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MicroRNA-155 Expression with *Brucella* Infection *in vitro* and *in vivo* and Decreased Serum Levels of MicroRNA-155 in Patients with Brucellosis

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ABSTRACT

Infection by *Brucella* is characterized by the inhibition of host immune responses. MicroRNA-155 (miR-155) has been implicated in the immune response to many diseases. In this study, miR-155 expression during *Brucella* 16M infection of macrophages and mice were analyzed. Expression of miR-155 was significantly induced in macrophages at 24 hours post infection. Analysis of infected mice showed that miR-155 was inhibited at 7 and 14 days, but induced at 28 days. Very interestingly, the induction or inhibition trend was reversed at 7 and 14 days in 16M△virB-infected mice. This suggested that decreased expression of miR-155 at an early stage of infection was dependent on intracellular replication. In humans with brucellosis, serum levels of miR-155 were significantly decreased compared to those without brucellosis and healthy volunteers. Significant correlations were observed between serum level of miR-155 and serum anti-*Brucella* antibody titers and symptom of sweat. The decrease in miR-155 with *Brucella* infection contrasts with the increase in miR-155 observed in *Mycobacterium tuberculosis* infection. This contrasting effect suggests that *Brucella* interferes with miR-155-regulated immune responses through a unique mechanism. Taken together, data from this study indicate that *Brucella* infection affects miR-155 expression, and that human brucellosis patients show decreased serum levels of miR-155.

Keywords: MicroRNA-155, brucellosis, immune response, type IV secretion system, *Brucella melitensis*

Introduction

Brucellosis is a zoonosis found worldwide that causes great economic losses and public health problems, and there is no effective vaccine against the disease. The re-emergence of brucellosis in many countries in recent years has spurred concern over this "old disease"[1].An outbreak of brucellosis occurred in Lanzhou, China infected more than 3000 people in 2019. *Brucella* harbors a set of virulence effectors that hijack host cells to facilitate its own survival and replication. It uses stealth to avoid inducing a significant immune response. These characteristics of *Brucella* make it a successful intracellular pathogen[2]. Human brucellosis is characterized by atypical symptoms, including fever, sweating, arthralgia/arthritis, and other constitutional symptoms, as well as hepatomegaly and splenomegaly[3]. Brucellosis is often misdiagnosed or its diagnosis is delayed, resulting in chronic infections that are difficult to cure. Nucleic acid detection can be used for early diagnosis and can increase the diagnosis window[4]. However, the low concentration of *Brucella* in clinical samples and inconsistency in levels of serum antibody and bacterial DNA make it difficult to evaluate the diagnostic and prognostic performance of nucleic acid-based assays. Therefore, biomarkers would be of great value in diagnosis and determining the prognosis of brucellosis.

MicroRNAs (miRNA) are endogenous 22-nucleotide RNAs that play important gene regulatory roles. As a class of small non-coding RNAs, miRNAs are highly conserved across various eukaryotic species and function as key regulators in gene expression at the post-transcriptional level by targeting mRNAs for translational repression or degradation[5]. MiRNAs also modulate innate and adaptive immune responses to pathogens. The application of miRNAs as diagnostic or prognostic biomarkers has been demonstrated for various diseases[6]. However, compared to their well-known role in cancer, the role of miRNAs in susceptibility and resistance to infectious diseases, especially those of bacterial origin, is still poorly understood. Several miRNAs have been reported to fine-tune the innate and adaptive immune responses to mycobacterial infection. *Brucella* infection is characterized by a weak immune response, which can be attributed to its immune evasion strategy. The correlation between *Brucella* infection and mRNA expression remains largely unknown. A recent study showed that *Brucella* infection of macrophage RAW264.7 cells significantly altered miRNA expression profiles, suggesting that miRNAs are involved in interactions between *Brucella* and its hosts[7]. The differences in miRNA expression patterns among human patients, however, remain to be evaluated.

MiR-155 plays a central role in immune responses, particularly innate immunity[8]. More specifically, MiR-155 is known to regulate immune responses to various infections. *Mycobacterium tuberculosis* infection was found to significantly induce miR-155 expression[9], and MiR-155 has found to promote autophagy to eliminate intracellular *M. tuberculosis*[10]. Whether miR-155 is involved in the immune response to *Brucella* infection, remains largely unknown. To probe the possible roles of miR-155 in *Brucella* infection, in the present study, we examined miR-155 expression during *Brucella* infection of macrophages and mice, and analyzed serum levels of miR-155 in patients with brucellosis.

