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Original Article

Relative expression of pro-inflammatory cytokine genes in Holstein dairy cows naturally affected by *Escherichia coli* mastitis

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Abstract

Background: Bovine mastitis, the most common and costly disease, is characterized by mammary gland inflammation. In dairy cattle, *Escherichia coli*, one of the main causes of mastitis and its lipopolysaccharide (LPS), is a prominent virulence factor. The LPS is responsible for stimulating the expression of pro-inflammatory cytokines that are key components of the early response of the host's innate immunity and plays an important role in the subsequent inflammatory response to eliminate the infection. **Aims:** This study aimed to investigate the expression profiles of some pro-inflammatory cytokine genes (*IL-6*, *IL-8*, *TNF-α*, *IFN-γ*, and *GM-CSF*) in milk somatic cells of healthy cows and naturally infected cattle with *E. coli* in their first lactation. **Methods:** Milk samples were aseptically collected from six healthy cows and six cows with clinical mastitis. In animals with mastitis, those in which the cause of mastitis was only *E. coli* bacteria were selected for further research. Total RNA was extracted from the somatic cells of milk, the first strand cDNA was synthesized and real-time PCR was performed for the studied genes. As reference genes, *β-actin* and *GAPDH* were used to normalize the data. The REST and SAS programs analyzed the real-time data for significance. **Results:** Pro-inflammatory cytokine genes were expressed in all healthy cows and in cows with clinical mastitis. The expression profiles of cytokine genes showed significantly higher expression of the *IL-8*, *TNF-α*, *IFN-γ*, and *GM-CSF* ($P<0.01$) in cows with clinical mastitis compared with animals free of infection. Expression correlations were widely varied between all pairs of genes in healthy animals and those affected by mastitis. In healthy animals, a significant positive correlation was found between the mRNA expression of *IL-6* and *IL-8* genes ($P<0.01$). In addition, the mRNA levels of the GM-CFS showed a significant positive correlation with the expressions of both *IL-6* and *IL-8* genes ($P<0.01$). In cows suffering from clinical mastitis, an interesting finding was the presence of significant positive correlations between the mRNA levels of the *GM-CFS* and the expression levels of *IL-6*, *IL-8*, *IFN-γ*, and *TNF-α* genes ($P<0.05$). **Conclusion:** The study suggests that the *IL-8*, *GM-CFS*, *IFN-γ*, and especially *TNF-α* genes could be strong indicators of the early response of the immune system in the mammary gland of dairy cows naturally infected by *E. coli*. However, further studies should be conducted to confirm the findings of this study.

Key words: Cow, Cytokine, *E. coli*, Gene expression, Mastitis

Introduction

Mastitis is the most prevalent disease in dairy cows that greatly reduces the quality and quantity of milk and may eventually cause heavy tissue damage in the mammary gland, which can lead to an increase in culling rates (Burvenich *et al*., 2003; Vangroenweghe *et al*., 2005; Cobirka *et al*., 2020). Although the mastitis dynamics are influenced by the pathogen factors (Burvenich *et al*., 2003), the extent of the inflammatory response depends mainly on the individual cow factors such as cow's age, the lactation stage, and parturition (Burvenich *et al*., 2003; Wenz *et al*., 2006; Vangroenweghe *et al*., 2020). *E. coli* infection is considered to be the most common cause of clinical mastitis in dairy cattle (Bradley and Green, 2001; Schukken *et al*., 2011). The pathogenicity of *E.coli* can be due to the presence of virulence factors such as toxins, lipopolysaccharides (LPSs), hemolysins, and adhesins (Kaper *et al*., 2004; Steimle *et al*., 2016; Chen *et al*., 2017). In mammary glands infected by *E. coli*, the immune response begins with the interaction of *E. coli* with leukocytes and epithelial cells (Paape *et al*., 2002). The leukocytes are the first line of defense that is significantly involved in activating and regulating the innate immune response. These cells contain pathogen

