PROINFLAMMATORY CYTOKINE EXPRESSION AS A EARLY DIAGNOSTIC MARKER FOR DETECTION OF SUB CLINICAL ENDOMETRITIS IN REPEAT BREEDER CROSSBRED COWS

KALYAAN U.S. *[1], KULASEKAR.K [2], CECILIA JOSEPH[3], VAIRAMUTHU.S[4], RAJA P.[5] AND SENTHIL KUMAR T.M.A.[6]

Department of Veterinary Gynecology and Obstetrics,

Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai.

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ABSTRACT

A clinical study was carried out in a group of crossbred cattle with the history of repeat breeding. Ten apparently healthy cows having a normal calving history and clear estrual mucus discharge were selected as Control (Group I).Ten repeat breeder cows with watery,cloudy, copious discharge suspected for sub clinical endometritis (SEND) were selected for the study (Group II). These cows were subjected to gene expression studies of pro inflammatory cytokines IL – 8 and TNF α from endometrial biopsy samples using qPCR and their expression was found to be increased compare to control groups, thus indicating that pro inflammatory cytokines such as IL8 and TNF α can be used as a diagnostic indicator for SEND.

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Key words: Repeat breeders,SEND, Inflammatory cytokines

INTRODUCTION

Sub clinical endometritis (SEND) in cattle is considered as one of the major causes of repeat breeding leading to failure of conception and prolonged calving interval. However accurate diagnosis of the condition seldom happens and ample research are being executed to explore a diagnostic marker for this condition. The present study was undertaken to determine the presence of pro inflammatory cytokines as a diagnostic marker in sub clinical endometritic cows.

MATERIALS AND METHODS

Pleuriparous cross bred cows which were brought to Large Animal Gynaecology ward of Madras Veterinary College Teaching Hospital and crossbred cows of licensee unit, Madhavaram Milk Colony, Chennai-51 were utilized for the study. Apparently healthy, Pleuriparous crossbred cows aged 4 - 7 years, 60 -120 days postpartum with a history of normal calving, regular cycling and free from any palpable abnormality of the reproductive tract were selected for this study. Ten regularly cycling cows with normal estrual discharge, with no palpable abnormalities of the reproductive tract and with no failure of conception were taken as control (Group I). Ten repeat breeding cows that had copious and watery discharge suspected to have sub clinical endometritis were classified as group II. Both group of animals were screened with white side test and leucocyte esterase strip test for SEND. Albuchine's biopsy catheter which was autoclaved and disinfected with povidone iodine as described by Katagiri and Takagashi (2004) was used for collecting endometrial biopsy samples. The vulval region was cleaned with disinfectant before inserting the catheter and samples were collected from the body of the uterus. The biopsy samples were preserved in RNA later for expression studies to detect the inflammatory markers, IL-8 and TNF_{α} by real time polymerase chain reaction.

1- Junior Research Fellow, Dept of Veterinary Gynecology and Obstetrics, MVC, Chennai – 7

2, 3 – Professor, Dept of Veterinary Gynecology and Obstetrics, MVC, Chennai – 7

4- Professor and Head, Centralized Clinical Laboratory, MVC, Chennai – 7

5- PhD scholar, 6- Professor, Dept of Animal Biotechnology, MVC, Chennai - 7

RNA isolation and cDNA synthesis

RNA isolation was done as stated by Kasimanickam et al (2013). The cDNA was synthesized using the qScript cDNA Synthesis Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. Briefly, 500 ng of total RNA was reverse transcribed by adding 4 µL of qScript reaction mix, 1.0 µL of qScript RT and nuclease free water to a final volume of 20 µL. The cDNA was prepared in the GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA) using the following program: 1 cycle at 22 °C for 5 min, 1 cycle at 42 °C for 30 min and 1 cycle at 85 °C for 5 min. At the end of the run, samples were stored at −20 °C.

IL-8, TNFα and GAPDH genes as internal housekeeping genes of Bos taurus were amplified using the specific primers as shown in table 1.

To standardize the PCR, optimum concentration of each reagent were setup as follows.

cDNA	2.0 µL
10X Redtaq Master Mix	12.5 µL
Forward Primer – F – 10 pmol/ µL	1.0 µL
Reverse Primer – F – 10 pmol/ µL	1.0 µL
Nuclease Free Water (NFW)	8.5µL
Total volume	25 µL

The reaction was carried out using the following cyclic conditions

Initial denaturation	94 °C	2 min	1 Cycle
Final denaturation	94 °C	30 sec	
Annealing	55 °C	1 min	40 cycles
Extension	72 °C	15 min	
Final Extension	72 °C	7 min	1 cycle
Hold	4 °C	Infinity	

Confirmation of IL-8, TNFα and GAPDH genes amplicon was carried out 2% agarose gel electrophoresis

The standardized PCR conditions were optimized for IL-8 and TNFα gene along with endogenous control GAPDH gene of *Bos taurus* using the SYBR green technology. The quantitive real time (qPCR) was carried out using the SYBR green jump start (Sigma Aldrich, Bangalore). For standardization of qPCR the first strand cDNA synthesized from the healthy control of Bos taurus was used. Optimization of the qPCR for IL-8, TNFα genes along with endogenous control GAPDH gene of Bos taurus was carried out by employing the different concentrations (5-10 pmol) of Bos taurus primers as well as different annealing temperatures varying from 54°c to 64°c. The reaction was carried out as depicted as follows.