Results

Expression level of MiR-155 in macrophages were altered by Brucella infection

To test whether expression of miR-155 was affected by *Brucella* infection, macrophage cells were infected with *B. melitensis* 16M or phosphate buffered saline (PBS). Compared with the uninfected PBS control, expression of miR-155 was significantly induced at 24 hours in the 16M-infected group (Figure 1A). Expression of miR-155 did not change significantly from 0 to 24 hours post infection (p.i.) in the PBS group, but increased 2 fold at 48 hours. Compared with expression in the PBS control, expression of miR-155 was not significantly different at 0 hours in the 16M group. However, the expression levels of miR-155 in the 16M group were significantly increased at 24 hours and decreased at 48 hours p.i., relative to levels in the control. miR-155 expression in the 16M-infected group was increased 17 fold relative to that in the PBS group at 24 hours p.i. (Figure 1B).

Brucella wild-type strain 16M affected the expression of miR-155, suggesting that miR-155 might be involved in intracellular survival of *Brucella*. Macrophage cells were also infected with the *Brucella* type IV secretion system (TFSS) isogenic mutant 16M△virB, and expression levels of miR-155 were compared with those of the wild type and control. Expression levels of miR-155 in the 16M△virB group were significantly increased at 0, 24 and 48 hours, relative to those of the control (Figure 1A and 1C), and expression levels of 16M△virB compared to those of 16M were increased up to 15.4 and 5.6 folds at 0 and 48 hours (Figure 1D).

Expression levels of miR-155 in mice infected with B. melitensis 16M

To further characterize the effect of *Brucella* infections on miR-155, miR-155 expression in *Brucella*-infected mice was analyzed. At time points of 7, 14, and 28 days p.i., the relative expression level of miR-155 in the PBS control group was 1, 0.48, and 0.31, respectively (Figure 2A). Compared with the uninfected control, expression levels of miR-155 in the 16M-infected group were significantly decreased at 7 and 14 days. At 7 days p.i., the ratio of the expression levels of the 16M group to those of the PBS group were decreased to 0.1, and at 14 days, 0.72. At 28 days, however, the ratio was increased to 1.39 (Figure 2B).

Compared with the PBS group, miR-155 expression in the 16M△virB group was significantly increased at day 7 and 28 (Figure 2A and 2C). At 7 days p.i., the ratio of the expression level of miR-155 of 16M△virB to that of the 16M group was increased up to 22.6, but decreased to 0.64 at 28 days (Figure 2D). A comparison between the PBS, 16M, and 16M△virB groups showed that 16M infection resulted in an miR-155 decrease in mice at day 7, whereas 16M△virB infection resulted in the induction of miR-155 at the same time point. This suggested that the effect of *Brucella* infection on expression of miR-155 at 7 days p.i. is dependent on bacterial replication.

The serum MiRNA-155 level was decreased in brucellosis patients

To further analyze the expression of miR-155, blood samples were collected from patients suspected of having brucellosis. After confirmation of the diagnosis, the study participants were divided into two groups: brucellosis and non-brucellosis. A total of 56 patients, 47 non-brucellosis and 20 healthy

volunteers were enrolled. Demographic characteristics of the enrolled persons were compared. No significant differences were observed in sex or age distribution. Among the enrolled patients, the most common symptom was fever (67.85%), followed by fatigue (60.71%), sweat (48.21%), joint pain (48.21%), and leg pain (44.64%) (Table 1).

To quantify miR-155 expression, a standard curve was created using synthesized mimics. As shown in Figure 3A, the qPCR assay could detect as less as 1 fmol/L of miR-155, and the linear curve ranged over five orders of magnitude (Figure 3A). With this standard curve, serum concentrations of miR-155 were calculated for each sample and compared between brucellosis and non-brucellosis groups. Concentrations of miR-155 in the serum of brucellosis group were significantly lower than those in non-brucellosis group ($P < 0.001$) and healthy volunteers ($P < 0.001$) (Figure 3B). Serum concentrations of miR-155 among patients with brucellosis ranged from 0.24 to 165.9 (95% CI: 11.81–30.99), whereas for non-brucellosis and healthy volunteers, concentrations ranged from 1.39 to 1363 (95% CI: 95.57–282.4) and from 20.59 to 953.3 (95% CI 53.13-244.8) (Table 2).

