immediately placed in ice and transported to the laboratory within 1 h of collection. The uterine flushings were collected by intrauterine infusion using 50mL phosphate buffer saline and then the fluid was aspirated from the uterus by negative pressure using a 10 mL syringe. Approximately 2 mL fluid was recovered from the uterus, collected in a microcentrifuge tube, placed on ice, and transported to the laboratory within 1 h of collection.

Collection of pelleted cells

Blood samples were centrifuged at 2050 g for 10 min to separate the buffy coat layer into a microcentrifuge tube and RBC cell lysis buffer (Merck, US) 0.5mL was added, incubated for 5 min and centrifuged at 5000rpm for 6 min at room temperature. The supernatant was removed completely and the pelleted cells were mixed with 0.75-1 mL TRIzol ™ reagent (Thermo Fischer Scientific, Karnataka) and used for RNA isolation. The lavage collected was centrifuged at 5000 rpm for 6 min at room temperature. The pelleted cells were collected and mixed with 0.75-1mL of TRIzol reagent and used for RNA isolation.

RNA isolation and cDNA synthesis

The RNA was isolated from pelleted cells obtained from blood and uterine flushings using the TRIzol chloroform method. The isolated RNA was checked for its purity and quantity using a Nanodrop spectrophotometer (Thermo Scientific, USA). The quality of the RNA sample was assessed by electrophoresis using 1% agarose gel. It was then used for cDNA synthesis using a cDNA kit (Thermo Scientific, USA).

Quantitative Real-time PCR

The primers of genes for PIC except for *TNF-α* and APPs were designed using Primer quest and oligoanalyzer using the mRNA sequences downloaded from sequences available in NCBI Databases. Published primers sequences for amplifying *TNF-α* (Manimaran *et al*., 2019) were custom synthesized from Sigma Chemicals Co. (St. Louise, M.O., USA). The sequence of the primers and annealing temperature (Tm) used in the present investigation and the size of the PCR amplified products are detailed (Table 1).

The PfaffI technique was used to compute the fold change of the target genes between the control and SCE groups, and Actin-beta was used as the housekeeping gene for the expression analysis using the following formula:

ΔCT (of Target gene) = CT (target gene) in control group – CT (target gene) in treatment group

ΔCT (of Reference gene) = CT (Reference gene) in control group – CT (Reference gene) in treatment group

Where, CT: - Cycle threshold

Fold expression level of genes was calculated as follows:

$$\text{Expression Ratio} = \frac{E\ (\text{Target gene})^{\Delta CT}}{E\ (\text{Reference gene})^{\Delta CT}}$$

Statistical analysis

Prism 9 (GraphPad Software, Inc) was used to conduct an independent t test for the statistical analysis of gene expressions in RT-qPCR.

Results and discussion

Gene expressions of PICs and APPs vary according to the inflammatory conditions of the uterus. Average CT values are presented in Table 2. Average CT values of *β-actin* in blood were 26.58 and 25.10 in control and SCE group, respectively whereas in uterine washings it was 14.33 and 15.27, respectively (p= 0.4 and 0.3). There was no significant difference in the gene expression of *β-actin* noticed between control and SCE groups. The results showed the stability of *β-actin* expression in the present study in both blood and uterine washings and acknowledge the gene applicability as a reference (Fig.1).

Table 1. Primer sequences and product size

Primer name	Sequence	Tm	Size (bp)
BovIL6-FP	GCAATGAGAAAGGAGATATGTGAGAA	63.9	128
BovIL6-RP	TGATTGAACCCAGATTGGAAG	63.2	
BOV-IL-β-FP	ATGAACCGAGAAGTGGTGTTCTG	64.8	72
BOV-IL-1β-RP	CCACAGGAATCTTGTTGTCTCTTTC	64.5	
BOV-TNF-α-FP	CTCTTCTGCCTGCTGCACTTC	66.9	205
BOV-TNFα-RP	CCATGAGGGCATTGGCATACG	71.6	
BOV-SAA3-FP	CCACAGGATGAACCTTTCCAC	64	146
BOV-SAA3-RP	AGTTGGCTTCTTTCATGTCTTGGT	64.5	
BOV-HP-FP	CCTGAATGTGAAGAAGATGACAGC	64.5	73
BOV-HP-RP	GAACCAAGTACTCCACGTAGCCA	64.7	
BOV-ACTB-FP	GATGAGGCTCAGAGCAAGAGAG	63.0	205
BOV-ACTB-RP	ACATGATCTGGGTCATCTTCTCAC	63.8	

