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Cytokine genes expression in uteri of *Bubalus bubalis* associated with endometritis infection

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Abstract

Fertility deficiency is one of the major causes which lead to a decrease in river buffalo's production that has great economic importance in Egypt. Infection of the endometrium with different types of bacteria leads to inflammations causing infertility in severe conditions. The difference in cytokines expression profiles has a role in the early detection of subclinical endometritis-infected buffalo. The present study aimed to assess some cytokine gene expressions in endometritis-infected buffaloes using RT-qPCR. The uteri samples were collected from 60 animals with inflammation signs and other 60 healthy animals. RNA was extracted from uteri samples and cDNA was synthesized from extracted RNA. Gene expressions were detected by real-time PCR using SYBR Green PCR Master-Mix and primers specific for tested genes (*IL-1α, IL-1β, IL-6, IL-10* and *TNF-α)*. Cycle threshold (Ct) mean values of triplicate samples were utilized for analysis. Chi-square test was utilized to determine the significant differences ($P<0.05$) in gene expression of tested genes using *GAPDH* as a house-keeping gene for normalization. Bacterial examination of uteri from infected buffaloes showed the presence of bacterial contamination with *Escherichia coli, Klebsiella pneumonia* and *Proteus vulgaris,* whereas the uteri of apparently-healthy animals did not show any of the previous pathogenic bacteria. The RT-qPCR results revealed that the gene expression of three tested cytokines; *IL-1α. IL-1β* and *IL-6*increased in endometritis-infected buffaloes with 1.3, 1.7 and 5 folds, respectively compared to their expression in healthy animals. On the contrary, the expressions of two other genes: *IL-10* and *TNF-α* showed down regulations with 0.2 and 0.4 folds, respectively in infected buffaloes when compared with those in uninfected animals.
It can be concluded that the tested cytokine genes in endometritis-infected buffaloes showed different responses to the endometritis infection, where three of them: *IL-1α. IL-1β* and *IL-6* were up-regulated, whereas *IL-10* and *TNF-α* were down-regulated with different levels of significant values.

Keywords: Endometritis, Buffalo, Cytokines gene, RT-qPCR, Gene expression

1. Introduction

Buffalo is the most economically important livestock in many developing countries including Egypt where it is considered to be one of the main sources of milk and meat. Any loss in its production leads to a valuable increase in the gap between increased population and the demand for this important foodstuff. Fertility deficiency is one of the major causes leading to a decrease in buffalo's production (Mohammed, 2018).

The uteri in mammals are a sterile environment, but always - especially during coitus or parturition - they are ready to infection with different types of bacteria (Benko *et al.* 2015). This bacterial contamination leads to inflammation ranging from pelvic disease to chronic endometritis and infertility (Park *et al.* 2016). The infection of the endometrium with *E. coli* is associated with the impact on female fertility (Dahiya *et al.,* 2018).

Cytokines are natural proteins produced by immune cells and act importantly in host defense against infection and participate in specific and nonspecific immunity.

Depending on the type of simulated cells and the nature of the antigenic stimulus, the cytokine type is released from the cell. Cytokines equalize the leukocytes to respond to bacterial and microbial stimuli, and they are classified into the following six groups; L1 superfamily, TNF superfamily, IL-6 superfamily, IL-17 family, type I superfamily and type II superfamily (Muñoz Carrillo *et al*., 2018).

Several researches reported the variations in cytokine expression in inflammatory disorders (Audet *et al.,* 2014). Sensitive detection techniques are required to observe their expression and secretion under various physiological conditions. Many techniques allow quantitation of cytokine expression at the protein level like ELISA and at the mRNA level like reverse transcriptase polymerase chain reaction (RT-PCR) (Skalnikova *et al.,* 2017).

Subclinical endometritis is difficult to detect, the animals appear healthy but they are carrier of bacteria that cause infection to other animals. Therefore, the control of subclinical endometritis is the best way to reduce the harmful effect of this disease (Molina-Coto and Lucy, 2018). Since the inflammatory responses were known to be inducted by the cytokines during the infection, the

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difference in cytokines expression profiles became with valuable impact in the early detection of subclinical endometritis-infected buffalo. The present study aimed to investigate the difference in some cytokines gene expressions between healthy and endometritis-infected buffaloes reared in Egypt using RT-qPCR.

