T-bet, GATA3 and FOXP3 Transcription Factors Are Differentially Expressed in Acute and Subacute Brucellosis

Saeed Jalilian[1], Ali Reza Janbakhsh [2], Amirhooshang Alvandi [3], Farhad Salari[4], and Ali Gorgin Karaji[4, 5].

1. MSc., Department of Immunology, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran. E-mail: saeedjalilian64@yahoo.com.

2. M.D., Department of Infectious Disease, School of Medicine, Kermanshah University of Medical Sciences Kermanshah, Iran. E-mail: ajanbakhsh@kums.ac.ir

3. Ph.D., Department of Microbiology, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran. E-mail: ah_alvandi@kums.ac.ir

4. Ph. D., Department of Immunology, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran. E-mail: f.salari@kums.ac.ir

5. Corresponding Author. Postal address: Department of Immunology, School of Medicine, University Street, Shahid Shiroudi Boulevard, Kermanshah, Iran. Postal Code: 6714869914 E-mail: a_gorginkaraji@kums.ac.ir

Abstract

Back ground: Human brucellosis appears to be acute, subacute, or chronic, which may be due to differences in immune responses. The aim of this study was to explore the levels of T cell linage-specific transcription factors in the peripheral blood cells of acute and subacute brucellosis.

Methods: RNA was extracted from the peripheral blood cells of patients with acute and subacute brucellosis and healthy controls. Extracted RNA was transcribed into complementary DNA (cDNA). The cDNA of the target genes (T-bet, GATA3, and FOXP3) were amplified using Syber Green real-time PCR master mix and specific primers.

Results: Our results showed that the T-bet copy number in acute brucellosis patients was significantly higher than that in subacute patients and healthy controls. However, both acute and

subacute patients showed the same FOXP3 copy number; hence, the ratio of FOXP3/ T-bet in subacute patients was higher than that in acute patients and healthy controls.

Conclusions: Taken together these results revealed that in the acute phase of brucellosis, the expression of both T-bet and FOXP3 increased, but as the disease progressed to the subacute phase, T-bet expression decreased while the expression of FOXP3 did not change.

Key words: Acute Brucellosis; Subacute Brucellosis; T –bet; GATA3; FOXP3

1. Introduction

Brucellosis, also known as “undulant fever”, “Mediterranean fever” or “Malta fever” is known as the second most common zoonotic disease in the world (1). This disease is caused by bacteria of the genus *Brucella,* which have a wide range of animal reservoirs (2, 3). Human infection is caused by direct or indirect contact with infected animals or their products, and manifests itself as an acute, subacute or chronic disease.

Brucella species are facultative intracellular pathogens that can survive and multiply within host phagocytic cells. The immune response against *Brucella* species depends on cell-mediated immunity, which mainly involves activated macrophages, CD4+T cells, and CD8+ CTLs (4, 5). Naive T helper (Th) cells mainly differentiate into one of two subsets, Th1 or Th2, depending on the cytokine milieu in the local microenvironment (6). Th1 cells are essential for the clearance of *Brucella* infection (7), and the pivotal role of the Th1 cytokine (IFN-γ) in the outcome of *Brucella* infection has been well established in both mice and humans (4, 8). In contrast, Th2 cells produce IL-4 and IL-10 that inhibit macrophages and facilitate persistent and development of *Brucella* infection (9, 10). In addition to Th1 and Th2 cells, regulatory T cells (Tregs) are

involved in the immunopathology and orientation of immune responses against pathogens. It is obvious that CD4+ CD25+ Treg cells are not only directly involved in suppressing the immune response to self-antigens and preventing autoimmunity, but also play an essential role in regulating the immune response to foreign antigens (11, 12).

The differentiation of Th1, Th2, and Treg cells is driven and regulated by subset-specific transcription factors T-bet, GATA-3, and FOXP3, respectively (13-15). These factors cross-regulate each other and are selectively expressed in corresponding cell populations. T-bet modulates GATA-3 function; Th2-related cytokines block Th1 differentiation, and GATA-3 inhibits FOXP3 transcription and Treg formation (16, 17). In contrast, Treg cells suppress Th1 and Th2 cells differentiation and function (18). As mentioned above, Th1 cells are essential for *Brucella* clearance, and because they are suppressed by Th2 and Treg cells, we assume that the relative frequency of these T cell subpopulations might play a key role in the control of *Brucella* infection and the clinical appearance of human brucellosis.