recognition receptors (PRRs) on their membranes that recognize the pathogen-associated molecular patterns (PAMPs) on the invading pathogens. Toll-like receptors (TLRs) and lipopolysaccharides (LPSs) are the best-known classes of PRRs and PAMPs. In bovine mastitis caused by *E. coli*, the main pathway of immune response stimulation is binding the LPS with the TLR4, myeloid differentiation protein 2 (MD-2), and CD14. This binding leads to the activation of some cell signaling pathways such as the NF-κB (nuclear transcription factor-kappa B) cascade (De Schepper *et al*., 2008), and MAPKs (mitogen-activated protein kinase) that trigger the pro-inflammatory cytokines production (Guo *et al*., 2017; Jiang *et al*., 2017). Pro-inflammatory cytokines are mainly produced by activated macrophages and play a major role in inflammation regulation. The important pro-inflammatory cytokines are IL1-α, IL1-β, IL-6, and TNF-α. Other pro-inflammatory cytokines include IL-8, IL-11, IL-12, IL-17, IL-18, IL-20 family, IL-33, IFN-γ, GM-CSF, TGF-β, and a variety of other chemotactic cytokines (chemokines) that control the migration and residence of inflammatory cells (Özaktay *et al*., 2006; Smith *et al*., 2012). Various attempts confirmed that the expression of cytokine genes displayed considerable variation in the milk somatic cells of healthy and infected mammary glands in dairy cattle (Leutenegger *et al*., 2000; Alluwaimi *et al*., 2003; Fonseca *et al*., 2009; Hassan and Torky, 2016). In addition, the immune response of the mammary gland to *E. coli* infection has been intensively studied in dairy cattle. For example, the up-regulation of genes associated with immune response was observed following experimental intramammary infection of mammary gland quarters in the microarray method (Mitterhuemer *et al*., 2010; Buitenhuis *et al*., 2011). In both studies, most up-regulated genes were mainly in the chemokine and cytokine signaling-associated pathways groups in the first 24 h of the inflammatory response. This early cytokine and chemokine response was shown to be a critical mechanism during *E. coli* mastitis and is known to play an important role in the inflammatory process (Bannerman *et al*., 2004). In response to the infection of the mammary glands, the increase in the concentration of pro-inflammatory cytokines such as interleukin IL-1β, IL-8, IL-12, IFN-γ, and TNF-α in milk is well documented. Thus, this increase in cytokines is often observed in animals infected by Gram-negative bacteria such as *E. coli* (Bannerman *et al*., 2004; Rambeaud *et al*., 2006; Kauf *et al*., 2007; Vitenberga-Verza *et al*., 2022). In addition, *in silico* analysis and experimental validation confirmed that IL-8 is a putative early diagnostic marker for mastitis in dairy cattle (Huma *et al*., 2020). To date, there has been no report on the cytokine genes expression profile in cows naturally infected by *E. coli* in the first lactation. Therefore, this study aimed to characterize the expression profile of pro-inflammatory cytokine genes (*IL-6*, *IL-8*, *TNF-α*, *IFN-γ*, and *GM-CSF*) in milk somatic cells of healthy dairy cows, and cows naturally infected by *E. coli* immediately after the onset of clinical signs and before any drug treatment, in their first lactation.

Materials and Methods

Animals and sample preparation

Six healthy first-lactation Holstein cows were selected 7-10 days after parturition. In addition, six dairy cows in their first lactation with clinical mastitis, right before the starting treatment, were also used in this experiment. The selection criterion in healthy cows was SCC <350,000/ml. In healthy cows, one liter of milk sample, representing all four quarters, was collected in sterile tubes. The milk samples from cows with clinical mastitis were taken from the affected quarter immediately after the onset of clinical signs and before antibiotic treatment. In cows with clinical mastitis, aliquots of 3 ml were used to identify the major pathogens of mastitis by the Hucker method (Hucker, 1933). The remainder of the milk was centrifuged for 20 min at 1500 g at 4°C. The cell pellet was washed in PBS pH 7.4 twice and centrifuged for 20 min at 4°C and 220 g. The obtained pellets were lysed with 500 µL PBS-EDTA and kept at -80°C until RNA extraction.