2. Materials and Methods:

2.1. Samples and bacterial identification

The uteri samples were obtained from 120 Egyptian buffaloes; **60** infected with endometritis and **60** normal ones. Buffaloes with endometritis showed abnormal secretions with signs of inflammation such as swelling, redness and hardness in the uterus.

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.

2.2. RNA extraction and cDNA synthesis

RNA was extracted from uteri samples using a total RNA purification kit (Jena Bioscience, Germany), according to the 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 the 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 the RevertAid First Strand cDNA Synthesis kit (Fermantas) according to the manufacturer's instructions.

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

Gene expressions were detected by real-time PCR, which was performed using the 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 include 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. Data analysis

A 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 (Livak and Schittgen, 2001). 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 information of tested genes and the housekeeping gene

Gene	Primer sequences	Amplicon size (bp)	References
Interleukin-1 alpha	F: AGAGGATTCTCAGCTTCCTGTG	224-bp	
(*IL-1α*)	R: ATTTTTCTTGCTTTGTGGCAAT		
Interleukin-1 beta	F: GAG GAG CAT CCT TTC ATT CAT C	229-bp	
(*IL-1β*)	R: TTC CTC TCC TTG TAC GAA GCT C		Herath *et al.*,
Interleukin-6	F: ATG ACT TCT GCT TTC CCT ACC C	180-bp	(2009)
(*IL-6*)	R: GCT GCT TTC ACA CTC ATC ATT C		
Interleukin-10	F: TACTCTGTTGCCTGGTCTTCCT	178-bp	
(*IL-10*)	R: AGTAAGCTGTGCAGTTGGTCCT		
Tumor necrosis factor alpha	F: CGG TGG TGG GAC TCG TAT G	352-bp	Coussens *et al*.,
(*TNF-α*)	R: CTG GTT GTC TTC CAG CTT CAC A		(2004)
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	F: CCT GGA GAA ACC TGC CAA GT	214-bp	Buza *et al*.,
	R: GCC AAA TTC ATT GTC GTA CCA		(2003)

3. Results and Discussion

Two immunity systems are considered the key factors for controlling infectious diseases; innate and adaptive systems. Cytokines play an orchestral role in the balance between these two systems (Silva-Barrios and Protozoan, 2017). Cytokines are involved in the regulation of tissue repairing during inflammation (Grignani and Maiolo, 2000). They are secreted by immune cells and act on different target cells with major effects on the function of many immune genes including other cytokines (Carson and Kunkel, 2017). Many reports discussed the effect of uterine infection at postpartum on the ovarian function, embryo development and the decrease of the frequency of successful pregnancy. These fertility disorders lead to economic loss in livestock production, especially in buffalo (Patra *et al.*, 2013). In this respect, Osman *et al.*

(2018) suggested the existence of novel polymorphic sites in TLR4 gene in the river buffalo Egyptian breed and their accompanied with occurrence of endometritis disease. This study was considered to assess the differential expression of some cytokine genes in endometritis-infected buffalo reared in Egypt. Five cytokine genes: *IL-1α*, *IL-1β*, *IL-6*, *IL10* and *TNF-α* in addition to *GAPDH* (as a housekeeping gene) were included in this study.

Bacterial examination of uteri from 60 endometritis-infected buffaloes showed the presence of bacterial contamination in these samples; 33 samples with *E. coli*, 21 samples with *P. Klebsiella pneumonia* and 6 samples with *P. vulgaris*. The sixty apparently-healthy buffaloes did not show any sign of endometritis symptoms, and their uteri did not show any pathogenic bacteria.

The quantitative gene expressions of these cytokine genes were evaluated in one hundred-twenty buffaloes; sixty with endometritis infection and other sixty apparently healthy animals. Ct was calculated in triplicate for each sample and the mean was recorded for each sample.

IL-1α is a member of the interleukin 1 family, and it is related to the inflammation production and fever promotion. So, the inhibition of this immune gene contributes in treatment of these disorders. *IL-1α* is produced mainly by macrophages, neutrophils and epithelial cells. This cytokine has a major role in the regulation of the immune system through different physiological and hematopoietic activities including increasing of blood neutrophils and activation of fibroblasts and neutrophils proliferations (Dinarello, 2017).