Table 2. Average Ct values of genes in treatment and control groups

Sample collected	Gene expression	Average Ct values of control group	Average Ct values of treatment group
Blood	*IL-1β*	24.56	25.85
	IL-6	29.69	30.12
	TNF-α	20.48	20.37
	SAA	32.22	32.22
	HP	18.79	20.31
Uterine washings	*IL-1β*	28.05	21.48
	IL-6	30.02	25.51
	TNF-α	27.32	29.05
	SAA	16.9	35.07
	HP	29.82	34.38

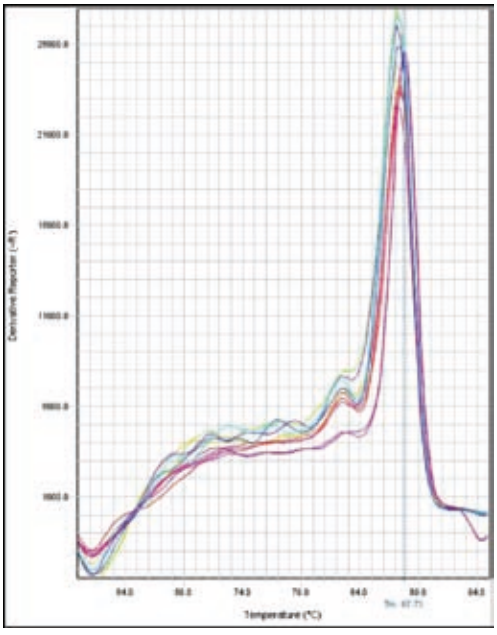

Fig. 1. Melt curve of β-actin in tested samples

In the present study the mean ± SE of Ct values of *IL-1β* in the blood and uterine washings were 1.02 ± 0.03 and 38.78 ± 1.74, respectively. The fold change noticed for *IL-1β* in the blood and uterine washings were 1.31 and 41.5 (Table 3). The endometrial mRNA expression of *IL-1β* was found to be significant in uterine washings ($p<0.05$) and it was non-significant in blood ($p>0.05$). Peter *et al*. (2015) observed *IL-1β* was highly expressed in SCE as 15-fold and 20-fold at 24-30 and 45-51 dpp in the cytobrush samples from the uterus. The higher fold change noticed in this study might be due to different cut off levels taken for PMN cells per cent. Most of the studies conducted adopted the per cent of PMN cells between five to ten at 60 dpp and hence, the cytokines expression might not be enhanced much as reported by Ghasemi *et al*. (2012) and Fagundes *et al*. (2019). Genes of cytokines were highly expressed in the case of PMN cells above a certain threshold (Gabler *et al*., 2010; Galvao *et al*., 2011; Ghasemi *et al*., 2012; Fagundes *et al*., 2019). According to Roach *et al*. (2002), *IL-1β* induced the production of *IL-8*, which attracted neutrophils and monocytes to the pathogen. The higher number of neutrophils found in the present study might be due to increased expression of *IL-1β*.

The mean ± SE of *IL-6* in blood and uterine washings were 0.94 ± 0.05 and 34.90 ± 1.01, respectively. The gene expression of *IL-6* was found to be significant in uterine washings ($p<0.05$) and it was non-significant in the blood ($p>0.05$). The fold change observed for *IL-6* were 34.54 and 0.99 in the uterine washings and blood, respectively (Table 3). The production of *IL-6* was stimulated by various factors like LPS, *IL-1β*, and *TNF-α*. In the present study, *IL-6* was up-regulated in the uterine washings might be due to the higher expression of *IL-1β*. The findings of this study were in harmony with Ghasemi *et al*. (2012) who perceived increased gene expression for *IL-6* (30-fold) in the endometrial tissue collected by cytobrush technique with a threshold of more than 18 per cent PMN cells in SCE.