Real-time PCR

Total RNA was isolated using DENAzist total RNA extraction kit (DENAzist Asia, Iran) according to the manufacturer's protocol. The extracted RNA samples were treated with DNase I (CinnaGen, Tehran, Iran) to remove any DNA contamination. The quality and quantity of extracted RNA samples were assessed by agarose gel electrophoresis and spectrophotometric readings. The first strand cDNA was synthesized with AccuPower® RocketScriptTM RT PreMix kit and random hexamer primers (Bioneer Company, Korea) according to the manufacturer's instructions. The final volume was adjusted to 50 µL with RNase-free water. The amplified cDNA samples were then stored at -20°C until further analysis. To evaluate gene expression, we used the primers previously reported by Lee *et al*. (2006). In addition, *GAPDH* (Leutenegger *et al*., 2000) and *β-actin* (Lee *et al*., 2006) genes were selected as the reference genes for the calculation of dCp (Table 1). Real-time PCR was performed using CFX96 Real-time System (Bio-Rad, USA) and HotTaq EvaGreen qPCR kit (CinnaGen, Tehran, Iran), as described by the manufacturer. All real-time PCR reactions were conducted in duplicate. Amplification conditions were 95°C for 15 min; 50 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 5 to 25 s (depending on the product length, 5 s per 100 bp). Then, the presence of nonspecific products and primer dimmers were assessed by dissociation curve analysis of samples (melting curve by 95°C for 5 s, 65°C for 15 s, and 95°C for 0 s).

The qPCR amplification efficiency was investigated using standard curve construction for each primer. To do so, a 10-fold dilution series were produced over six points starting from obtained cDNA samples. The standard qPCR was performed for all the primer pairs in

Table 1: Primers details for bovine cytokines and reference genes

Gene	Primer	Sequence (5′-3′)	Length	Accession
IL-6	IL-6.f209 IL-6.r313	TCATTAAGCGCATGGTCGACAAA TCAGCTTATTTTCTGCCAGTGTCT	105	NM173923
IL-8	IL-8.f251 IL-8.r355	CACTGTGAAAATTCAGAAATCATTGTTA CTTCACAAATACCTGCACAACCTTC	105	NM173925
IFN-γ	IFN-γ.f296 IFN-γ.f480	TCATTAAGCGCATGGTCGACAAA TCAGCTTATTTTCTGCCAGTGTCT	185	M29867
TNF-α	TNF-α.f2377 TNF-α.r2794	TCTTCTCAAGCCTCAAGTAACAAGC CCATGAGGGCATTGGCATAC	103	XM0275241
GM-CFS	GM-CFS.f170 GM-CFS.r250	AGTAATGACACAGAAGTCGTCTCTG GCCGTTCTTGTACAGCTTCAGG	87	U22385
β-actin	β-actin.f38 β-actin.r428	CCTTTTACAACGAGCTGCGTGTG ACGTAGCAGAGCTTCTCCTTGATG	391	AH00130
GAPDH	GADPH.463f GADPH582r	GGCGTGAACCACGAGAAGTATAA CCCTCCACGATGCCAAAGT	120	AF022183

duplicate and Cp values were determined. The standard curve is constructed by plotting log template concentrations against Cp values. The slope (b), in linear regression, is used to estimate qPCR efficiency (Brankatschk *et al*., 2012). Then, the qPCR optimization was evaluated using the coefficient of determination (R^2) or Pearson's correlation coefficient (r).

Statistical analysis

Real-time quantitative PCR (RT-qPCR) was analyzed by the 2-ΔΔCT method with the REST© program (Pfaffl *et al*., 2002), which uses the pairwise fixed reallocation randomization test to compare differences in gene expression across groups. The 2-ΔΔCT or comparative method has been extensively used to investigate the relative changes in gene expression from RT-qPCR data (Livak and Schmittgen, 2001). The real-time PCR data were analyzed with Furthermore, SAS's PROC CORR was applied to calculate Pearson correlation coefficients in expressions between all pairs of genes in healthy and mastitis-affected conditions separately, using the normalized Cp values. The Pearson correlation coefficient (r) is the most common method measuring the direction and strength of linear relationship between two variables (Asuero *et al*., 2006).

Results

In order to investigate the expression of pro-inflammatory cytokine genes in cows suffering from clinical mastitis, the cows were selected, in which *E. coli* was the only cause of infection based on the pathogen identification results. In addition, the selected animals in the healthy and mastitis groups were both in the first lactation. The standard curves for *IL-6*, *IL-8*, *IFN-γ*, and *TNF-α*, *GM-CFS* and two housekeeping genes (*β-actin* and *GAPDH*) are shown in Fig. 1. The amplification efficiencies were 1.82 (*β-actin*), 1.86 (*GAPDH*), 1.89 (*TNF-α*), 1.90 (*IL-8*), 1.94 (*IFN-γ*), 1.96 (*GM-CFS*) and 1.97 (*IL-6*), respectively. In addition, the coefficient of determination (R2) varied between 0.95 and 0.99 for different amplicons.

