Original Research Article

Expression assessment of some immunity-related genes in buffalo infected with endometritis

ABSTRACT

Background and aim: Despite the economic importance of buffalo as a main source of milk and meat, only little attention has been directed to its immune and reproductive performance. The early diagnosis of subclinical endometritis may reduce the economic loss of buffalo's production. The difference in expression profiles of immunity-related genes has an important role in the early detection of subclinical endometritis. This study aimed to assess the expression of five immunity-related genes: *TGFBR1*, *PTGER2*, *PTGER4, HP* and *CXCL5* in endometritis-infected buffaloes.

Materials and Methods: Total RNA was extracted from 120 buffalo uterine samples; 60 infected with endometritis and 60 healthy ones. Qt-PCR was performed on cDNA synthesized from extracted RNA using Sybr green and *GAPDH* as a house-keeping gene.

Results: The results showed the up-regulation of two tested genes; *TGFBR1* and *CXCL5* in endometritis-infected buffalo compared to healthy animals by 7.9 and 4.3 folds, respectively at a significance level of $p<0.05$. The other three tested genes; *PTGER2*, *PTGER4* and *HP* were down-regulated in buffalo during endometritis infection at different levels; *PTGER2* and *HP* (0.6 folds, $p<0.05$) and *PTGER4* (0.4 fold, $p= 0.2$).

Conclusions: It is to be concluded that the assessment of expression of inflammation-related immunity genes may have an effective role on the detection of endometritis infection in buffalo during its early stages and this early diagnosis can reduce the economic loss of buffalo production and reproduction.

Short running title: Expression of immunity genes in endometritis-infected buffalo

Keywords: Endometritis, Buffalo, *TGFBR1*, *PTGER2*, *PTGER4, HP* and *CXCL5*

1. INTRODUCTION

The low reproductive performance in farm animals can be considered as one of the factors leading to the economic loss around the world [1]. Most of dairy animals suffer the uterine contamination with different types of bacteria during parturition [2]. This infection leads to the complete infertility in acute cases or at least sub-fertility in chronic cases [3]. One of the undesired effects of uterine contamination is the reduction of conception rate due to the increasing interval between calving to conception [4].

The development of uterine disease is associated with the immune response of the animals [5]. The defense's first line against the infection with bacteria is the endometrium that ascends the genital system in animal after parturition. Clinical endometritis is an inflammation of the endometrium associated with the presence of mucopurulent discharge detected in the vagina [6]. The early diagnosis of subclinical endometritis may reduce the economic loss of buffalo's production at dairy farm. There are different methods for diagnosis of endometritis like uterine biopsies and swabs but these methods lead to the irritation and distortion of cells [7]. Because inflammatory responses are regulated by the immune genes during the infection, the difference in expression profiles of immunity-related genes has an important role in the early detection of subclinical endometritis [8].

Buffaloes are the main source of good quality meat and milk in Egypt and some other developing countries, despite this species is mostly reared under harsh socioeconomic conditions and shows low reproductive potentials [9]. The increasing resistance against fertility-related diseases leads to solving some reproductive discouragements in this economically important species. The immune genes that are related to reproductive diseases can be identified as being expressed differently between high and low responders [10]. This work aimed to assess the gene expression of five immunity-related genes in buffalo infected with endometritis using real-time qt-PCR.

2. MATERIALS AND METHODS

2.1. Samples and bacterial identification:

The uterine samples were obtained from 120 Egyptian buffaloes; **60** infected with endometritis and **60** uninfected ones. Buffaloes with endometritis had signs of abnormal secretions and inflammation such as swelling, redness and hardness in uterus. The uterine samples were collected in slaughterhouse from animals after sacrificing under normal condition without any special requirement, so it is not needed to any ethical permission.

Collected samples were streaked on the Blood agar, Mac-Conkey agar and mannitol salt agar plates. All samples were incubated aerobically and anaerobically. Aerobic plates were incubated at 37°C for 24 h, whereas anaerobic plates were incubated in an anaerobic jar using anaerobic system (BD) at 37°C for 84-72 h. Plates were examined for colony characters, cellular morphology and the purity of the culture.

2.2. RNA extraction and cDNA synthesis:

RNA was extracted from uteri samples using total RNA purification kit (Jena Bioscience, Germany), according to manufacturer's instructions. An aliquot of RNA was diluted in RNase free water to estimate RNA quantity. The concentration of RNA samples was determined using Nanodrop spectrophotometer and the purity of RNA was assessed by 260/280 nm ratio.

cDNA synthesis was performed on extracted RNA, which was treated with DNase to remove any possible DNA contamination. One µl of DNase and 1 µl buffer were added to 1 µg RNA and the

volume was completed to 10 µl by DEPC water and incubated at 37°C for 30 min., 1 µl of EDTA was added and incubated at 70°C for 10 min. The DNase-treated RNA was reverse transcribed into first strand cDNA using RevertAid First Strand cDNA Synthesis kit (Fermantas) according to the manufacturer's instructions.