In the present study, *TNF- α* was up-regulated in the blood and uterine washings of SCE cows ($p<0.05$), respectively. The mean ± SE of *TNF- α* expression in blood and uterine washings were 1.10 ± 0.14 and 6.63 ± 0.80

Table 3. Gene expression profiles of cytokines and acute phase proteins in the blood and uterine washings of SCE cows

Sample collected	Gene expression	Fold changes between control and SCE	± SEM	p value
Blood	*IL-1β*	1.31	0.033	0.522^{ns}
	IL-6	0.99	0.051	0.262^{ns}
	TNF-α	1.42	0.1483	0.025*
	SAA	0.93	0.093	0.902^{ns}
	HP	1.68	0.186	0.265^{ns}
Uterine washings	*IL-1β*	41.5	1.742	0.001***
	IL-6	34.54	1.008	0.001***
	TNF-α	7.52	0.806	0.001***
	SAA	1.24	0.096	0.03*
	HP	1.72	0.083	0.001***

*ns- non-significant * Significant at 0.05 level ***significant at 0.0001 level*

in SCE compared to control animals. The fold change observed for *TNF-α* in the blood and uterine washings were 1.42 and 7.52 (Table 3). The expression of *TNF- α* noticed by Ghasemi *et al*. (2012) was 20-fold in the uterine washings that was higher compared to the present study (7.52- fold), which may be due to changes in the sample collection method, post-partum days, and techniques used for studying gene expression. Endometrial epithelial cells, which are the main targets for bacteria (Herath *et al*., 2009), could be collected using cytobrush technique however uterine washings were employed in the present study to examine gene expression. These could be the cause of comparatively low expression of *TNF- α* compared to other cytokines found in this study.

SAA and *HP* expression change was non-significant in the blood ($p>0.05$) and it was found to be significant in the uterine washings ($p<0.05$). The mean ± SE of *SAA* and *HP* in the blood were 1.01 ± 0.93 and 1.22 ± 0.18, respectively. The expression of *SAA* and *HP* in the blood was 0.93 and 1.68 folds. The mean ± SE of *SAA* and *HP* in the uterine washings of SCE animals were 1.94 ± 0.08 and 1.23 ± 0.09, respectively. The fold change noticed for *SAA* and *HP* were 1.24 and 1.72 in the uterine

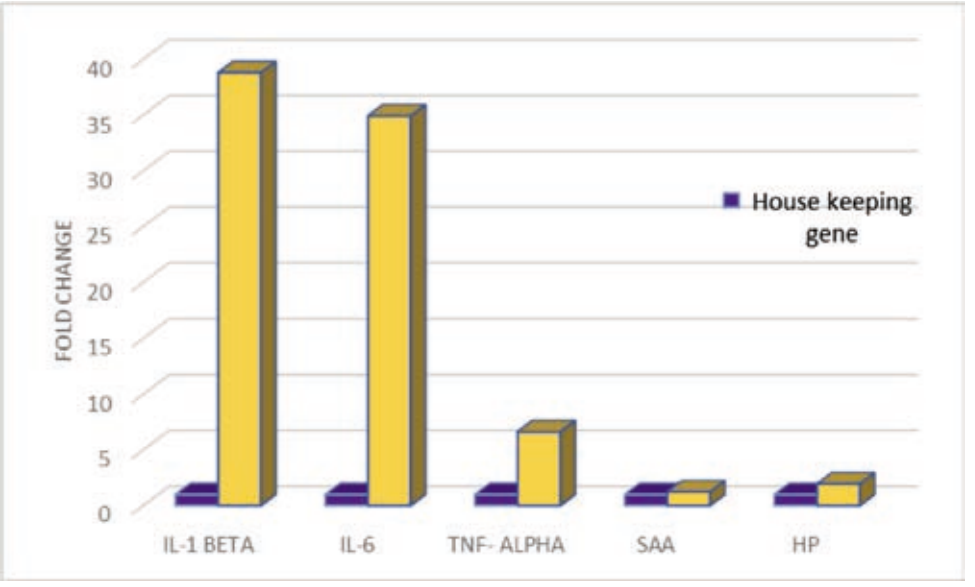

Fig. 2. Expression profile of PICs and APPs in the uterine washings of SCE cows

washings (Table 3). In the present study, *HP* was significantly up-regulated in the uterine washings of SCE cows compared to *SAA*. In clinical endometritis, Manimaran *et al*. (2019) found that the fold change of *HP* (1.65-fold) was similar to our findings. The expression of *SAA* and *HP* was up-regulated in the present study might be due to the increased expression of PIC in the endometrium. Serum amyloid A was released in response to the expression of *TNF-α* and *HP* due to *IL-6* (Tothova *et al*., 2014). Hence, increased expression of HP rather than *SAA* was seen in the current investigation. This could be because *IL-6*, an inflammatory mediator, is expressed at a higher level than *TNF- α*. Serum amyloid A may not constantly rise; rather, it only seems to do so in postpartum cows under the circumstances of the accompanying stress and acute uterine infection (Chan *et al*., 2010). Fig.2 depicts the expression profile in uterine washings.