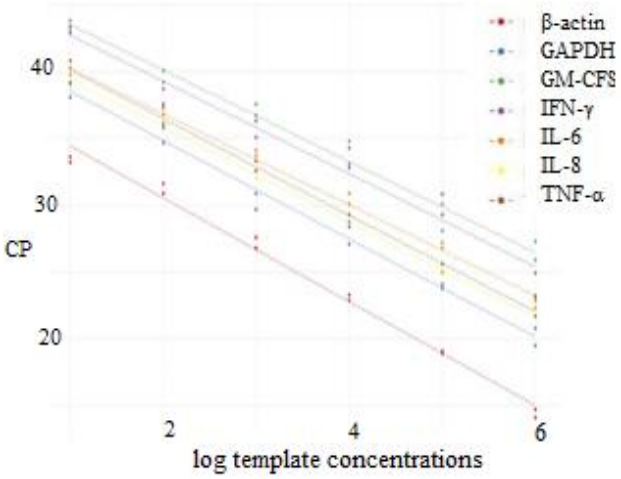

Fig. 1: Standard curves for *IL-6*, *IL-8*, *IFN-γ*, *TNF-α*, *GM-CFS*, *β-actin* and *GAPDH* genes

In this study, the expression of five cytokine genes including *IL-6*, *IL-8*, *IFN-γ*, and *TNF-α*, and *GM-CFS*, were observed in the milk cells of healthy cows and in cows with clinical mastitis. The mRNA expression of pro-inflammatory cytokine genes in cows with clinical mastitis was represented as the induction of folds compared with the healthy gland, and they showed large variations among the animals in terms of magnitude (Table 2). The results show no significant difference in the mRNA levels of *IL-6* in cows with clinical mastitis relative to free-infected cows, but the expression of other cytokine genes were significantly higher in animals with clinical mastitis compared with healthy animals ($P<0.01$). However, in the infected cows, the expression of IL-6 was 2.11 times higher than in healthy cows, but this difference was not significant ($P=0.34$). A striking difference was found in the mRNA expression of *TNF-α* between infected and healthy animals. The infected cows

showed a ~8754.65-fold increase in *TNF-α* transcript compared with the control group. In addition, a high expression difference was observed in the *IFN-γ* gene, so its mRNA expression in animals with clinical mastitis was 405.2 times higher than in healthy animals. The expression levels of *GM-CFS* and *IL-8* in cows with clinical mastitis were 140.47 and 75.02 folds higher than in healthy cows, respectively (Table 2).

Table 2: Cytokine gene expression levels in the animals with clinical mastitis compared with healthy cows

Gene	Expression fold	P-value	Results
IL-6	2.11	0.34	-
IL-8	75.02	0.007	Upregulated
IFN-γ	405.20	0.002	Upregulated
TNF-α	8754.65	0.004	Upregulated
GM-CFS	140.47	0.001	Upregulated

We computed the correlation coefficients in expressions between all pairs of genes in healthy and mastitis-affected cows (Table 3). Correlations between the expression of genes were completely discriminated in healthy animals and those affected by clinical mastitis. In healthy animals, there is a significant positive correlation between the expression of *IL-6* and *IL-8* genes (P<0.01). In addition, *GM-CFS* gene expression showed a significant positive correlation with both *IL-6* and *IL-8* genes (P<0.01). In animals with clinical mastitis, a very interesting finding was the existence of significant positive correlations between the mRNA level of the *GM-CFS* gene and the expressions of *IL-6*, *IL-8*, *IFN-γ*, and *TNF-α* genes (P<0.05). In contrast, no significant correlation was observed between other genes.

