Research Article

Expression changes of CD177 and MPO as novel biomarkers in lung tissue of CLP model rats

Azadeh RASOOLI[1,*] 📷, **Elham GHAFARI**[1] 📷, **Hamed SAEDI**[2] 📷, **Saba MIRI**[3] 📷
[1]Department of Biochemistry, Faculty of Sciences, Payame-e-Noor University, Tehran, Iran
[2]Department of Biology, Damghan Branch, Islamic Azad University, Damghan, Iran
[3]National Institute of Scientific Research (INRS-ETE), University of Quebec, Quebec City, Canada

Background/aim: Sepsis is an unregulated systemic response to microbial invasion that can lead to multiple organ failure. This study aims at investigating the relationships among myeloperoxidase (MPO) and CD177 in major organ systems including whole blood, liver, and lung tissues in septic rats.

Materials and methods: Sepsis was induced by cecal ligation and puncture (CLP) in female Wistar rats. Whole blood, liver, and lung samples were obtained from rats of 3 groups (n = 10 for each group, n total = 30: control as a wild-type group, laparotomy group (LAP), and CLP). Gene expression of MPO and CD177 in targeted tissues was determined by real-time PCR after CLP. MPO activity was also determined by ELISA method for the result validation of the real-time PCR.

Results: Expression levels of MPO increased significantly in all targeted organs in the CLP group, while CD177 expression was upregulated only in lung tissue in response to sepsis (P < 0.05). The results obtained with ELISA analysis also show that MPO level was significantly increased in all the targeted tissues in the CLP group (P < 0.05).

Conclusion: A high level of MPO as an inflammatory enzyme can be a potentially novel biomarker for sepsis in all organs. On the other hand, CD177 may be a marker in lung tissue.

Key words: Sepsis, gene expression, myeloperoxidase, CD177

1. Introduction

Sepsis is a life-threatening and complex inflammatory response to infection with a variety of comorbidities. After sepsis, the immune response is associated with a major inflammatory response leading to shock, organ dysfunction, and the development of immune alterations that can directly or indirectly impair the function of almost all types of immune cells (1,2). Some key inflammatory enzymes, including myeloperoxidase (MPO), are involved in the disease in question (3). The measurement of CD177 mRNA levels has also become a useful diagnostic tool for distinguishing some diseases (4).

Neutrophils have various functions in the immune system in some diseases such as sepsis, circulating lipopolysaccharide (LPS) released from bacteria that may activate both neutrophils and monocytes in whole blood. The activated neutrophils release reactive oxygen intermediates such as MPO with strong oxidative activity (5). CD177 is an important neutrophil gene that encodes the glycoprotein of the membrane and the expression of

this gene is increased during bacterial infections, burns, and pregnancy (6–8).

Neutrophils are sequestered from the blood during systemic inflammation, a process that is accompanied by organ and tissue infiltration, including the liver and lungs (9,10). The mechanisms mediating neutrophil infiltration appear to be tissue-specific (11). Because of the lack of knowledge on the relationship between CD177 and MPO as a biomarker for sepsis, we performed a gene expression study on these genes in the response of systemic inflammation in 3 main involved organs including whole blood, the liver, and the lungs.

2. Materials and methods
2.1. Animal treatment

Female Wistar rats weighing between 120 and 150 g were maintained at room temperature (22–23 °C) and 50% humidity in a controlled environment. Water and food were available ad libitum. Animal experimentation was performed according to ethical committee and

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* Correspondence: a.rasouli_57@yahoo.com

institutional animal care and ethical guidelines. The animals were divided into 3 groups (control, laparotomy (LAP), and cecal ligation and puncture (CLP)). There were ten rats in each group (n total = 30). Control groups did not receive any surgical intervention. For LAP groups the cecum was minimally handled without ligation and puncture due to a combination of the effect of anesthesia, laparotomy, mobilization of abdominal contents, and postoperative analgesia (12).