Correlations between serum levels of miR-155 and antibody titer and clinical symptoms

The decreased levels of miR-155 in brucellosis patients compared to those of non-brucellosis indicated that it might be a potential biomarker for diagnosis of brucellosis. This prompted us to test whether there was any correlation between the serum concentration of miR-155 and SAT titer and clinical symptoms. SAT antibody was tested at dilutions of 1:100, and ≥1:200. A significant correlation was observed between SAT titers and miR-155 levels (Figure 4A). Mean serum level of miR-155 of the 1:100 group was 6.29 fmol/L (95%CI 1.29-11.29), being lower than 27.88 (95%CI 14.73-41.02) of the ≥1:200 group. The concentrations of miR-155 were compared between patients with or without typical symptoms. Significant correlation was observed between serum level of miR-155 and symptom of sweat (Figure 4B). No correlations were observed between serum level of miR-155 and symptom of fatigue (C), fever (D), leg pain (E), and headache (F).

Discussion

MicroRNAs play important roles in regulation of host immune responses. miR-155, specifically, has been found to be associated with many biological processes[8,11,12]. Recent studies have also shown that miR-155 plays a role in both bacterial and viral infections. Further, miR-155 has been shown to regulate the innate immune response, which is an important defense against invading pathogens[13]. Here, in the present study, we found that expression of miR-155 is affected by *Brucella* infection in macrophages and in mice, whereas its expression was inhibited in human patients with brucellosis. Analysis of macrophage infections showed that miR-155 was induced at 24 hours p.i. Contrary to cell experiments, miR-155 was inhibited at days 7 and 14, but induced at day 28. Compared with the 16M group, the 16M△virB group showed significant induction of miR-155. Therefore, miR-155 may be involved in responses to *Brucella* infection.

As a key microRNA molecule, miR-155 has been studied widely. Studies have shown that miR-155 is mainly expressed in activated macrophages, dendritic cells, and B and T lymphocytes[8,11,12,14], and that miR-155 is upregulated by a variety of inflammatory mediators and pathogens. miR-155 has been implicated in the immune responses to several bacterial pathogens, including *Helicobacter pylori* and

M. tuberculosis [9,15]. Interestingly, miR-155 expression is enhanced by mycobacterial infection both in vivo and in vitro. Recent studies have revealed that *M. tuberculosis* purified protein derivative highly induces the expression of miR-155 in peripheral blood mononuclear cells from patients with active tuberculosis[16]. *Brucella* has many characteristics that are similar to those of *M. tuberculosis*. Both are intracellular bacteria that cause chronic infections refractory to treatment. However, contrary to what has been observed in *M. tuberculosis*, miR-155 was significantly inhibited in infections of mice and patients with brucellosis. The mechanism of miR-155 inhibition by *Brucella* infection remains largely unknown. On the other hand, we also observed the induction of miR-155 in macrophages at 24 hours p.i. We only tested serum levels of miR-155 in this study. It will be interesting to test whether miR-155 is also inhibited in PMMC cells from patients with brucellosis.

Brucella contains multiple virulence factors that contribute to its intracellular survival. The TFSS encoded by the *vir*B operon is one of the most important virulence factors[17]. Inactivation of the TFSS results in reduced survival of the mutant and its inability to establish a chronic infection. Results from 16M△virB-infected mice showed that expression of miR-155 was greatly induced. Because 16M△virB had lost its capacity to survive intracellularly and to establish a chronic infection, it can be inferred that wild-type strain 16M is able to inhibit the induction of miR-155 and that this inhibition is essential for establishment of chronic infection. At present, we do not know whether this inhibition of miR-155 is mediated by virB or intracellular bacterial replication. If it is mediated by virB, it is possible that the TFSS effector proteins are involved in this inhibition, because the function of the TFSS is mainly mediated by its effector proteins. Many TFSS effector proteins have been identified; however, the functions of these effector proteins remain unknown. Analyzing the roles of effector proteins in regulating miR-155-mediated immune responses will be of great value in understanding the interaction between *Brucella* and its hosts.

In vivo studies using miR-155-deficient mice have demonstrated that miR-155 is required for normal immune function of T- and B-lymphocytes and dendritic cells. miR-155 has also been reported to promote the development of T helper 1 (Th1) and Th17 cell subsets and to attenuate the Th2 cell response[18]. These studies together suggest a potential role for miR-155 in the cellular immune response, which is the major arm of immunity in anti-bacterial defenses. A Th1 response is essential for controlling *Brucella*. *Brucella* has the ability to interfere with host development of Th1 and Th2 responses[19]. Therefore, it is possible that miR-155 mediates host responses to *Brucella*. Further, *Brucella* has the ability to interfere the polarity in a TFSS-dependent manner. This also implies that TFSS effector might be involved in interference of miR-155 in immune responses.