Discussion

Mastitis is a bacterial infection of the udder tissue that leads to inflammation in the mammary gland. In response to invading bacteria, innate and specific immunity are two distinct defense mechanisms in the mammary gland. The somatic cells of milk contain several immune cell types such as neutrophils, macrophages, and lymphocytes. The macrophages are the major cell type in the healthy mammary gland whereas neutrophils are the predominant cell population in the course of early inflammation. Following the bacterial invasion, mainly macrophages activated by bacterial virulence factors such as LPS induce neutrophil recruitment in infected mammary glands by inflammatory mediators. Several pro-inflammatory cytokines participate in inducing the acute phase response and allowing leukocyte accumulation at the infection site (Riollet *et al*., 2002). In the present study, we evaluated the expression of some pro-inflammatory cytokine genes including *IL-6*, *IL-8*, *IFN-γ*, *TNF-α*, and *GM-CFS*, in milk somatic cells of healthy and naturally infected cows, by *E. coli* in their first lactation, right before any treatment. IL-6 is a pro-inflammatory cytokine produced by various cells and involved in T-cell activation and differentiation and inhibition of TNF production (Diehl and Rincón, 2002). Il-8 is a neutrophil chemotactic cytokine produced by an array of cell types (Remick, 2005), and plays an important role in attracting neutrophils to the infected bovine mammary gland by blocking the neutrophil chemotactic activity with anti-IL-8 antibodies (Rabot *et al*., 2007). The IFN-γ induces macrophage functions such as antigen presentation and increasing lysosome activity. In addition, IFN-γ stimulates the differentiation of Th1 cells and concomitantly suppresses the Th2 activity (Schukken *et al*., 2011). TNF-α cytokine is produced by the immune and non-immune cells in inflammatory and infectious conditions (Aggarwal *et al*., 2002; Flavell, 2002). Therefore, TNF-α is a potent pro-inflammatory cytokine that has pleiotropic effects on various cell types (Bradley, 2008). GM-CSF belongs to the family of hematopoietic cytokines that stimulate the antibacterial functions of neutrophils and monocytes (Hamilton, 2008).

In this study, transcriptions of the selected cytokine genes were observed in all animals of two groups. However, except for *IL-6*, the expression levels of *IL-8*, *IFN-γ*, *TNF-α*, and *GM-CFS* genes showed a significant increase in milk somatic cells of the naturally *E. coli* infected cows compared with the healthy cows. Generally, neutrophils migration from the bloodstream to the site recruitment is IL-8 dependent (Kehrli and Harp, 2001). In addition, IL-8 also has an important role in activating neutrophils during early inflammatory processes (Galligan and Coomber, 2000). Milk

Table 3: Correlation coefficients between the transcriptional activity of the target genes in healthy and mastitis-affected animals

Gene	Animals	Correlation coefficients			
		IL-6	IL-8	IFN-γ	TNF-α
IL-8	Healthy	0.99 (0.001)	-	-	-
	Infected	-0.27 (0.59)	-	-	-
IFN-γ	healthy	0.23 (0.66)	0.21 (0.68)	-	-
	Infected	0.69 (0.13)	0.19 (0.17)	-	-
TNF-α	Healthy	-0.29 (0.57)	-0.27 (0.60)	-0.17 (0.75)	-
	Infected	0.52 (0.29)	-0.12 (0.82)	0.50 (0.31)	-
GM-CFS	Healthy	0.96 (0.002)	0.97 (0.002)	0.43 (0.39)	-0.35 (0.49)
	Infected	0.81 (0.04)	0.95 (0.03)	0.81 (0.04)	0.86 (0.03)