2.2. Cecal ligation and puncture (CLP) model

The CLP model is described in Figure 1. First, the rats were anesthetized by injections (i.p.) of a mixture of ketamine (90 mg/kg b.w.) and xylazine (10 mg/kg b.w.). A small midabdominal incision (2–3 cm) was made, as shown in Figures 1a and 1b, and the cecum was exposed. A distended portion of the cecum just distal to the ileocecal valve was isolated (as shown in Figure 1c), filled with fecal content, and tied with a 3-0 silk suture in a manner so as to not disrupt bowel continuity (Figure 1d). The ligated portion of the cecum was punctured twice with a 20-gauge needle (Figure 1e). The cecum was then replaced in its original position within the abdomen and the abdomen was then closed with a 3-0 suture in two layers (Figure 1f). Then the animals were allowed to recover. Immediately after surgery,

normal saline (3 mL/100 g b.w.) was subcutaneously given to all rats as fluid resuscitation.

2.3. Sample collection

Blood samples were obtained from the hearts of the rats. Heparinized blood was used for RNA extraction. Immediately after death, the lungs and liver were excised and washed with normal saline to remove blood, and then were used for RNA extraction.

2.4. RNA extraction and reverse transcription

Total RNA was isolated from fresh peripheral whole blood and lung and liver tissue. Total RNA was prepared with an RNA Total Kit (BioBasic BS584, Canada). To ensure that the sample of extracted RNA was not contaminated with DNA, DNase treatment was carried out using DNase I (SinaClon Co., Iran). cDNA was then synthesized using the PrimeScript RT reagent kit (TaKaRa, Japan). Total RNA was quantified by a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The same amount of RNA (ng) in each reaction for cDNA synthesis was used. Primers for real-time PCR were designed with the Gene Runner software version 3.05 and BLASTn searches were used to check primer specificity. Primers used in this study are listed in the Table. The specific fragments were amplified and then checked by 2% agarose gel electrophoresis
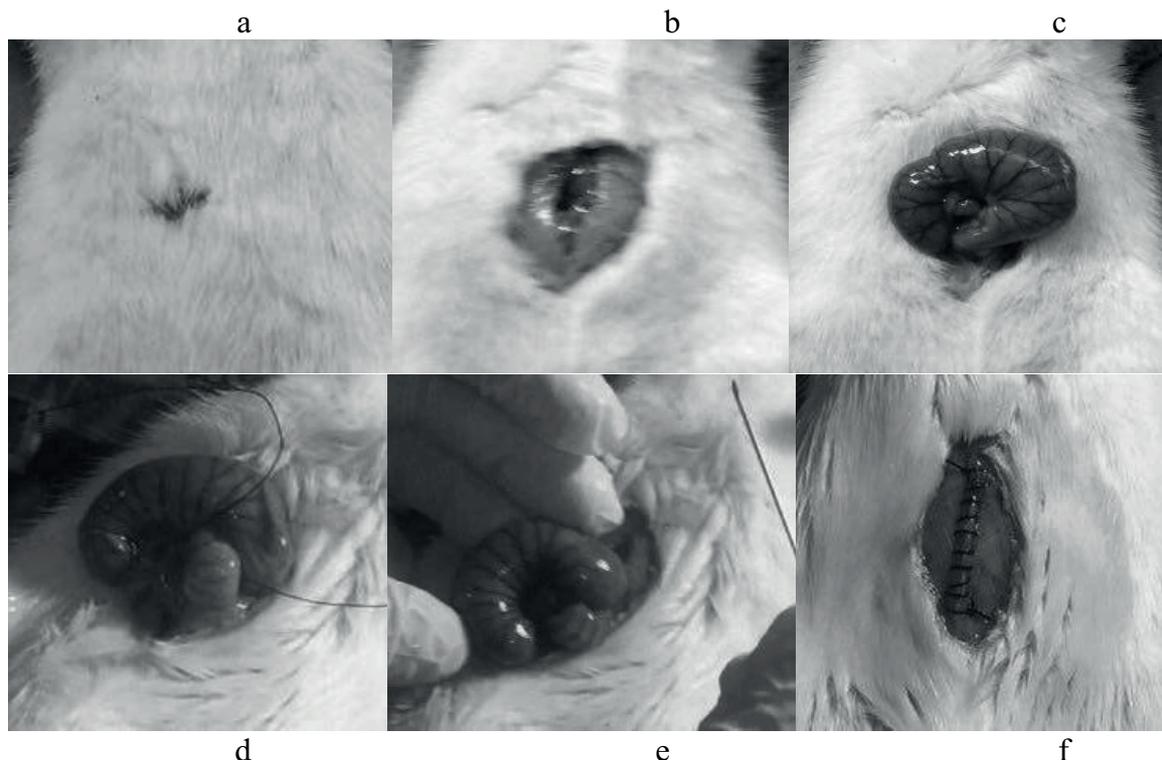
Figure 1. CLP procedure. A small midline incision (2–3 cm) is made through the skin (a, b). The cecum is identified and exposed (c). The cecum is tightly ligated with a 3.0 silk suture at its base, below the ileocecal valve (d). The cecum is punctured twice with a 20-gauge needle (e). The cecum is returned to the peritoneal cavity and the abdomen is stitched up with 3.0 silk sutures in two layers (f).