The Cts for 60 infected buffaloes ranged from 19.79 to 31.77 with a mean value of 25.44, whereas their values ranged from 22.21 to 31.15 with a mean of 26.14 in normal buffaloes. The calculated -ΔΔCt was 0.37; which means that the expression of this gene is up-regulated with 1.3 folds (Fig. 1). The p-value of the up-regulation of *IL-1α* gene expression is 0.017 (<0.05) as a significant level.

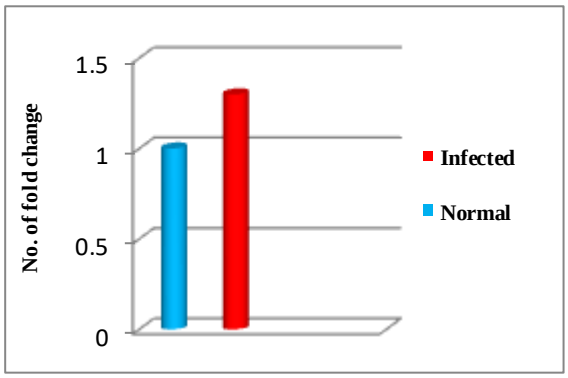


Figure 1: No. of fold changes in *IL-1α* gene expression between normal and infected animals

Gene expression profiles of this cytokine were extensively studied in livestock infected with different diseases. Using of *Yersinia enterocolitica* as a model enteric pathogen, Dube *et al.* (2001) identified the role of *IL-1α* for induction of pathologic inflammation during bacterial infection. Aho *et al.* (2003) recorded the enhanced expression of *IL-1α* in Johne's disease-infected cattle compared with healthy animals using cDNA microarray and RT-qPCR. On the other hand, the pro-inflammatory mediators' expressions as *IL-1α* was reported to be higher during the first week post partum as well as in infertile than fertile animals (Herath *et al.*, 2009). In the same respect, mRNA of *M. avium* subsp. *paratuberculosis* infected-animals expressed elevated measures of many interleukins including IL-1α comparing to the similar tissues from normal cattle (Coussens *et al.*, 2004 and DeKuiper and Coussens, 2019).

The cytokine interleukin-1β (*IL-1β*) has a major role in the inflammatory response, where its secretion is essential for the response and resistance of infected host to pathogens (Lopez-Castejon and Brough, 2011). The pro-inflammatory cytokine *IL-1β* is expressed by several cells which include monocytes, macrophage, neutrophils and NK cells. The *IL-1β* secretion and its continuity depends on the strength of stimuli and the requirement for it (Chan and Schroder, 2020).

In this study, the differential expression of this *IL-1β* immune gene was assessed in endometritis-infected buffalo. The threshold cycles of infected animals ranged from 17.46 to 32.68 with a mean value of 23.01. The Ct values in normal animals ranged from 18.17 to 34.34 with a mean of 24.10. Relative to Ct values of *GAPDH*, the -ΔΔCt was 0.76, which means; the *IL-1β* expression was elevated in infected buffaloes with 1.7 folds (Fig. 2) compared to healthy animals. The p value of up-regulation of *IL-1 β* gene expression is 0.032 (<0.05) as a significant level.

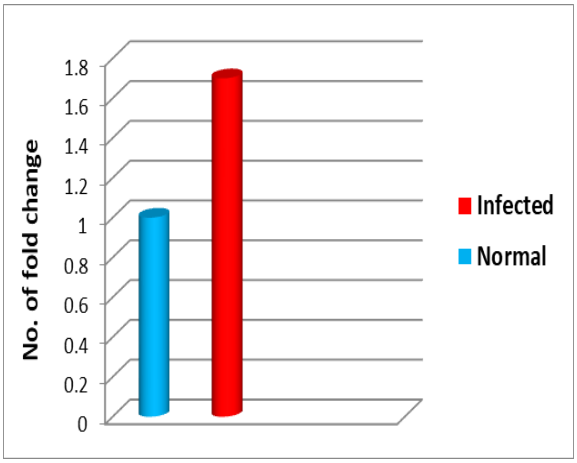


Figure 2. No. of fold changes in *IL-1β* gene expression between infected and normal animals

The up-regulation of *IL-1β* gene expression was recorded in some infected livestock compared with healthy ones. By comparing the expression in sheep ileal tissue for thirteen genes with three different forms of paratuberculosis, Smeed *et al*. (2007) has reported higher levels of tested cytokines including *IL-1β* compared to normal's tissues. Confirming the hypothesis that postpartum cattle suffer from endometritis and infertility as a result of the limited inflammatory response to uterine bacterial infection, Herath *et al.* (2009) declared the elevation of *IL-1β* expression in infected and infertile animals than that in healthy cattle. Abdel Aziz *et al*. (2014) analyzed the expression profile of three cytokine genes in response to bovines infected with *Babesi*a. The results showed highly significant up-regulation of *IL-1β* gene in *Babesia*-infected cattle compared to non-*Babesia* infected animals.