2.3.3. Real-time polymerase chain reaction (Real-time PCR):

Gene expressions were detected by real-time PCR, which was performed using Rotor-Gene Q system (Qiagen Company). A 25 µl reaction mixture consisted of 12.5 µl SYBR Green PCR Master-Mix (applied Biosciences, USA), 0.5 µl of each primer (10 PMole) (**Table 1**), 1 µl cDNA (50 ng) and 10.5 µl RNase free water.

The optimum amplification conditions were chosen empirically according to each tested gene. Generally, the amplification conditions included: initial incubation, then 40 cycles of amplification with denaturation, annealing and extension steps. Mean cycle threshold (Ct) values of triplicate samples are used for analysis. The Ct value indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold.

2.4.9. Data analysis:

The chi-square test was used to evaluate the significant differences ($P<0.05$) in gene expression of tested genes. Data from real-time PCR were analyzed using $2^{-\Delta\Delta Ct}$ method [11]. Data were represented as the fold change in target gene expression normalized to a House-Keeping gene (HKG) and relative to the control (uninfected animals). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a house-keeping gene to normalize input RNA amount, RNA quality and reverse transcription efficiency.

Table 1: Primer sequences of tested genes

Gene	Primer Sequence	Product size (bp)	Anneal temp	Reference
Transforming growth factor beta receptor (*TGFBR1*)	F: CAGGTTTACCATTGCTTGTTCA R: TGCCATTGTCTTTATTGTCTGC	243-bp	56°C	12
Prostaglandin E2 receptor (*PTGER2*)	F: GTTCCACGTGTTGGTGACAG R: ACTCGGCGCTGGTAGAAGTA	246-bp	56°C	
Prostaglandin E4 receptor (*PTGER4*)	F: TCGTGGTGCTCTGTAAATCG R: CTCATCGCACAGATGATGCT	226-bp	56°C	
Haptoglobin (*HP*)	F: TGG TCT CCC AGC ATA ACC TC R: TTGATGAGCCCAATGTCTACC	217-bp	60°C	13
Chemokine CXC ligand 5 (*CXCL5*)	F: TGA GAC TGC TAT CCA GCC G R: AGA TCA CTG ACC GTT TTG GG	193-bp	61°C	
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	F: CCT GGA GAA ACC TGC CAA GT R: GCC AAA TTC ATT GTC GTA CCA	214-bp	60°C	14

3. RESULTS AND DISCUSSION

The incidence of uterine infection with different types of bacteria at postpartum prevents the restoration of ovaries and uteri's functions and consequently the failure of the fertilization and

conception [15]. Postpartum endometritis is considered one of the most common disorders in dairy animals, especially cattle and buffalo, leading to the high economic loss due to the elongation of interclving intervals [5]. The frequency of uterine infection in buffalo is higher than that in cow, where it ranges from 10 to 50% in cow dairy cattle [16] and from 20 to 75% in dairy buffaloes [17]. Due to the difficulty of subclinical endometritis detection, where the animals are reservoirs of bacteria despite their healthy appearance, the infection can spread among the whole herd animals [8]. So, the early diagnosis of animals with subclinical endometritis is considered the best effective way for endometritis control in buffalo and it reduces the economic harm effect of this disease [18].

Determining the immune status of buffalo in relation to the occurrence of endometritis may assist to improve some strategies for effective reproductive management. Although more than 70% of cows clear uterine bacteria via innate immune responses, 17 to 37% of cows develop clinical endometritis, whereas 14 to 53% develop subclinical endometritis [19,20]. The expression of mRNA of inflammatory-related genes in uterine tissue was related to the development of bovine clinical or subclinical endometritis [21,22]. The elevation of immunity gene expression is a sensitive indicator for endometritis incidence in cows [21,23,24]. The aim of this study was to elucidate the expression of five immunity-related genes during endometritis-infected buffaloes compared them with those of healthy animals. The five tested genes are *TGFBR1*, *PTGER2*, *PTGER4, HP* and *CXCL5*.

