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Differential expression of *TGF-β* and *IL-1R2* genes during endometritis infection in Egyptian buffalo

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ABSTRACT

Contamination of the uterine lumen with bacteria occurred in bovid within the first week after parturition. The bacterial infection causes the persistent inflammation in the endometrium leading to the infertility and huge economical loss in animal production. *TGF-β* and *IL-1R2* genes are involved in innate immune recognition of pathogens and the inflammatory response. This study aimed to compare the expression of these two genes in uteri samples of endometritis-infected and apparently healthy buffaloes using QT-PCR. The uteri samples were collected from endometritis-infected and normal buffaloes. Bacterial examination of uteri from endometritis-infected buffaloes showed the presence of bacterial contamination with *E. coli*, *P. Klebsiella pneumonia* and/or *P. vulgaris*. RNA was extracted from uteri of infected and normal animal, and cDNA was synthesized for QT-PCR. Using *GAPDH* as a housekeeping gene, the gene expression of two tested genes was assessed and the results showed that the expression of *TGF-β* and *IL-1R2* genes was up-regulated in infected animals compared to control by 11.39 and 12.99 folds, respectively and this increase of gene expressions was highly significant. In conclusion, the gene expression assessment of important innate immune genes—like *TGF-β* and *IL-1R*2 genes can help to establish new approaches for the improvement of the immune response of buffalo through marker-assisted selection of animals characterized by superior innate immunity system.

Keywords: Buffalo, Egyptian, Endometritis, *IL-1R2*, QT-PCR, *TGF-β*

River buffalo occupy an important part and has an essential role in Egyptian social life where its milk and meat production is considered the principal food stuff for most of Egyptians. Buffalo's productivity suffers a huge loss due to the infection of animals with different diseases including endometritis (Peters *et al*. 2013). Transforming growth factor beta (*TGF-β*) is a member of transforming growth factor superfamily and it is a multifunctional cytokine. *TGF-β* through the binding and activation of different substrates and protein induce the transcription of different genes which are responsible for differentiation, proliferation and activation of different immune cells including macrophage (Massagué 2012). Interleukin-1 receptor (*IL-1R*) is a cytokine receptor which binds interleukin-1, two forms of *IL-1R* are present, *IL-1R*1 and *IL-1R2*. *IL-1R1* is primarily responsible for transmitting the inflammatory effects of interleukin-1 whereas *IL-1R2* acts as a suppressor of *IL-1* activity by competing for *IL-1* binding. *TGF-β* and *IL-1R2* genes are involved in innate immune recognition of pathogens and the inflammatory response. So this study aimed to compare the expression of these two genes in uteri samples of endometritis-infected and apparently healthy buffaloes using QT-PCR.

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MATERIALS AND METHODS

Sampling: The uteri samples were collected from 100 Egyptian buffaloes, 50 infected with endometritis and 50 apparently normal ones. The uterine samples were collected in slaughter house from animals after sacrificing under normal condition without any special requirement. Hence, ethical permission was not required. Buffaloes with endometritis had signs of abnormal secretions with signs of inflammation such as swelling, redness and hardness in uterus. A swab from each infected uterus was separately collected in transport medium and sent to the lab in an ice box for bacterial analysis. The uteri samples were frozen in –80°C till RNA extraction.

Bacterial identification: Collected samples were streaked onto 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 and the suspected colonies were identified.

RNA extraction: Total RNA was extracted from uteri samples using Direct-zol™ RNA MiniPrep Kits (Zymo

Table 1. Primer information of tested genes and the housekeeping gene

Gene	Primer sequences (5′–3′)	References
TGF-β	F: CTG AGC CAG AGG CGG ACT AC R: TGC CGT ATT CCA CCA TTA GCA	Coussens *et al*. (2004)
IL-1R2	F: ATC CCA TGT AAG GTG TTT CTG G R: TGA CAG GAT CAA AAA TCA GTG G	Hearth *et al*. (2009)
GAPDH	F: CCT GGA GAA ACC TGC CAA GT R: GCC AAA TTC ATT GTC GTA CCA	Buza *et al*. (2003)