The decreased serum level of miR-155 in patients with brucellosis also suggests that miR-155 could be used as an auxiliary biomarker for the diagnosis of brucellosis. Before miR-155 can be used as a biomarker, a much larger sample needs to be evaluated. Furthermore, whether there is a correlation between serum levels of miR-155 and infection stages or disease outcomes needs to be examined. With this information, it may be possible to use miR-155 as an auxiliary biomarker to monitor *Brucella* infection and determine disease prognosis.

In summary, in this study, we analyzed the expression of miR-155 during *Brucella* infection in macrophages, mice, and human patients. The results showed that miR-155 was inhibited by *Brucella* infection in mice. In humans, serum levels of miR-155 were decreased when compared with those in healthy volunteers. Very interestingly, the inhibition of miR-155 was reversed in the *vir*B mutant, suggesting that the TFSS or intracellular survival might be involved in interference by *Brucella* in miR-155-regulated immune responses. Further studies are needed to define this interaction between

miR-155 and *Brucella*.

Materials and Methods

Ethics Statement

Suspect brucellosis patients were enrolled at Brucellosis Hospital of Plague and Brucellosis Prevention and Control Base. This study was carried out in accordance with the approved by ' Ethics Committee of Plague and Brucellosis Prevention and Control Base' with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the ' Animal Ethics Committee of Beijing Institute of Disease Control and Prevention '. All animals experiments methods are reported in accordance with ARRIVE guidelines.

Brucellosis patient diagnosis and blood sample collection

Brucellosis patients were diagnosed based on serological assay standard agglutination test (SAT), clinical symptom and epidemic information. Cut-off value for SAT assay was 1:100. Epidemic information, clinical symptom, and serum samples were collected at Brucellosis Hospital of Plague and Brucellosis Prevention and Control Base. Non-brucellosis patients were persons with similar symptoms but without Brucella antibodies. Samples were collected from Sex and age matched blood donor and used as healthy volunteer control. Blood samples were stored at -20°C until use.

Quantitative RT-PCR

B. melitensis 16M and its derivative were routinely cultured in rich medium Tryptic Soy Broth (TSB) or Tryptic Soy Agar (TSA). A *vir*B inactivation mutant 16M△virB was constructed previously (Zhong et al., 2009)[20]. Total RNA was extracted from blood sample, spleen of infected mice or macrophage cells using Trizol reagent (Invitrogen) as recommended by the manufacturer. RNA samples were then treated with DNAse I (Promega) to remove any contaminating genomic DNA. RNA quantity and quality were assessed using ND-1000 Spectrophotometer Nanodrop (Technologies). The treated total RNA was reversed transcribed into cDNA by miRcute miRNA First Strand cDNA Synthesis Kit (Tiangen, Beijing) as recommended by manufacturer. The cDNA samples were amplified with qPCR using 2×SYBR Green I Master Mix (Takara Biochemicals). Primer sequences for amplification of miR-155 and U6 snRNA was listed in supplementary table. Standard curve of miR-155 was made by amplification of serial dilution of synthesized control and plot of Ct value to concentration. Relative expression levels of miR-155 were calculated by using U6 snRNA as housekeeping miRNA. The average expression levels and standard deviations were calculated using data from two triplicates from independent experiments.

Macrophage cell and mouse infection

Murine macrophage-like RAW264.7 were infected with 16M, 16M△virB, or PBS as negative control to assess survival capability and expression of miR-155 essentially as described previously [21]. In brief, monolayers of macrophages were seeded in 24 well plates 1 day prior to infection at 1×10^5 cells per well. Macrophages were infected with bacterial suspension at a MOI of 50. At 45 min post-infection, the cells were washed by PBS in three times and then incubated with 50 μg/mL of gentamycin for 1 h

to eliminate extra-cellular bacteria. Then replaced the cultures by DMEM with 20 μg/mL of gentamycin. At 0, 24 and 48 hours post-infection, discarded the supernatant and the cells were lysed, and the live bacteria were enumerated by plating in duplicate on TSA plates with or without kanamycin or ampicillin. Cell lysates were subject to total RNA isolation and miR-155 expression analysis. For mouse infection, groups of ten 8-week-old female BALB/c mice intraperitoneally infected with an inoculum (2×10^6 CFU/mL) of 16M or 16M△virB. At 7, 14 and 28 days post the inoculation, the infected mice were sacrificed by cervical dislocation and spleens were collected aseptically and homogenized with PBS containing 0.1% Triton X-100. Serial dilutions of spleen homogenates were prepared and plated in duplicate on TSA plates, and the CFU were counted after 4 days of infection at 37°C. Homogenized spleen cells were subject to total RNA isolation and miRNA expression analysis.