Transforming growth factor beta receptor I (*TGFBR1*) gene encodes a membrane-bound receptor protein which is one of the TGF beta superfamily of signaling ligands. This protein bounds with TGF beta receptors to form a complex transition of the TGF-β signal from the cell surface to the cytoplasm [25]. Much research showed the important role of *TGFB* receptors in the behavior and function of genital system in human and animals and the mutations of *TGFBR1* gene were detected to be responsible for fertility problems [26,27]. In this study, the relative gene expression of *TGFBR1* gene was assessed in endometritis-infected and healthy buffaloes. The means of threshold values were 22.65 and 26.97 in infected and healthy animals, respectively. This up-regulated expression of *TGFBR1* gene in endometritis-infected buffaloes with 7.9 folds (**Fig. 1**) was statistically significant at $p<0.05$.

The expression of some receptors including *TGFBR1* was examined in cow infected with cystic ovarian disease [1,12]. They reported the high expression of *TGFBR1* in granulose cells of cysts from infected cows compared to that in tertiary follicles from the control group. In contrast to our results and Matiller's finding, the expression of this receptor gene did not differ significantly between cattle infected with postpartum uterine disease and healthy cow.

Prostaglandins are physiologically-active compounds having action like hormones in animals. The differences in the prostaglandin's structures are responsible for their different biological activities where there are four principal prostaglandin compounds [28]. Prostaglandin E2 exhibits its effect by acting on G-protein-coupled receptor group [29]. Prostaglandin E_2 is the most abundant prostaglandin which exerts its inflammatory response by acting through the prostaglandin E receptors, EP2 and EP4 that are encoded by the genes *PTGER2* and *PTGER4*, respectively [30].

Due to the relation between these receptors and inflammation responses, we assessed in this study the relative expression of both *PTGER2* and *PTGER4* genes in endometritis-infected buffaloes compared with those in healthy animals.

The means of threshold values were 23.34 and 24.92 for *PTGER2* and *PTGER4* genes, respectively in infected buffaloes whereas their values were 22.84 (for *PTGER2*) and 23.86 (for *PTGER4*) in healthy animals. After the normalization of CT values with those of *GAPDH* as a normalized gene and comparing them with CT values in healthy animals, the expression of *PTGER2* and *PTGER4* genes was assessed as down-regulation by 0.6 and 0.4 folds, respectively in endometritis-infected buffaloes (**Fig. 1**). The statistical analysis showed that the down regulation of *PTGER2* expression was statistically significant ($P<0.05$), whereas this was not the case for *PTGER4* expression ($P=0.2$).

The endometrial mRNA expression of prostaglandin-endoperoxide synthase 2 (PTGS2) was investigated in the primiparous cows postpartum period using RT-PCR [10]. They reported a significantly higher PTGS2 mRNA content in samples from cows with an inflamed endometrium compared with those from healthy endometrium cow. Unlike the Gabler's findings, the expression of genes encoding prostaglandin E2 receptors (PTGER2 and PTGER4) did not differ significantly between infertile and fertile animals after the first week postpartum [12]. Our results did not match with the above-mentioned ones, where we declared that the expression of *PTGER2* and *PTGER4* genes in healthy animals was assessed as down-regulation by 0.6 and 0.4, respectively in endometritis-infected buffaloes. The down regulation of *PTGER2* and *PTGER4* expression in endometritis-infected buffaloes may be interpreted by the inhibition of *PTGER2* and *PTGER4* production activated Th1 responses of bovine leukemia virus *in vitro* as evidence for the enhanced T cell proliferation and Th1 cytokine production and consequently the reduction of BLV proviral load in vivo [38].

Haptoglobin (Hp) is an α2-globulin protein which is synthesized in liver and its concentration is increased in serum during acute infections [32]. This protein was reported as a regulator of lipid metabolism in farm animal like cattle [33] and also acts as immunomodulator in cases of inflammation and infection [34,35]. The diagnostic potential role of Hp for mastitis was developed and validated by ELISA technique which was sensitive to its subclinical concentrations in both blood and milk [36]. The difference in milk whey protein was reported in haptoglobin isoform for serum from subclinical cases [37] and this finding was supported by RT-PCR confirming the role of Hp as a diagnostic biomarker. *Hp* concentration is significantly increased in milk of cattle after the intra-mammary administration of endotoxin or bacteria [38].

The relative expression of *Hp* gene in endometritis-infected buffalo in comparison with its expression in healthy animals was measured in this study using Qt-PCR. The results showed that the threshold value mean was 27.90 in infected buffalo, whereas it was 27.49 in healthy animals. It means that the expression of *Hp* is down-regulated in buffalo during endometritis infection by 0.6 folds (**Fig. 1**) with a statistical significant level ($p<0.05$).

Endometrial cells have a role in embryo/maternal communication as well as support the immune response during defending against pathogen's infection. The association between expression of inflammatory factors including *Hp* and signs of clinical or subclinical endometritis were evaluated [13] and they found no correlation between the uterine health and *HP* transcripts.