Research, USA), following manufacturer's instructions. An aliquot of total RNA was diluted in RNase free water was set aside to estimate RNA quantity and integrity and the remaining sample was stored at –80°C until gene expression analysis. The concentration and purity of the RNA samples was determined using Nano-Drop® ND-1000 Spectrophotometer (Thermo Scientific, USA). Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis and all samples maintained a 28S/18S rRNA ratio range from 1.85 to 2.05.

cDNA synthesis: cDNA synthesis was carried out on RNA extracted from uteri samples from 100 animals, 50 endometritis-infected and 50 apparently normal buffaloes. The RNA of these uteri samples was treated with DNase (Fermentas) to remove any possible DNA contamination according to the manufacturer's instructions. The DNase-treated RNA was reverse transcribed into first strand cDNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions.

Real-Time PCR (*RT-PCR*): Real-time PCR was performed for quantification of *TGF-β* and *IL-1R2* gene expression. Real-time PCR was performed using Rotor-Gene Q (Qiagen, Germany). Intron-spanning gene-specific primers that were short enough to ensure optimum amplification were chosen from published references (Table 1). The 25 µl reaction mixture consisted of 12.5 µl SYBR Green PCR master mix (Thermo Scientific, USA), 0.5 µl of each primer (10 pmol), 1 µ cDNA (400 ng) and 10.5 µl RNase free water. For each gene of interest, negative and positive controls were included. Two housekeeping genes were tested in this study: (GAPDH and β-Actin) and GAPDH was used as a housekeeping gene for validation because it gave more suitable result. For *GADPH* and the tested genes (*TGF-β* and *IL-1R2*), 40 cycles of amplification with denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 60°C for 30 sec, were performed after an initial incubation at 95°C for 5 min. For each sample, the melting curve was generated after completion of amplification and analyzed in comparison to the positive and negative controls. Mean cycle threshold (Ct) values of duplicate samples were used for analysis.

Data analysis: Data from the Real-time PCR were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgrn 2011) where:

$\Delta\Delta Ct$ = (Ct, Target gene – Ct, House-keeping gene) infected samples – (Ct, Target gene – Ct, House-keeping gene) control samples.

By using the $2^{-\Delta\Delta Ct}$ method, the data are represented as the fold change in target gene expression normalized to the *GAPDH* as a house-keeping gene to normalize input RNA amount, RNA quality and reverse transcription efficiency. Statistical significant was evaluated using the Student's *t*-test. P-value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Contamination of the uterine lumen with different strains of bacteria—especially *E. coli*, *P. Klebsiella pneumonia* and *P. vulgaris* occurred in bovid within the first week after parturition (Williams *et al.* 2007, Sheldon *et al*. 2009). The bacterial infection causes the persistent inflammation in the endometrium leading to the infertility of about 30% of the infected animals (Gilbert *et al*. 1998). The endometrium is considered the first defense line against bacterial infection that infects the female genital tract after parturition. The endometrial cells have an essential role in innate immune defense in bovines (Herath *et al*. 2006). This defense of endometrium against bacteria and microbes depends on the innate immune system including interleukines and cytokines (Herath *et al*. 2009, Peters *et al*. 2013).

Variation in gene expression in endometrium is subject to the effects of genetic variation. Understanding regulation of gene expression in this tissue is important because the endometrium is essential for fertility. Common genetic effects alter expression of many genes and are known as expression quantitative traits (eQTLs). The eQTLs play an important role in mediating effects of genetic factors increasing risk for common diseases. The genetic effects may be tissue specific or influence expression across multiple tissues, and may interact with other factors including changing hormonal environments (Fung *et al*. 2018).

The economic loss in buffalo production as a result of endometritis infection orientates us to focus on the enhancement of buffalo innate immunity system though the marker-assisted selection depending on the animals with strong immune system. The first step toward this target is the assessment of differential expression of genes related to the endometritis in Egyptian buffalo. This work aimed to evaluate the differential expression of two cytokines, *TGF-β* and *IL-1R2* in endometritis-infected buffalo comparing to apparently normal ones using Qt-PCR and *GADPH* as a housekeeping gene.