This study aimed to evaluate the levels of T-bet, GATA-3, and FOXP3 mRNA expression in the peripheral blood cells of patients with acute and subacute brucellosis compared to healthy controls. By measuring the copy number expression of these transcription factors, the relative frequency of relevant T cell subsets in acute and subacute patients was determined, and the relative role of Th subpopulations in the development of brucellosis was clarified.

2. Materials and Methods

2.1. Subjects and blood samples

This study enrolled 30 adult (mean age 35.7 years) patients who had been diagnosed with brucellosis after informed consent was obtained. Brucellosis patients were diagnosed based on clinical sign and symptoms (joint pain, fever, sweating, chronic fatigue, and splenomegaly) and

positive serological Wright agglutination test results. Brucellosis subjects were classified based on the duration of the disease as follows: less than 3 months for acute and 3-12 months for sub-acute. These patients included 12 (40%) females and 18 (60%) males, of which 17 (57%) were in the acute phase and 13 (43%) were in the subacute stage, 10 (33%) were urban and 20 (67%) were rural. The control group consisted of 18 healthy adult volunteers (mean age 37.6 years), in which the absence of brucellosis and some other diseases was confirmed based on a clinical questionnaire and negative standard Wright test. Peripheral blood samples were collected in EDTA containing and serum separation tubes. Serum was used for performing Wright agglutination tests, and whole blood was used for RNA extraction. The signs and symptoms of the patients were also recorded using a questionnaire.

2.2. RNA isolation and cDNA synthesis

Isolation of RNA from whole blood was carried out according to the QIAamp RNA blood mini kit protocol (Qiagen, USA), and the extracted RNA was stored at -70 ° C. The extracted RNA was transcribed into complementary DNA (cDNA) using first- strand cDNA synthesis reverse transcriptase (Parstous, Mashhad, Iran), according to the manufacturer's instructions. The reactions were performed using a Thermo cycler (C1000 touch, Bio-Rad, USA). The synthesized cDNA was stored at -70 º C until use.

2.3. Preparation of PCR Standards

To prepare standard for PCR, pooled cDNA was prepared from 10 healthy control volunteers. PCR was performed for all three genes (FOXP3, T-bet and GATA3), and the PCR products were electrophoresed. The PCR products were purified using an AccuPrep ® PCR purification kit (Bioneer, USA), according to the manufacturer's protocol.

The purified PCR products were quantified using Nanodrop 2000 (Thermo Scientific, USA), and the copy number values of all three genes were determined by bioinformatics analysis at Cels.uri.edu/gsc/cdna.html. Based on the information obtained from the bioinformatics analysis, six copy number standard values, from 10^6 to 10 copy numbers, were prepared by serial dilution.

2.4. Real-time PCR

cDNA of the intended genes was amplified using SYBR Green real- time PCR master mix (Parstous, Mashhad, Iran), according to the manufacturer's recommendations. The reaction was run on a Light Cycler 96 Real-Time PCR Detection System (Roche, Basel, Switzerland). The following primer sequences were designed using bioinformatics analysis (NCBI genome data bank and primer BLAST tool): T-bet forward: CAGGAAACCGCCTGTACG, T-bet reverse: GACTGGAGCACAATCATCTG; GATA-3 forward: GATGGCACGGGACACTACC; GATA-3 reverse: GTGGTCTGACAGTTCGCAC; FOXP3 forward: CCAGAGAGAGATGGTACAGTC; FOXP3 reverse: GGATGATGCCACAGATGAAG. Real-time PCR conditions included an initial step at 94 ° C for 3 min, followed by 45 cycles of 94 ° C for 30 s, annealing at 55 ° C for 20 s, and extension at 70 ° C for 10 s.

2.5. Statistical analysis

Statistical analysis was performed using SPSS 16. Student's t-test (normality test passed with Kolmogorov–Smirnov) or the Mann–Whitney non-parametric test (normality test failed) was used to determine significant differences between the individual groups. Statistical significance was defined as a p-value < 0.05.

3. Results

3.1. Patient's signs and symptoms

Thirty brucellosis patients, who presented clinical manifestations and positive Wright test results, were classified based on illness period as acute or subacute brucellosis, and 18 age and sex- matched healthy volunteers who had negative Wright test results were chosen as the healthy control group. The mean ages of participants were 31.60 ± 17, 39.8 ± 17.2 and 37.6 ± 17.8 years for acute brucellosis, subacute brucellosis and healthy control individuals, respectively. The most common complaints in both groups were joint pain, sweating, and fever. By contrast, fever, shivering, and splenomegaly were more frequent in the acute group than in the subacute group (Table 1).