The endometrial mRNA expression of haptoglobin in the postpartum period was investigated in cow [16] using RT-PCR. They reported that *Hp* mRNA expression was correlated significantly with the proportion of polymorphonuclear neutrophils suggesting the role of this protein in inflammatory process. The elevation of serum amyloid and haptoglobin levels was observed in blood serum in ruminant viral diseases [28]. Therefore, it is possible to use the levels of these proteins for diagnosing infections especially in sub-clinical cases. The same finding was reported [39], who investigated the significant increase in serum concentrations of both SAA and Hp in Foot and Mouth-infected animals. The levels of serum haptoglobin, SAA and ceruloplasmin were significantly elevated in cattle with FMD compared with those in healthy animals [40]. These findings supported the importance of the role of this protein in immune response of animals towards the infection with different viral diseases. These results contradict the ones obtained in our study, which showed the down regulation of *Hp* transcripts in buffalo infected with endometritis suggesting the difference of *Hp* expression regulation between bacterial and viral infections.

Chemokine CXC ligand 5 is a cytokine protein belonging to the family of chemokines. This protein is produced during the inflammatory stimulation [41]. The biological functions of chemokines that are related to immune response and their role in host defense were reviewed [42]. The relation between some potential candidate genes - including *CXCL5* and *Hp* - with the physiological and pathological features in bovine endometrium was reported [6]. Due to the clear role of chemokines in innate immunity response towards different infections, this work aimed to assess the expression of one of this group - *CXCL5* - in endometritis-infected buffalo and comparing it with that in healthy animals.

The results declared that the expression of *CXCL5* in infected animals was up regulated compared to that in non-infected ones, where the mean of threshold values in infected buffalo was 31.82 while it was 34.24 in healthy animals. The statistical analysis showed that the upregulation in *CXCL5* expression in endometritis-infected buffalos was by 4.3 folds (**Fig. 1**) with insignificant statistical level. The alteration in the expression of immunity genes has an effective role in the early diagnosis of subclinical as well as clinical with any stage of endometritis infection.

The significant higher expression of these pro-inflammatory factor transcripts in the endometrium of cows with subclinical or clinical endometritis compared to healthy animals was reported [13]. The time-dependent endometrial mRNA expression of some factors involved in the inflammation process and infection of cow's uterus during postpartum was investigated [6]. They observed significantly higher *CXCL5* mRNA expression in cows with inflamed endometrium

compared to cows with a healthy endometrium. The above-mentioned results agreed with our findings related to the upregulation of *CXCL5* expression during endometritis infection in buffalo.

4. CONCLUSION

The assessment of gene expression of some immunity genes related to the inflammation in endometritis-infected buffaloes has an important role in reducing the loss of buffalo's production and reproduction. This goal can be achieved through the early diagnosis of sub-clinical endometritis, where the animals appear to be healthy while they are reservoirs of bacteria that lead to infections to other animals.

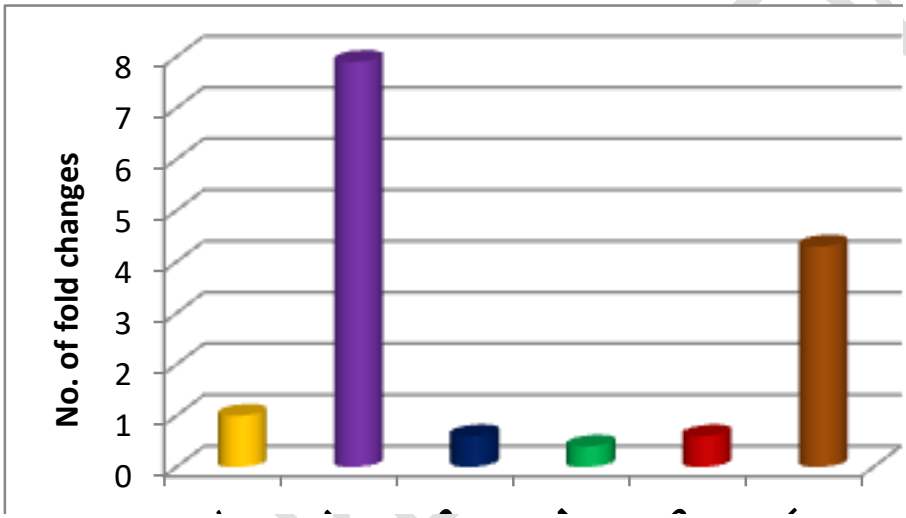

Fig. 1. No. of fold changes in expression of tested genes between healthy and infected animals

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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