Bacterial examination: Bacterial examination of uteri from 50 endometritis-infected buffaloes showed the presence of bacterial contamination in these samples as follows, 26 samples with *E. coli*, 12 samples with *P. Klebsiella pneumonia* and 12 samples with *P. vulgaris*. The 50 apparently healthy buffaloes did not show any sign of endometritis symptoms and their uteri did not show any pathogenic bacteria.

Gene expression analysis: Fifty samples each for endometritis-infected buffaloes and apparently healthy

animals were subjected for the gene expression quantification of two cytokines genes, *TGF-β* and *IL-1R2* and *GAPDH* as a housekeeping gene using real-time PCR with SYBR Green master Mix. All tested genes presented a single peak in the PCR melting curve, which indicates absence of primer-dimer formation during the reaction and specificity of the amplification. Gene expression for each sample was quantitatively estimated in a duplicate and the means of cycle threshold (Ct) were calculated. For *GAPDH* gene, the mean cycle threshold was 15.32 in endometritis-infected buffaloes whereas it was 15.49 in apparently healthy animals.

Transforming growth factor beta (*TGF-β*): The quantitative gene expression of this cytokine gene was evaluated in 100 animals (50 endometritis-infected and 50 healthy animals). The mean of cycle threshold in infected animals was 17.87 with the standard deviation 0.98 whereas in healthy or control animals, the cycle threshold mean was 21.55 with the standard deviation 0.87. ΔΔCt was calculated as follows:

$$\Delta\Delta Ct = (17.87–15.32) – (21.55–15.49) = 2.55–6.06 = –3.51$$
$$\text{No. of folds} = 2^{-\Delta\Delta Ct} = 2^{-(-3.51)} = 11.39 \text{ folds.}$$

Hence, the expression of TGF-β was up-regulated in infection animals by 11.39 folds. The Student's *t*-test was used to evaluate the significance of the number of fold changes in the expression of TGF-β gene in endometritis-infected buffaloes as compared to healthy animals. The results showed that the N-fold change was highly significant (P<0.001) (Table 2).

TGF-β is reported to increase in the peritoneal fluid, serum, ectopic endometrium and peritoneum of female endometriosis compared to female without endometriosis, and *TGF-β*-null mice have reduced endometriosis lesion growth when compared to their wild-type controls. Studies in mice had indicated that increasing levels of *TGF-β* ligands are associated with decreased immune cell activity within the peritoneum, together with an increase in ectopic endometrial cell survival, attachment, invasion and proliferation, during endometriosis lesion development (Vicky *et al*. 2017).

Transforming growth factor beta is considered as an immune-regularity cytokine which has essential role in host immune response against different pathogens and microbes. Gomez-Laguna *et al.* (2012) examined the role of this gene in porcine reproductive disease resulted from PRPS virus infection. An increase of *TGF-β* antigen was detected in the lung and lymphoid organs of PRPSV-infected pig when compared with the control confirming the role of this cytokine in the immune response and differentiation of T cells.

Coussens *et al.* (2004) examined the differential expression of many genes including *TGF-β* in PBMCs, intestinal lesions, and mesenteric lymph nodes of cattle naturally infected with *M. avium* subsp. *paratuberculosis.* They reported that the expression of *TGF-β* in infected cattle was greater than its expression in comparable tissues from control uninfected cattle. The expression patterns of *TGF-β*, *IFN-γ* and *IL-1β* in response to infection with *B. bovis* and *B. bigemina* in bovines was analyzed by Abdel Aziz *et al.* (2014). The results revealed that infected cattle showed highly significant up-regulation of *IL-1β* and *TGF-β* and down-regulation of *IFN-γ* when compared with healthy animals. This up-regulation of expression of *TGF-β* in different diseases is in agreement with the finding of this work where the expression of *TGF-β* increased by more than 11 folds in endometritis-infected buffalo compared with healthy ones.