Interleukin 6 (*IL-6*) is acting as a pro-inflammatory cytokine as well as anti-inflammatory myokine, and it is an important mediator of the acute phase response and fever

(Tanaka *et al*., 2014). In the innate immune system macrophages secrete *IL-6* responding to pathogen-associated molecular manner linked to a group of recognition receptors pattern. These receptors, which exist on the cell surface, stimulate intracellular signaling cascades that induce inflammatory cytokine production (Woo *et al*., 2017). This interleukin can change the body's temperature setpoint through initiating Prostaglandin E2 synthesis in the hypothalamus after crossing the blood brain barrier, and it induces energy mobilization in fatty tissue and muscle that increases the body temperature (Zampronio *et al*., 2015).

The differential expression of *IL-6* in healthy and endometritis-infected buffaloes was studied in this work. The range of threshold cycles for infected animals was detected between 18.49 and 33.11 with a mean value of 25.43, whereas it was from 16.48 to 37.3 with a mean of 28.09 in healthy buffaloes. The -ΔΔCt value of this gene was 2.33, which means that the expression of *IL-6* is increased in infected animals by 5 folds (Fig. 3) compared to healthy buffaloes. The p value of up-regulation of this immune gene expression is 0.001 as a highly significant level.

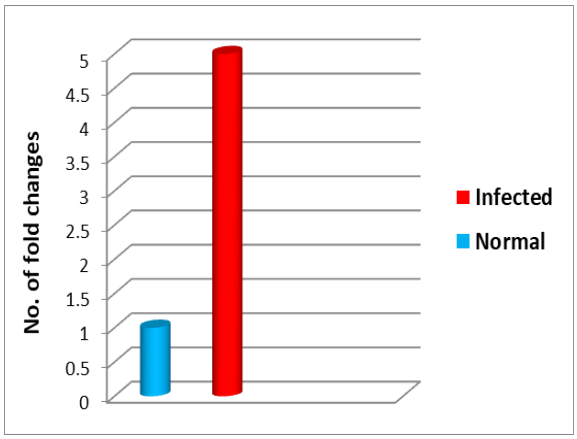


Figure 3: No. of fold changes in *IL-6* gene expression between normal and infected animals

Harris *et al.* (2000) reported the significant elevation of some cytokine molecules number including *IL-6* in gastric tissue infected with *Helicobacter pylori* comparing with the pre-infection numbers. *IL-6* mRNA expression in *M. avium* subsp. *Paratuberculosis*-infected cows was significantly greater ($P<0.05$) than that in healthy animals, where it was more than 6.5-fold higher in infected animals (Coussens *et al.*, 2004). On the other hand, Glass and Jensen (2007) detected the gene expression of some pro-inflammatory cytokines in cattle infected with *Theileria annulata* and reported the non-significant increase of *IL-6* in infected animals compared to controls. Maranga *et al.* (2013) reported the significant ($p<0.05$) upregulation of *IL-6* expression in experimentally *Trypanosoma brucei*-infected monkeys compared with uninfected animals. The upregulation of this cytokine expression was observed in VSV-infected swine suggesting a strong correlation between *IL-6* and the infection of pig with vesicular stomatitis virus (Velazquez-Salinas *et al*., 2019).

Interleukin 10 (*IL-10*) is a cytokine that has strong anti-inflammatory characters and plays a major role in response to infected-host against pathogens. Therefore, the disturbance in the expression of this immune gene leads to the increased risk for infection with autoimmune diseases (Iyer and Cheng, 2012). During the infection, *IL-10* does not only inhibit the activity of some immune cells including macrophages, which is important for pathogen clearance, but also it has a role in tissue damages. This controversial protein is produced with different types of cells, and its source may vary between different tissues and during the response to the same infection (Couper *et al.*, 2008). Upon infection, the unclear behavior of this gene was one of the main reasons for this study to shed the light on this important cytokines in order to assess its expression in endometritis-infected buffaloes.