Statistical analysis

Unpaired Student's t test (Man-Whitney test) or two-way ANOVA analysis of variation was used to determine the significance of the results. Data were considered statistically significant at $P<0.05$, P value of <0.01 was considered greatly significant.

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Author contributions

X. Zhang and J. Chen performed the experiments and analyzed the data, H. Zhang, H. Cheng，Q. Dong and J. Zhu participated the experiments, Z. Chen and X. Zhang draft the manuscript, H. Zhang and Z. Chen conceived and designed the study.

Conflicts of interests

The authors declare no conflicts of interests.

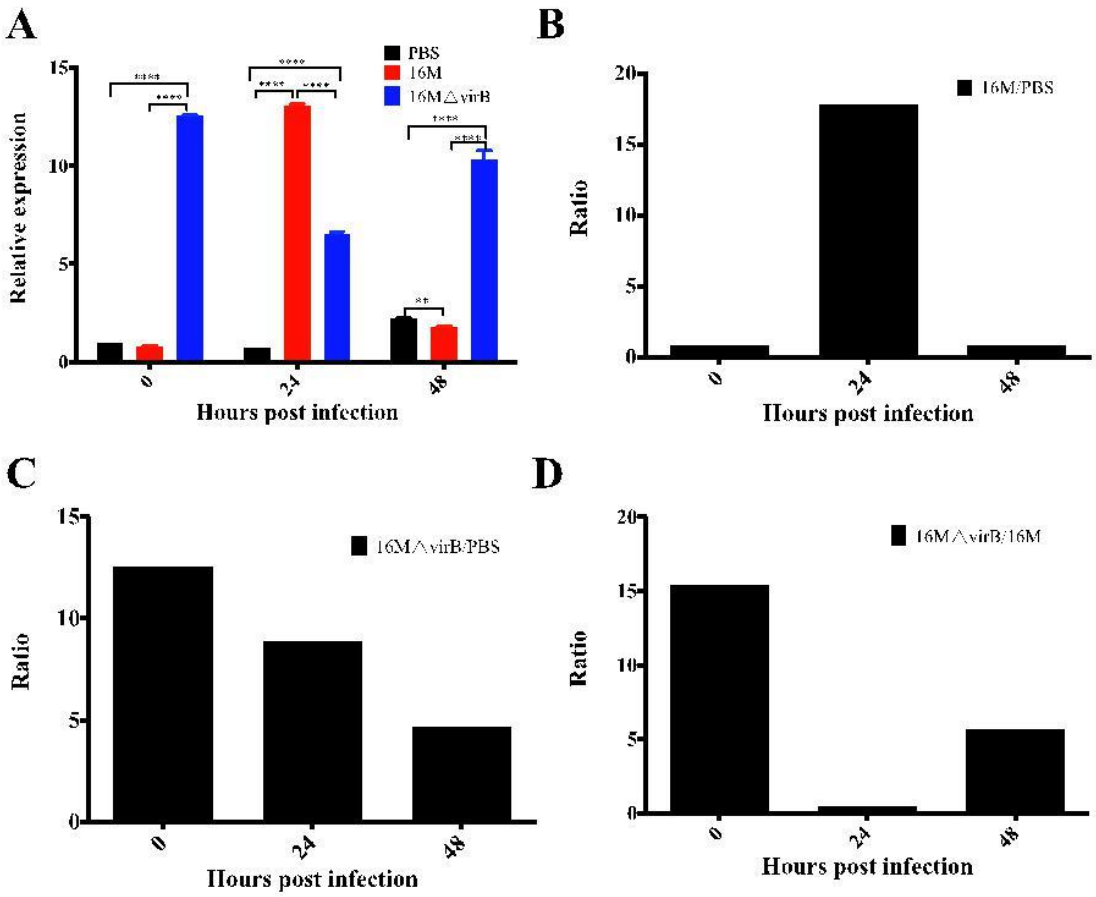

Figure 1. Altered expression levels of miR-155 in macrophages following infection with *Brucella melitensis*. Macrophage RAW 264 cells were infected with *B. melitensis* 16M, 16M△virB, or phosphate buffered saline. The expression level (A), ratio of 16M to PBS (B), 16M△virB to PBS (C), and 16M△virB to 16M (D) of miR-155 were analyzed. The expression level of miR-155 was significantly altered in 16M△virB group at 0, 24, and 48 hours and in 16M group at 24 and 48 hours post infection. **, P<0.01, ****, $P < 0.0001$.