Conclusion

Subclinical endometritis is often diagnosed late due to the absence of clinical signs and is commonly detected when AI turns out to be ineffective. In the present study, animals with SCE was diagnosed with more than eighteen per cent PMN cells at 60 dpp. This study showed that endometrial cytology using the cytobrush technique is an accurate diagnostic approach for identifying SCE at 60 dpp. The mRNA expression of PICs except *TNF-α* and APPs was found to be non-significant in blood whereas that of PICs and APPs (*IL-1β, IL-6, TNF-α, SAA*, and *HP*) were found to be significant in the uterine washings of post-partum dairy cows diagnosed with SCE. So, evaluating PICs and APPs in uterine washings could be done to detect SCE.

Acknowledgement

The financial support provided by Kerala Veterinary and Animal Sciences University is acknowledged.

References

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. *J. Immunol*. **168**: 4620-7.

Kasimanickam, R.T.F., Duffield, 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. *Canad. Vet. J*. **46**: 255-259.

Sheldon, M., Lewis, G.S., Leblanc, S. and Gilbert, R.O. 2006. Defining postpartum uterine disease in cattle. *Theriogenology* **65**: 1516-1530.

Sheldon, I.M., Cronin, J., Goetze, L., Donofrio, G. and Schuberth, H.J. 2008. Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. *Biol. Reprod*. **81**:1025-32.

Herath, S., Lilly, S.T., Fischer, D.P., Williams, E.R., Dobson, H., Bryant, C.E. and Sheldon, I.M. 2009. Bacterial lipopolysaccharide induces an endocrine switch from prostaglandin F2alpha to prostaglandin E2 in the bovine endometrium. *Endocrinology* **150**:1912–1920.

Chan, J.P., Chang, C.C., Hsu, W.L., Liu, W.B. and Chen, T.H. 2010. Association of increased serum acute-phase protein concentrations with reproductive performance in dairy cows with postpartum metritis. *Vet. Clin. Pathol*. **39**:72-78.

Gabler, C., Drillich, M., Fischer, C., Holder, C., Heuwieser, W. and Einspanier, R. 2010. Endometrial expression of selected transcripts involved in prostaglandin synthesis in cows with endometritis. *Theriogenology* **71**: 993-1004.

Galvao, K.N., Santos, N.R., Galvao, J.S. and Gilbert, R.O. 2011. Association between endometritis and endometrial cytokine expression in postpartum Holstein cows. *Theriogenology* **76**: 290-299.

Ghasemi, F., Gonzalez, P., Canob, G., Griebelb, P.J. and Palmera, C. 2012. Proinflammatory cytokine gene expression in endometrial cytobrush samples harvested from cows with and without subclinical endometritis. *Theriogenology* **78**: 1538 -1547.

Tothova, C., Nagy, O. and Kovac, G. 2014. Acute phase proteins and their use in the diagnosis of diseases in ruminants: a review. *Vet. Med*. **59**:163-180.

Peter, S., Michel, G., Hahn, A., Ibrahim, M., Lubke-Becker, A., Jung, M., Einspanier, R. and Gabler, C. 2015. Puerperal influence of bovine uterine health status on the mRNA expression of pro-inflammatory factors. *J. Physiol. Pharma*. **66**: 449-462.

Fagundes, N.S., Rezende, A.L., Alvarenga, P.B., Magalhaes, L.Q., Santos, R.M., Headley, S.A., Silva, M.J.B., Beletti, M.E. and Saut, J.P.E. 2019. Proinflammatory gene expression relative to the collection technique of endometrial samples from cows with and without subclinical endometritis. *J. Dairy Sci*. **102**:5511-5517.

Manimaran, A., Kumaresan, A., Sarkar, S.N., Boya, S., Sreela, L., Mooventhan, P. and Wankhade, P.R. 2019. Differential expression of bovine major acute phase proteins, cytokines and metabolic indicator genes in clinical endometritis cows. *Indian J. Ani. Sci*. **89**: 402-406.

Gayathri, P., Shibu, S., Kurien, M.O., Bibin, B.B., Surej, J.B., Niyas, E. And V.L., Gleeja. 2020. Comparison of endometrial cytology and transrectal ultrasonography for the diagnosis of subclinical endometritis. *J. Vet. Anim. Sci*. **51**: 146-152. ■