Table. Primer sequences used in this study.

Primer	Sequence (5′ → 3′)	T_m^b (°C)	Product (bp)
Forward CD177	ATACCAGTGCTG ACCCTTCTG	59.44	
Reverse CD177	CCTCGCAGGTTT TCTCACC	60.25	145
Forward MPO	GCGATAGGTTTTGGTGGGAG	58.62	
Reverse MPO	AGCTCACAAAGTCTCGGGG	57.89	165
Forward GAPDH	TGCCAGCCTCGTCTCATAG	58.88	
Reverse GAPDH	ACTGTGCCGTTGAACTTGC	59.27	197

to ensure the specificity of primers and the size of PCR products.

2.5. Real-time PCR

The expression of the selected gene was carried out with a real-time PCR system (Rotor-Gene Q, QIAGEN, Germany). The reaction mixture contained 5 μL of SYBR Green Real-time PCR Master Mix (Rotor-Gene Q), which contains Taq DNA polymerase, dNTP, $MgCl_2$, SYBR Green I dye, 0.2 μL of a 10 mM solution of sense/antisense primer, 100 ng of template cDNA, and H_2O added to a total of 10 μL. Negative controls were also designed. Thermal cycling conditions were carried out with an initial denaturation stage at 95 °C for 2 min, followed by 40 cycles at 95°C for 15 s, (61.2 °C for CD177, 59 °C for MPO, 60 °C for GAPDH) for 20 s, and 72 °C for 20 s. At the completion of each run, melting curves for the amplicons were measured by raising the temperature by 0.3 °C from 57 to 95 °C while monitoring fluorescence. To determine the specificity of the PCR amplification, the melting curve for Tm, its symmetry, and the lack of nonspecific peaks were checked. All tests were conducted in triplicate. The expression ratio was recorded as the fold difference in the quantity of real-time PCR product from samples. Each mRNA expression value was normalized against the threshold cycle (C_T) of a housekeeping gene expression (GAPDH), and the fold change in the MPO and CD177 genes were calculated by the formula $2^{-\Delta\Delta Ct}$ as follows: $\Delta\Delta Ct = (\Delta Ct$ of the experimental test) – (ΔCt of control test). ΔCt was calculated by subtracting the Ct of the target gene (MPO or CD177) from the Ct of the reference gene (GAPDH).

2.6. Measurement of MPO by enzyme-linked immunosorbent assay (ELISA)

For qPCR validation, MPO levels were measured by the o-dianisidine-H_2O_2 method for 96-well plates (13). From every sample, 20 μL was added to 0.53 mmol/L o-dianisidine dihydrochloride (Sigma-Aldrich, USA), and then 0.15 mmol/L H_2O_2 in 50 mmol/L potassium phosphate buffer with pH 6.0. After 10 min of incubation at room temperature, the change in absorbance was measured at 460 nm. ELISA was performed with three replicates for each sample.

2.7. Statistical analysis

Data are presented as means ± standard error (SE). The results were subjected to one-way ANOVA followed by Tukey's honestly significant differences test using SPSS 22.0 (IBM Corp., USA). The significance was considered as $P < 0.05$.

3. Results

### 3.1. CD177 expression in whole blood, livers, and lungs by real-time PCR

The results of CD177 expression showed a significant difference in whole blood, livers, and lungs of the control and two other groups (LAP and CLP). One-way ANOVA revealed a significant effect of surgery by laparotomy compared to the control group ($P < 0.05$). Furthermore, in contrast with the CLP group ($P > 0.05$) in the liver, there was no significant difference in the laparotomy group. There were differences between LAP and CLP in whole blood and lung tissue. CD177 gene expression increased in response to surgery stress in the LAP group in all tissues (Figure 2). As a result, when the level of infection increased, we observed that the CD177 gene was expressed more only in lung tissue.