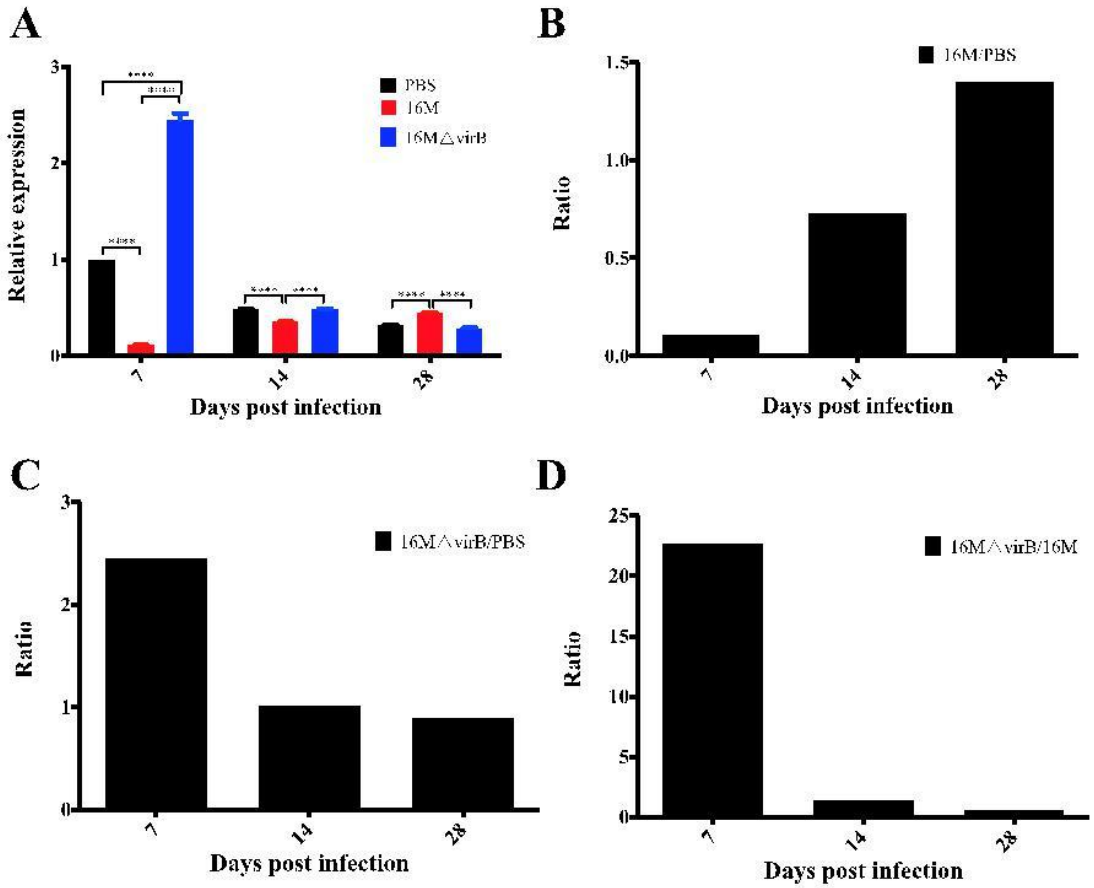

Figure 2. Altered expression level of miR-155 in mice during infection with *Brucella melitensis*. Balb/c mice were infected with *B. melitensis* 16M, 16M△virB, or phosphate buffered saline (PBS). The expression level (A), ratio of 16M to PBS (B), 16M△virB to PBS (C), and 16M△virB to 16M (D) of miR-155 were analyzed. Compared with uninfected PBS controls, the miR-155 expression level was altered in 16M and 16M△virB groups. **, P<0.01, ****, $P < 0.0001$.