3.2. Acute brucellosis patients express more T-bet transcript

A comparison of T-bet copy number values among acute and subacute brucellosis patients and healthy controls revealed that the T-bet copy number value in acute patients was significantly higher than in subacute patients and healthy controls ($P< 0.05$) (fig. 1). On the other hand, subacute patients showed a lower T-bet copy number than healthy controls, although this was not statistically significant. These results indicate that while acute brucellosis patients showed stronger Th1 response than normal group, subacute patients showed weaker Th1response than normal group.

3.3. Brucellosis patients showed more FOXP3 transcript

Similar to other immunologic responses, in brucellosis infection, concurrent with stimulation of the immune response, expression of immune inhibitory mechanisms, such as Treg cells, is provoked. As shown in Fig. 3, patients with brucellosis (both acute and subacute) expressed

higher FOXP3 copy number values than the healthy control group (P< 0.05). It appears that acute and subacute patients express relatively the same level of FOXP3 copy number values.

3.4. Acute brucellosis patients showed higher T-bet/GATA3 ratio

As depicted in fig. 2, acute brucellosis patients expressed higher GATA-3 copy number values than subacute brucellosis patients and healthy controls; however, the difference was not statistically significant. In contrast, the GATA-3 copy number in subacute patients was approximately equal to that in the control group.

Although acute brucellosis patients produced more GATA-3 copy number than subacute patients and healthy controls, as they produced many more Th1 cells (fig. 1), the ratio of T-bet/GATA-3 in acute brucellosis patients was significantly higher than that in subacute brucellosis and healthy controls (P< 0.05) (fig. 4). These results indicate that although the levels of both T-bet and GATA3 copy numbers were higher in acute brucellosis patients than in subacute patients and healthy individuals, an increase in Th1-related transcription factor was more prominent than Th2-related transcription factor in these patients.

3.5. Subacute brucellosis patients showed higher FOXP3/T-bet ratio

Acute and subacute brucellosis patient approximately produced the same level of FOXP3 copy number value (fig. 3), but since, at the same time, subacute brucellosis patients expressed lower T-bet copy number values than acute brucellosis patients (fig.1), the Treg/Th1 ratio in subacute patients was significantly higher than that in acute patients (p< 0.05) (fig. 5). On the other hand, subacute brucellosis patients expressed higher FOXP3 copy number values (fig. 3), but a slightly lower T-bet copy number value (fig. 1) than healthy controls, so the Treg/Th1 ratio in subacute patients was significantly higher than that in controls (p< 0.05) (fig. 5).

4. Discussion

Host protection against *Brucella* infection depends on cell-mediated immunity, which involves mainly activated macrophages and dendritic cells, $CD8^+$T cells and $CD4^+$ T subsets (4, 5). Among Th subsets, while Th1 is essential for the clearance of *Brucella* infection (7), Th2 and Treg have opposite effects and suppress Th1 responses. This study was performed to evaluate Th1-, Th2- and Treg- related lineage-specific gene expression in peripheral blood mononuclear cells of acute and subacute brucellosis patients.

The results of the present study showed that the level of Th1-lineage-specific gene (T-bet) expression in patients with acute brucellosis was higher than that in subacute patients and healthy controls. Consistent with this finding, it was previously shown that in human brucellosis, IFN-γ is released during the acute phase and patients with acute brucellosis display a Th1-orientated immune response (19). In addition, it has been reported that peripheral blood mononuclear cells of patients with acute brucellosis produce more Th1 cytokines (IFN-γ and IL-2), in response to *Brucella* cytoplasmic proteins, than chronically infected patients and healthy controls (20, 21).

Recognition and signaling by TLRs are crucial for the activation of macrophages and dendritic cells (DC) and priming of adaptive immunity (22). It has been shown that several *Brucella* antigens interact via TLR2 and TLR4 on macrophages, and by interaction via TLR9 within the endosomal compartment, activate these cells to produce IL-12, which in turn promotes differentiation of Th cells toward Th1 (23, 24). IFN-γ, as reviewed by Baldwin and Goenka, is a key player in controlling bacterial development within the host (25). However, despite IFN-γ production in the acute phase, the disease progresses to chronic brucellosis in some patients. Previous studies have suggested that the host's ability to mount an inflammatory response during

the initial stages of infection is not directly associated with protection (26). *Brucella* has been reported to displays several survival strategies to avoid the host's innate and adaptive immune responses (25, 27, 28). For instance, *Brucella* possesses a TIR domain-containing protein (Btp1/TcpB) that disturbs TLR2 and TLR4 signaling by impeding MyD88-induced activation of NF-κB and leads to destabilization of innate immune recognition and pro-inflammatory response. This stealthy strategy presents a low- quality response ability, which ultimately suppresses Th1 cytokines (IFN-γ, TNF-α and IL-2) (22). In addition, *Brucella* can interfere with IFN-γ signal transduction in human macrophages in vitro through an unknown mechanism (29).