A non template control was prepared by using NFW without c-DNA. Two replicates of reaction mixtures were taken and centrifuged @ 560 rpm to get rid of air bubble artifacts and the plate was loaded to the qRT PCR machine Eppendorf, USA)

Table 1: Primer Sequence used in this study for IL-8, TNF_{α} and GAPDH gene amplification

Gene	Primer sequence (5 – 3)	Accession number
IL-8	Forward CAAGAGCCAGAAGAAACCTGAC Reverse AGTGTGGCCCACTCTCAATAAC	EU276073
TNF_{α}	Forward CTCTTCTGCCTGCTGCACTTC Reverse CCATGAGGGCATTGGCATACG	EU276079
GAPDH	Forward GCATCGTGGAGGGACTTATGA Reverse CACTGTCCACGCCATCACTGCCA	BC102589

Standardization of qPCR qPCR reaction mixture composition and conditions

Reagents	Volume (µl)
SYBR green master mix	5.0
Forward primer (10 pm/µl)	1.0
Reverse primer(10 pm/µl)	1.0
Template cDNA	1.0
NFW	2.0
Total volume	10.0

The Ct values were documented for housekeeping gene (control) and desired genes. The data were accepted when no amplification was found for negative control.

Denaturation/ RT Inactivation	95 ºC	2 min	1cycle
Denaturation	95 ºC	30 sec	40 cycles
Annealing	59 ºC	30 sec	
Extension	72 ºC	45 sec	
Final Extension	72 ºC	7 min	1cycle
Denaturation	95 ºC	15 sec	
Annealing	68ºC	1 min	
Extension	4ºC	Infinity	

The ΔCt values indicate the differential expression of target genes with respect to control housekeeping gene. The $2^{-\Delta\Delta Ct}$ value gave the relative quantification of expressed genes.

RESULTS AND DISCUSSION

The expression pattern of IL-8 was compared between control (group-I) and SEND (group-II) groups. The expression pattern showed that SEND group had 52.64 fold increase of IL 8 than the control group. Overall comparison showed that there was a highly significant difference in the expression level of IL-8 in SEND group than that of the control group. The expression pattern of TNFα was compared between control and SEND group. The expression pattern showed that SEND group had 26.18 fold increase of TNF_{α} than the control group. Overall comparison showed that there was a highly significant difference in the expression level of TNFα in SEND group than that of the control group.

According to Bhattacharya *et al.* (2011) under field conditions, it was always difficult to differentiate normal animals from repeat breeders suffering from sub-clinical endometritis. Hence, the endometrial biopsy provided the most reliable statement of changes within the endometrium to explore the degenerative changes and to determine distribution and severity of inflammatory processes (Cocchia *et al.*, 2012). The inflammatory response in the uterus is mediated by pro-inflammatory factors including cytokines and the inflammatory mediators such as TNFα, histamine, interleukins producing varied effects to clear the infection (Bondurant, 1999) and soon after invading pathogens are detected by immune cells the pro inflammatory markers such as TNFα and cytokines including IL-6 and IL-8 are synthesized locally by the uterus (Young *et al.*, 2002). The pro-inflammatory cytokine served as chemo attractant causing influx of neutrophils to clear the pathogen (Burke *et al.*, 2010). The immune response is autocoded with the release of several inflammatory cytokines followed by mobilization of the neutrophils and that increased expression of pro inflammatory cytokines in bovine uterine tissue is related to the development of clinical / sub clinical endometritis (Galvao *et al.*, 2011). In this study, the pro inflammatory cytokines IL- 8 and TNFα increased to 53 and 27 fold respectively in sub clinical endometritis cows. The present study demonstrated that pro inflammatory cytokines were increased several fold in sub clinical endometritis which concurs with the reports of Bondurant (1999) and Young *et al.* (2002). From the present study it could be concluded that there is a differential expression of the pro inflammatory cytokines and their transcripts in the endometrial tissues of the subclinical endometritis cattle. Hence, the expression pattern of pro inflammatory cytokines IL 8 and TNFα could be used as one of the diagnostic marker for subclinical endometritis in cattle.

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