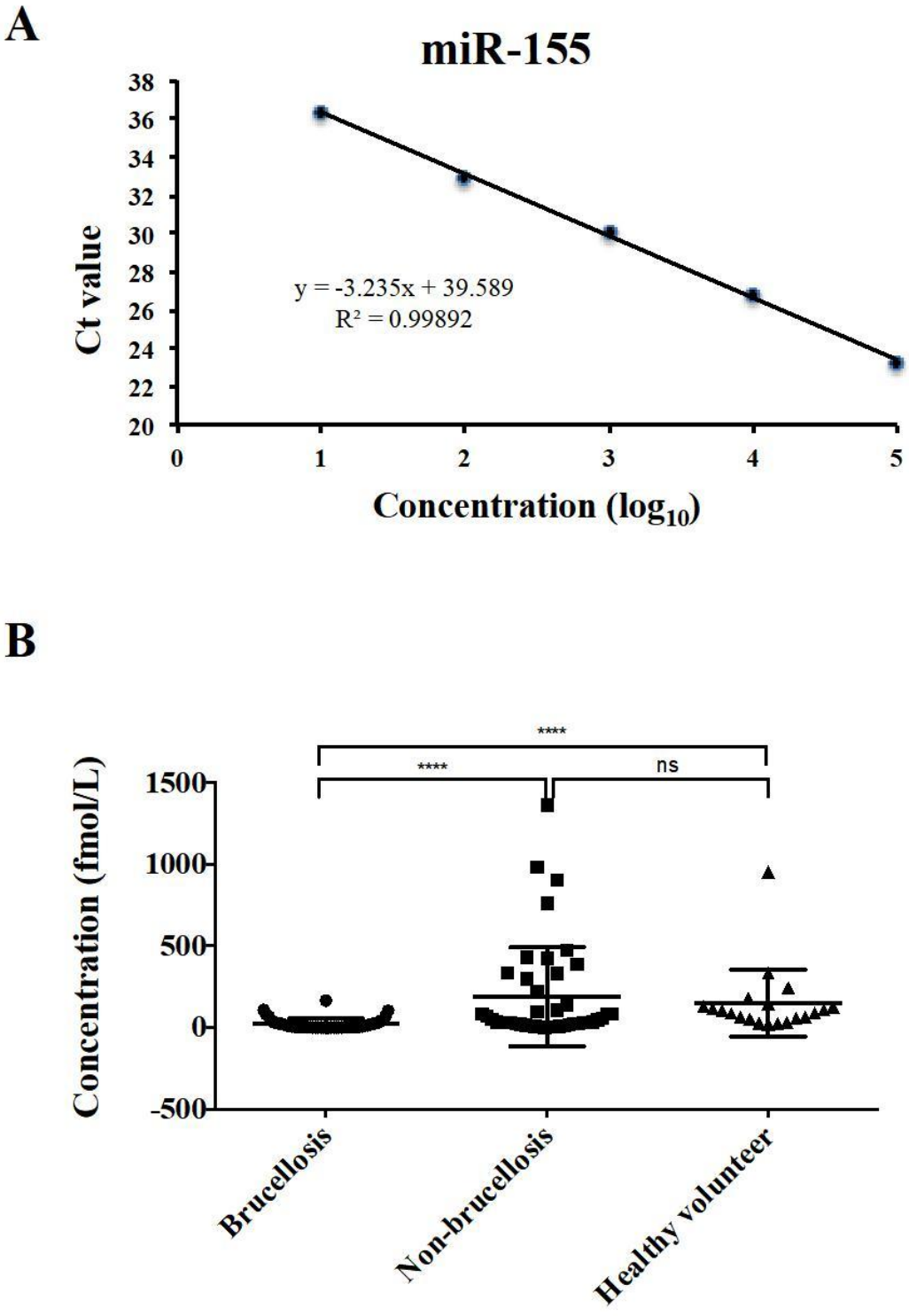

Figure 3. Expression levels of miR-155 in patients with brucellosis and healthy volunteers. (A) Quantification curve of miR-155; (B) Concentration distribution of miR-155 in patients and healthy volunteers； ****, $P < 0.0001$.