Numbers in parentheses are P-values

concentration of IL-8 has been observed to increase within 18 to 24 h of *E. coli*-induced infection and to reach ranging from 100 to 250 pg/ml (Shuster *et al*., 1997). In addition, an increase in *IL-8* mRNA expression was found in milk somatic cells isolated from *E. coli*-infected glands. However, *IL-6* mRNA expression was highly reduced in milk somatic cells isolated from quarters infected with *E. coli* (Ma *et al*., 2011). In cows with experimentally induced *E. coli* mastitis, *IL-8* mRNA level was increased in epithelial cells of the mammary gland, especially surrounding the alveoli, at all-time points (McClenahan *et al*., 2006). High concentrations of IL-8 and TNF-α were observed in the milk of mammary glands infected by Gram-negative bacteria, such as *Escherichia coli*, *Klebsiella pneumonia*, or *Pseudomonas aeruginosa*, but the concentrations of IL-8 and TNF-α were lower or undetectable in the cow's milk with udders infected by *Staphylococcus aureus* (Riollet *et al*., 2000; Bannerman *et al*., 2004). Significant increases were observed in *L-1β*, *IL-6*, *IL-8*, and *TNF-α* at mRNA levels in either milk or mammary tissues of cows that infected experimentally with *E. coli* (Lee *et al*., 2006). The milk concentrations of TNF-α, IL-1β, and IL8 were increased in *E. coli*-infected mammary glands (Waller *et al*., 2003). In addition, milk concentrations of TNF-α and IFN-γ were relatively high in the *E. coli* group (Safak *et al*., 2022). The mRNA transcription of *IL-6* was detected in mammary glands infected with *E. coli* as early as 14 h pi and earlier in endotoxin-infused mammary glands (Sbuster *et al*., 1993). Increases in mRNA levels of *IFN-γ* have been observed in milk somatic cells of mammary glands infected with *S. aureus* (Riollet *et al*., 2000) and *E. coli* (Lee *et al*., 2006). Milk concentrations of IFN-γ protein have been increased in naturally occurring mastitis and also in experimentally induced mastitis by *E. coli*, *Mycoplasma bovis*, *S. aureus*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Streptococcus uberis* (Hisaeda *et al*., 2001; Bannerman *et al*., 2004; Kauf *et al*., 2007). The expression levels of *TLR-2*, *IL-1β*, *IL-10* and *Hp* were found to be significantly higher in the milk somatic cells of cattle with subclinical mastitis as compared with healthy ones, while down-regulation was observed in the mRNA levels of *TLR-4*, *TNF-α*, *IFN-γ*, and *IL-6* (Singathia *et al*., 2023). The up-regulation of pro-inflammatory cytokine genes and higher production of IL-6 and IL-8 were observed in bovine punch-excised teat tissue during early infection of cattle by *E. coli* (Noleto *et al*., 2023).

Generally, the promoter region of most pro-inflammatory genes contains binding sites for the nuclear factor κB (NF-κB); thus, their expression partly depends on the NF-κB transcription factor. The high levels of active NF-κB complexes were always found in milk cells of cows with acute mastitis, whereas levels of NF-κB activity were undetectable in milk cells of healthy cows. The IL-8 and GM-CSF are NF-κB-dependent pro-inflammatory cytokines involved in initiating and perpetuating neutrophilic inflammation. The level of NF-κB activity was drastically correlated with the expression levels of *IL-8* and *GM-CSF* in milk cells of mastitis-affected cows (Boulanger *et al*., 2003). The present study showed significant increases in mRNA levels of *IL-8* and *GM-CSF* in milk cells of *E. coli*-infected animals compared with healthy cows. In addition, significant positive correlations were found between the transcriptional activity of *IL-8* and *GM-CSF* in both healthy and infected animal groups. However, obtained correlations were different between the expression of studied genes in healthy and infected animals. In another study, the correlation of transcriptional activity of cytokines genes including *IL-6*, *IL-8*, *IL-12*, *IFN-γ*, *TNF-α*, and *GM-CFS* showed a significant negative correlation between *IL-8* and *IL-12* in cows with subclinical mastitis in the Gir breed. In addition, *TNF-α* showed a positive significant correlation with *GM-CSF* and IFN-γ in crossbred cattle. The correlations of other cytokines were not significant in Gir and crossbred cattle (Bhatt *et al*., 2014). The discrimination in the correlations between cytokine gene expressions in two groups could be attributed to the mammary gland conditions in healthy and infected animals, which has resulted in differences in the expression of pro-inflammatory cytokines.

In general, the pathogen type and the host's conditions determine the early immune response of the mammary glands. *E. coli*, among Gram-negative bacteria, is the most dominant pathogen that leads to infection of the mammary cells in dairy cows. In bovine mastitis caused by *E. coli*, the stimulation of the immune response leads to the activation of some cell signaling pathways that regulate inflammation by producing pro-inflammatory cytokines. Our results showed that the expression of some pro-inflammatory cytokine genes such as *TNF-α*, *IFN-γ*, *GM-CFS*, and *IL-8*, was significantly higher in the milk cells of cows naturally infected with *E. coli* compared with healthy cows in their first lactation. Therefore, monitoring the expression and milk concentration of pro-inflammatory cytokines in the mastitis condition could be a potential marker for early diagnosis of mastitis caused by *E. coli* in dairy cattle.

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Conflict of interest

The authors declare that they have no conflict of interest.

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