Regarding its expression assessment, Ct values ranged from 24.01 to 32.71 with a mean of 26.74 in endometritis-infected animals, whereas the range of Ct values in healthy ones was 19-8-28.7 with a mean value of 24.86. The calculated -ΔΔCt of *IL-10* in this study was -2.21, which means that the expression of *IL-10* was down-regulated in infected animals by 0.2 folds (Fig. 4). The p value for down-regulation of *IL-10* gene expression is 0.15 as non significant level.

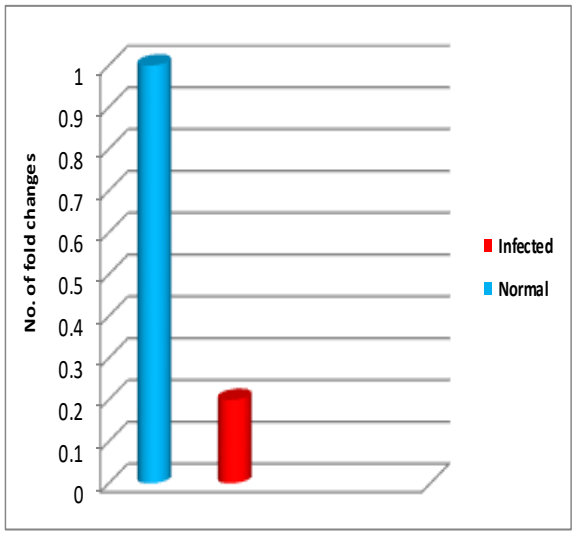


Figure 4. No. of fold changes in *IL-10* gene expression between normal and infected animals

Coussens *et al.* (2004) examined the expression of ten interleukins in addition to other three cytokines in cattle naturally-infected with Johne's disease. The differential expression of all tested genes did not require stimulation with the exception of *IL-10* which required enhancement by *M. avium* subsp. *paratuberculosis* stimulation of PBMCs from sub-clinically infected cattle. *IL-10* was not included with the cytokines whose expression was recorded to be increased during the first week postpartum (Herath *et al.*, 2009). They reported higher ratios of both interleukins 1 (α and β) mRNA expression to *IL-10* in endometritis-infected cattle. The *IL-10* expression during infection with *Fasciola hepatica* was investigated by Mendes *et al.* (2013) using Qt-PCR. They reported a synergism between *IL-10* and *IL-4* with Interferon gamma in the liver tissues of infected cattle and suggested the role of *IL-10* in modulating the immune response.

Tumor necrosis factor alpha (*TNF-α*) is a cell signaling protein which has a role in systemic inflammation process. The main source of its production is the activated macrophages in addition to other producers like neutrophils and eosinophils (Parameswaran and Patial, 2010). In addition to the role of *TNF-α* in immune system

functions like antitumor and antimicrobial activities, it has a role in physiological activities including appetite, fever and endocrine activity (Kushibiki, 2011). The disturbance of TNF regulation is implicated in some diseases including Alzheimer's disease (Swardfager *et al.*, 2010), cancer (Ma *et al.*, 2016) and bowel disease (Brynskov *et al*., 2002).

The comparison between *TNF-α* expression in endometritis-infected and healthy buffaloes was performed in this study. The mean of Ct values in endometritis-infected animals was 25.41, where the Cts ranged from 20.08 to 31.15. The range of Ct values in healthy buffaloes was observed between 19.44 to 27.31 with a mean value of 24.38. The -ΔΔCt was -1.36, meaning that the expression of *TNF-α* was down-regulated in infected buffaloes with 0.4 folds (Fig. 5) compared to healthy animals. The p value of this statistical down-regulation is 0.009 (<0.01) at a high significant level.

Using real-time RT-PCR, Smeed *et al.* (2007) reported the increased level of *TNF-α* expression in sheep's ileal tissue infected with three types of Johne's disease as indication of persistent inflammatory lesions, whereas asymptomatic animals had increased levels of *TNFα* and significantly decreased levels of *IL-18*.