### 3.2. MPO expression in blood, livers, and lungs by real-time PCR

Results showed that there was a significant difference between the control group and two other groups in blood, livers, and lungs. The CLP group significantly differed from the control and LAP groups ($P < 0.05$). Compared to control and LAP rats, the MPO of CLP rats showed a significant increase. There was a relationship between expression of the MPO gene and level of infection. We observed that MPO gene expression increased in response to infection in the CLP group in whole blood, the liver, and lungs (Figure 3).

3.3. Analysis by ELISA of MPO level after CLP

One-way ANOVA analysis identified differences between the increase of MPO activity and severity of sepsis in the control group and two other groups (LAP and CLP) in blood, livers, and lungs. The same significant increase was observed between LAP and CLP (Figure 4). The results of
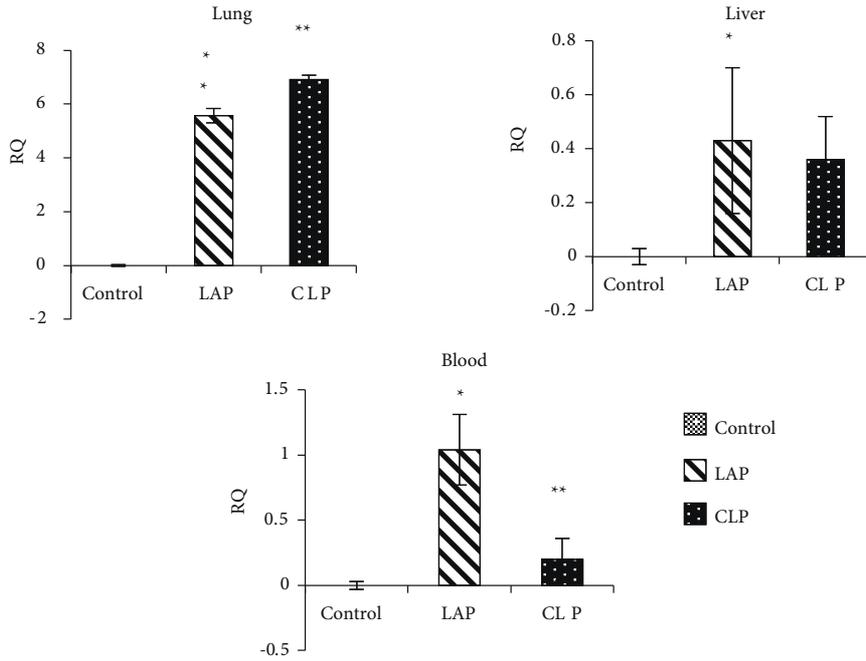
Figure 2. The gene expression of CD177. *P < 0.05 is considered significant between the control group and LAP group. **P < 0.05 is considered significant between the LAP group and CLP group. Data are presented as mean ± SD.
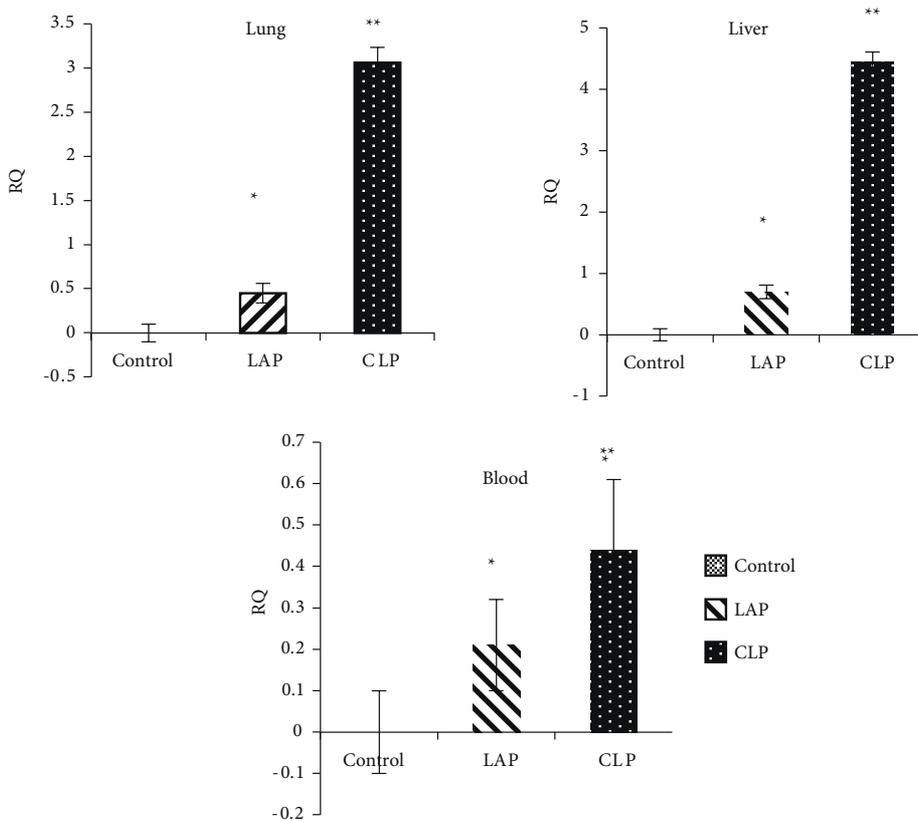
Figure 3. The gene expression of MPO. *P < 0.05 is considered significant between the control group and LAP group. **P < 0.05 is considered significant between the LAP group and CLP group. Data are presented as mean ± SD.