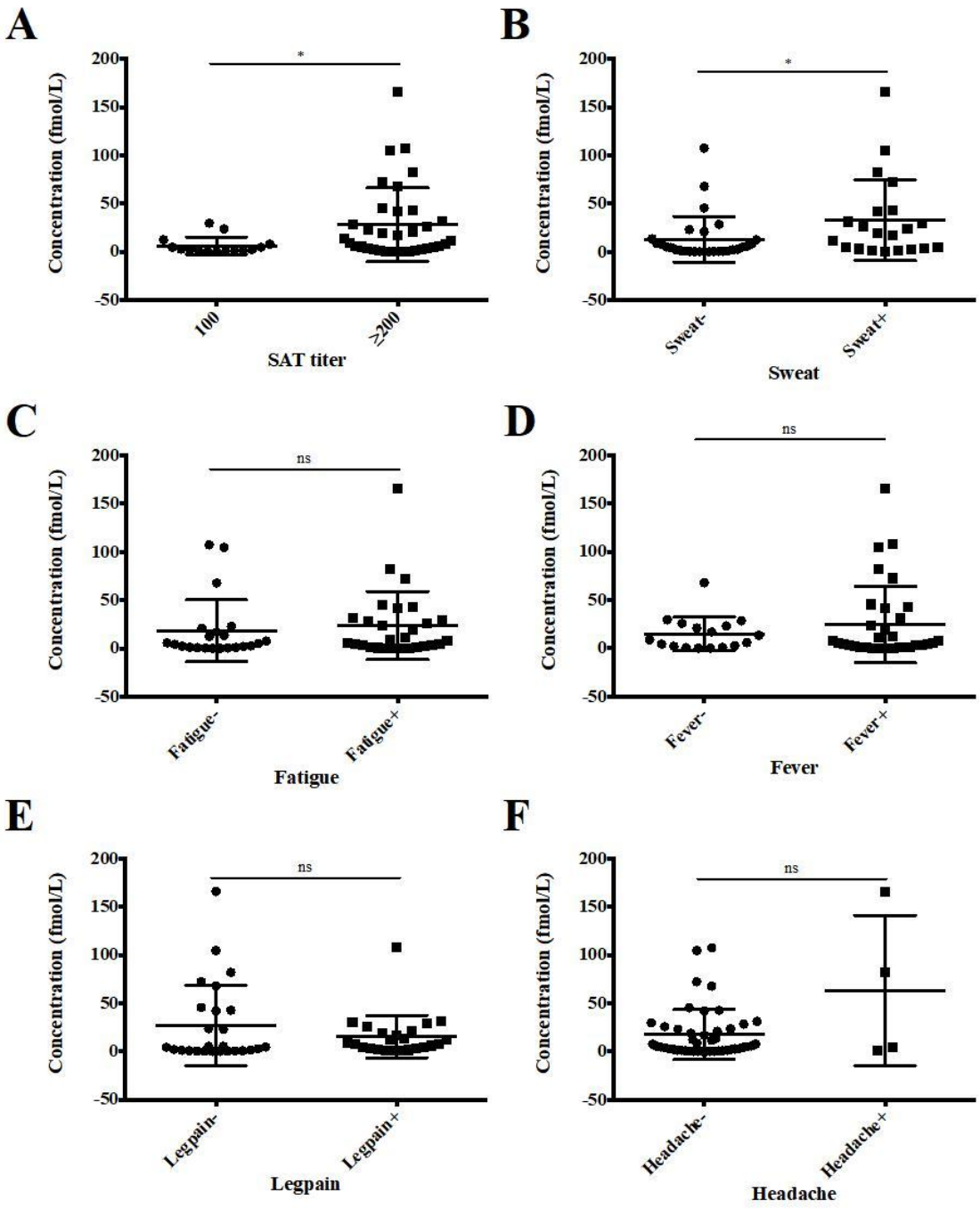

Figure 4. Correlations of miR-155 concentrations with antibody titers and symptoms. The concentration of miR-155 was calculated for each patient, and its correlations with antibody titer and symptoms were analyzed. Statistically significant correlations were observed between miR-155 concentrations and antibody titer (A) and sweat (B), but not between miR-155 concentrations and symptom of fatigue (C), fever (D), leg pain (E), and headache (F). *, $P<0.05$.

	Brucellosis (n=56)	Non-brucellosis (n=47)	Healthy volunteer (n=20)
Sex (NO, %)			
Female	12 (21.42%)	23 (48.93%)	9 (45%)
Age			
Mean	44.31	45.93	44.82
95% CI	40.76-47.64	41.23-50.60	39.64-51.26
SAT titer (NO, %)			
1:100	15 (26.78%)	-	-
1:200	41 (73.21%)	-	-
Symptom (%)			
Fever	38 (67.85%)	22 (46.8%)	-
Fatigue	34 (60.71%)	28 (59.6%)	-
Joint Pain	27 (48.21%)	19 (40.4%)	-
Leg Pain	25 (44.64%)	16 (34.0%)	-
Sweat	27 (48.21%)	19 (40.4%)	-

Table 1. Demographics of the enrolled brucellosis patients, non-brucellosis and healthy volunteers

Statistic Characteristics	Brucellosis (n=50)	Non-brucellosis (n=43)	Healthy volunteer (n=20)
Percentile			
Minimum	0.24	1.39	20.59
25% Percentile	1.413	20.52	54.64
Median	5.66	46.59	97.5
75% Percentile	26.5	297.8	143.3
Maximum	165.9	1363	953.3
Means			
Mean	21.4	189	149
Std. Deviation	33.76	303.5	204
Std. Error of Mean	4.774	46.29	45.78
95% CI			
Lower 95% CI	11.81	95.57	53.13
Upper 95% CI	30.99	282.4	244.8
Mean ranks			
Mean ranks	33.78	62.37	82.35

Table 2. Statistics of miR-155 concentrations of patients, non-brucellosis and healthy volunteers

Supplementary Files

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