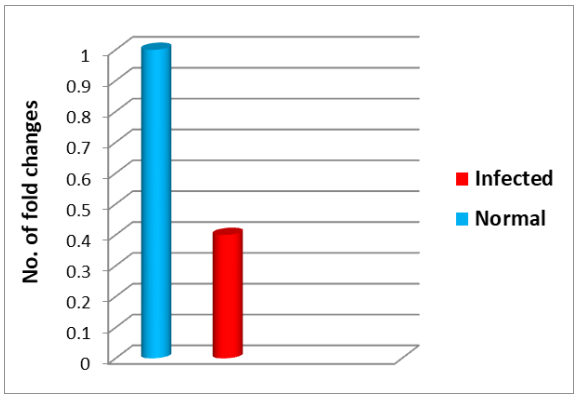
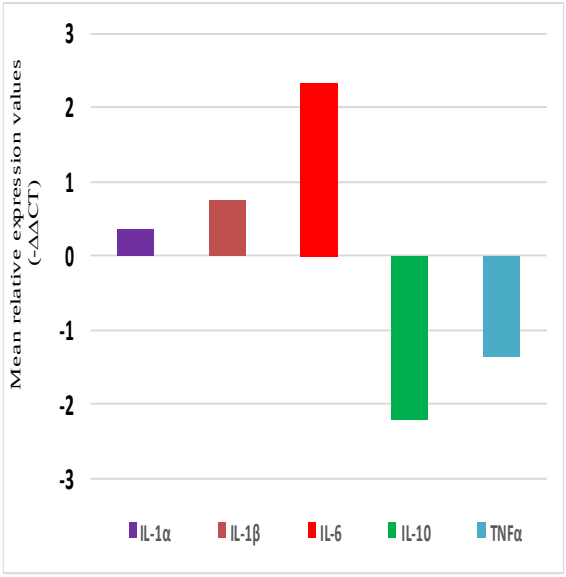


Figure 5: No. of fold changes in *TNF-α* gene expression between normal and infected animals

Aho *et al.* (2003) declared the significant high levels of gene expressions for some cytokines like *IL-1α* in tissues from infected cattle with Johne's disease. They also reported that increased levels of TRAF1 within the lesions of Johne's-infected cattle may result in cells that are highly resistant to TNF-*α* stimulated signaling. On the contrary to the above-mentioned results, Glass and Jensen (2007) registered the absence of differences in gene expression of some immune genes including *TNFa*, *IL-1β* and *IL-6* in infected cattle with *Theileria annulata*. Also, DeKuiper and Coussens (2019) argued that non-significant expression of cytokines like IFN-γ, *TNF-α* and *IL-17a* in CD4+ T cells from cows infected with Johne's disease may be due to the exhaustion of immune T cells. In the same context, Bojaroj *et al.*, (2016) declared that there is no significant difference in the percent of PBMCs-expressing *TNF-α* in cow infected with leukemia virus even with the presence of different *TNF*-alpha genotypes.

Finally, it can be concluded that tested cytokines genes in endometritis-infected buffaloes differ in their response to endometritis infection, where three of them (*IL-1α*, *IL-1β* and *IL-6*) were up-regulated, while the other two genes: *IL-10* and *TNF-α* were down-regulated with different levels of significant values. The mean relative expression values (-ΔΔCt) of the five tested genes in endometritis-infected buffaloes comparing to healthy animals were shown in Fig. 6.

Figure 6: Mean relative expression values (-ΔΔCt) of the tested genes in endometritis-infected buffaloes comparing to healthy animals



4. Conclusion

Innate and adaptive immune systems are considered the key factors for controlling infectious diseases. Cytokines play an orchestral role in the balance between these two systems, and they are involved in the regulation of tissue repairing during inflammation. This study was applied to determine the differential expression of some cytokine genes in endometritis-infected buffalo reared in Egypt. Five cytokine genes: IL-1α, IL-1β, IL-6, IL10 and TNF-α in addition to GAPDH (as a housekeeping gene) were included in this study. IL-1α, IL-1β and IL-6 were found to be up-regulated, while the other two genes: IL-10 and TNF-α were down-regulated with different levels of significant values. The identification of physio-pathological pathways underlying many complex diseases may be facilitated by studying the genetic factors involved in their occurrence, which will develop our understanding for the disease in its entirety and to define the risk of developing it. This will ease and pave the way to improve the disease resistance in herds by selective breeding as well as identify and synthesize innovative drugs.

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