ELISA also confirmed the data obtained by real-time PCR as MPO mRNA expression was upregulated in response to infection in the CLP group in whole blood, livers, and lungs (Figure 4).

4. Discussion

Sepsis and its sequelae remain the most common cause of morbidity and mortality among critically ill patients in intensive care units (14). Sepsis is initiated by immune cells such as neutrophils, monocytes, and macrophages (15).

Neutrophils have a key role in controlling an immune response to infection (16). During sepsis, reactive oxygen species (ROS) are produced by activated neutrophils and macrophages, which induce an inflammatory response that causes structural membrane damage (17). MPO is also one of the formations of ROS and oxidation of biological substances and is involved in this structure (18,19).

However, there is evidence that uncontrolled or excessive accumulating of neutrophils lead to the development of autoimmunity, the exacerbation of inflammation, and tissue damage (20).

In severe sepsis, MPO is released in a high percentage by activated neutrophils for antibacterial activities and causes the increase of degranulated neutrophils (21). Like others, we also observed that there was a relationship between expression of MPO and level of infection (Figures 3 and 4).

Previous studies described that MPO composition in myeloid-lineage cells usually increased as an antiinflammatory activity in response to infection. During the systemic inflammatory response, the aggregation of neutrophils may be associated with enhanced neutrophilic MPO activity (21). These observations confirm our result, which shows that the mRNA level of MPO significantly
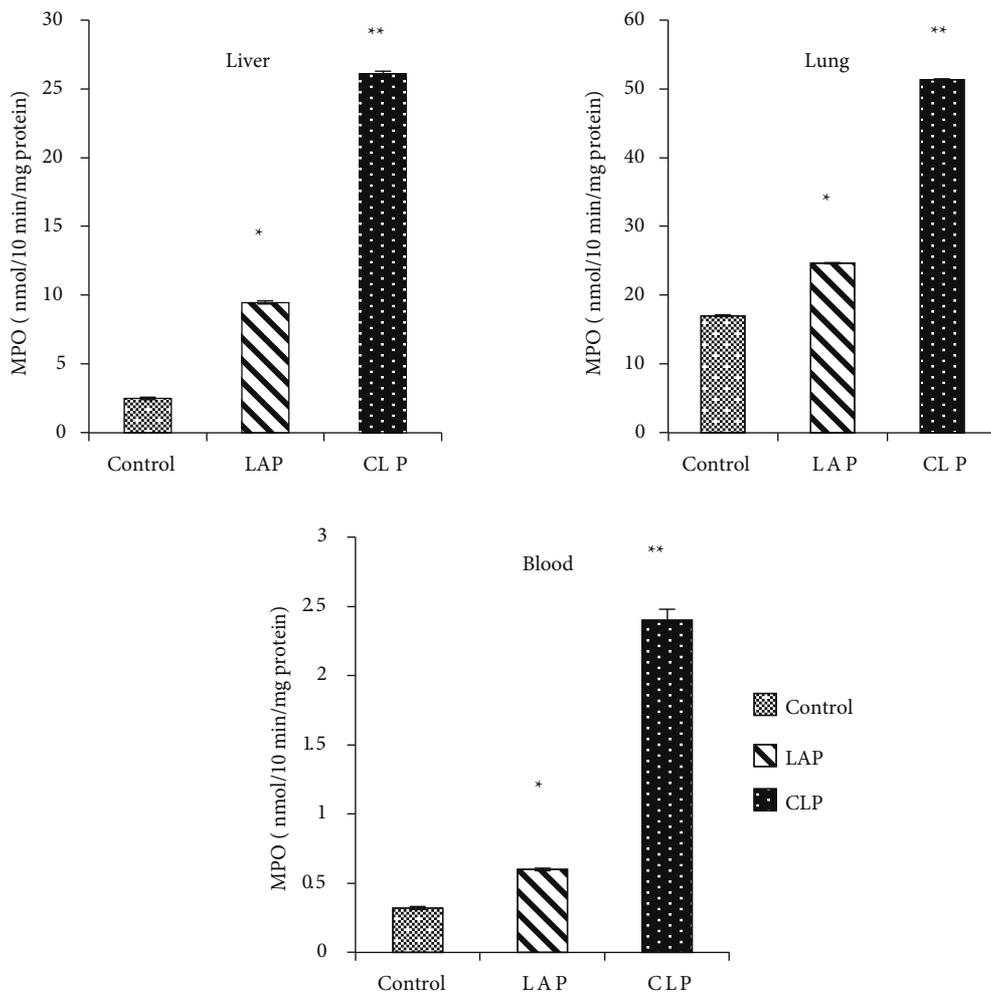
Figure 4. Validation of gene expression results of MPO by ELISA. *P < 0.05 is considered significant between the control group and LAP group. **P < 0.05 is considered significant between the LAP group and CLP group. Data are presented as mean ± SD.

increased in the CLP group in whole blood, livers, and lungs in response to infection (Figures 3 and 4).

In another context, Kothari et al. found that during oxidative stress neutrophil activation occurs and the number of cells increases during sepsis, and MPO is released into the blood. The results of that study showed that MPO activity may be a good biomarker for inflammatory responses in septic patients (5).