Table 2. N-fold change of *TGF-β* and *IL-1R2* gene expression in endometritis-infected buffalos

Gene	Animal status	CT (mean ±SD)	ΔΔCt	No. of folds	Significant level
TGF-β	Infected Healthy	17.87±0.98 21.55±0.87	–3.51	11.39	P>0.001***
IL-1R2	Infected Healthy	23.81±1.02 27.68±1.27	–3.7	12.99	P>0.001***

Using qRT-PCR, Wang *et al*. (2013) measured the levels of *TGF-β* and its receptors in the murine Alveolar echinococcos is model. They showed that *TGF-β* and its receptors were markedly expressed in the periparasitic infiltrate and also in the hepatocytes, close to and distant from AE lesions suggesting that *TGF-β* plays an important role in AE both in immune tolerance against the parasite and in liver fibrosis. Also the greater resistance to *S. typhimurium* infection in r*TGF-β*-treated mice is associated with different mechanisms in the protective response (Galdier *et al.* 1999). The widespread distribution of *TGF-β* and its receptors suggested the important role of this cytokine in these mechanisms depending on *TGF-β* concentration.

Interleukin-1 receptor 2 (*IL-1R2*): The gene expression of this cytokine receptor gene was evaluated in 50 endometritis-infected and 50 healthy animals. In the infected buffaloes, the mean of cycle threshold was 23.81 with the standard deviation 1.02 whereas in apparently-healthy animals, the cycle threshold mean was 27.68 with standard deviation 1.27. ΔΔCt was calculated as follows:

$$\Delta\Delta Ct = (23.81 – 15.32) – (27.68 – 15.49) = 8.49 – 12.19 = –3.7$$
$$\text{No. of folds} = 2^{-\Delta\Delta Ct} = 2^{-(-3.7)} = 12.99 \text{ folds}$$

Hence the expression of *IL-1R2* was up-regulated in infected buffaloes by 12.99 folds. The Student's *t*-test was used to evaluate the significance of the number of fold changes in the expression of *IL-1R2* gene in endometritis-infected buffaloes as compared to healthy animals. The results showed that the N-fold change was highly significant (P<0.001) (Table 2).

Contamination of the uterine lumen in cattle with different types of bacteria develops endometritis which causes fertility reduction in some animals whereas there is no uterine disease in others. This different response of animals to bacterial contamination is related to the differential expression of immune genes (Herath *et al.*

2009). Higher gene expression of *TLR4* as well as pro-inflammatory cytokines *IL1A* and *IL1B* with their receptor *IL1R2* had been detected in infertile than fertile animals. Using qtPCR assays, Lim *et al.* (2012) showed considerable variation in expression level among cattle infected with tuberculosis. They reported decreased gene expression of 12 immune genes whereas there was marked increase in expression of some genes including the *IFN-γ* and *IL-1R2*.

Xu *et al*. (2016) cloned the full-length cDNA and genomic DNA of two cytokines, *IL-1β* and *IL-1R2* in the Asian swamp eel. RT-PCR analysis indicated that these cytokines were expressed in all tissues, especially immune-related organs. *In vitro*, *IL-1β* and *IL-1R2* mRNA levels were up-regulated after the infection with *A. hydrophila*.

The transcript levels of *IL-1β* and *IL-1R2* were increased by 21.4-fold and 20.8-fold, respectively, relative to the control indicating the important role which *IL-1β* and *IL-1R2* play in inflammation and host defense. This increase in the gene expression of *IL-1R2* reported by Herath *et al.* (2009) and Lim *et al.* (2012) in infected-cattle and by Xu *et al*. (2016) in infected-eel confirmed our result for the 13-folds increase of this immune gene in endometritis-infected buffalo.

In human, *IL-1* is critical to the pathogenesis of a variety of human diseases. The functional significance of *IL-1R* is well documented due to the clinical utilization of the recombinant human *IL-1R*. *IL-1R2* is structurally similar to the type *IL-1R1* responsible for *IL-1* signal transduction. *IL-1R2* has been implicated in many diseases including endometriosis suggesting the important role of this cytokine in human immune system (Peters *et al*. 2013).

In conclusion, the assessment of gene expression for important innate immune genes like *TGF-β* and *IL-1R* genes can play an important role in the improvement of the immune response of bovid including buffalo through more efficient marker assisted selection of animals with superior immunity responses and recruit them in breeding programs to obtain herds whose animals possess a strong immune system.

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