In addition, CD177 is known as a neutrophil-specific molecule that is involved in severe antibody-dependent infections such as neonatal alloimmune neutropenia and respiratory infection (22). Although CD177 increase was formerly seen in the flow of neutrophils in response to LPS of bacteria (23), we did not find any relationship between expression of CD177 and level of infection in the liver (Figure 2). One study confirmed a role of CD177 in the neutrophil response to LPS of bacteria during neutrophil transendothelial migration and an adhesion in which neutrophil CD177 mRNA levels were increased, associated with increased neutrophil counts (8). We found increased CD177 mRNA expression in response to surgery in the LAP group in whole blood, livers, and lungs, while CD177 expression was elevated in response to infection in the CLP group in the lungs (Figure 2). These results suggest that CD177 may have a role in the aggregation of neutrophils in the lung tissue. If this process is not controlled, it can lead to organ damage and severe infection. Thus, in cases of respiratory infection that is worsened by uncontrolled neutrophilic infection, CD177 may have a potential contribution to treat respiratory inflammation (23). In airspace neutrophils, the high level of CD177 expression was identified as increased CD177-specific antibodies of blood in acute lung injury, suggesting a potential involvement of CD177 in neutrophil infiltration in infectious diseases (22,24). Xie et al. also reported that the number of neutrophils in a blood and skin infection model was decreased in CD177 knockout animals (23). Another interesting observation is that the MPO level was higher in CD177- neutrophils (25).

The results in this study validate MPO increasing in the CLP group in response to inflammation in all tissues, but the expression of CD177 was elevated in response to infection in the CLP group only in the lungs (Figures 2–4). The finding of a positive correlation between the increased expression of MPO and CD177 suggests that MPO and CD177 may have potential as novel sepsis biomarkers in the lungs, although further studies and assays such as flow cytometry and immunohistochemical staining are required to more clearly establish our results. Finally, this study underscores the powerful potential of using the real-time PCR method to determine involvement of genes in infectious diseases such as sepsis.

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