According to previous studies, there is a correlation between reduced Th1 response and chronic/relapsing disease (20, 30). Our results showed that the level of the Th1 lineage-specific gene (T-bet) transcript in subacute brucellosis patients was lower than that in patients with acute brucellosis. In addition, although it seems that patients with acute brucellosis expressed a slightly higher number of Th2 lineage-specific genes (GATA3) than subacute patients and healthy controls, at the same time, they produced more Th1 cells, and the ratio of Th1/Th2 in acute patients was higher than that in subacute and healthy controls.

However, according to our results, both acute and subacute brucellosis patients expressed higher levels of Treg lineage-specific gene (FOXP3) transcripts than healthy controls. Although acute and subacute patients produced approximately the same amount of FOXP3 (Treg cells), but since acute patients expressed more Th1 related transcription factor (T-bet) than subacute patients, the ratio of Treg/Th1 in subacute patients was significantly higher than that in acute patients.

Regulatory T cells are fundamental components of the immune response exerted by hosts against pathogens (11). Several lines of evidence suggest that the presence of Tregs is a

protective factor in that they preserve the host by the deleterious effect of an exaggerated immune response (31-33). However, their activity also influences the response to pathogens, reducing the capacity of effector cells to control infection and paving the way for the establishment of a chronic infection (34). Tregs have been shown to be directly involved in the immune response to *Brucella* infection, suppressing effector T cells (Th1) that contribute to infection control. In addition, mice lacking Treg cells were more resistant to infection and more effective in producing IFN-γ (35). Therefore, it is plausible that, during *Brucella* infection, Treg cells by suppressing Th1 cells contributing to prolonging brucellosis from the acute to subacute form.

It has been shown that *Brucella* has virulence factors that suppress immune responses. *Brucella* abortus lipopolysaccharide (LPS) significantly impairs the MHC class II presentation pathway in macrophages, acts as a down-regulator of T cell activation, and may explain the immunosuppression observed in individuals with chronic brucellosis (36). It has also been reported that *Brucella* LPS suppresses cellular immunity by inducing IL-10 production (37). In addition, *Brucella* abortus has a virulence factor, *prp*A (for Proline Racemase Protein A), which downregulates IFN-γ and TNF-α and upregulate TGF-β in vivo (38). *Prp*A, which is secreted during infection, has the capacity to induce T cell-independent B cell-nonspecific polyclonal activation concomitant with the secretion of IL-10 and is required for the establishment of chronic *Brucella* infection in mice (39, 40). The presence of IL-10, in addition to TGF-β, leads to an increased expansion of Tregs with enhanced CTLA-4 expression and suppressive capability (41). Therefore we can hypothesize that during the acute phase of brucellosis, *prp*A participates in the stimulation of B cell proliferation and IL-10 and TGF-β secretion, allowing *Brucella* to manipulate the balance between the proinflammatory and anti-inflammatory responses in its own

benefit, increasing the anti-inflammatory response through expansion of FOXP3+ Tregs and production of TGF-β and IL-10, thus generates a transient immune suppression that leads to the prolongation of the disease to the sub-acute phase.

In Conclusion, our results indicate that although all three sub-populations of Th cells (Th1, Th2, and Treg) increased in acute patients, the prominent response in acute patients was Th1. In contrast, the dominant response in subacute patients was Treg; however, the Th1 response in subacute patients also decreased. Therefore, there appears to be a correlation between an increase in CD4+Treg cells and disease progression from acute to chronic brucellosis.

Abbreviations

T-bet: T-box transcription factor expressed in CD4+ T lymphocytes committed to Th1 cells.

GATA-3: GATA- binding protein 3 is expressed in CD4+ T lymphocytes committed to Th2 cells.

FOXP3: Forkhead box Protein3 expressed in CD4+ T lymphocytes, is committed to regulatory T cells.

TLR: Toll like receptor

DC: Dendritic cells

NF-κB: Nuclear factor kappa beta

prpA: (for Proline Racemase Protein A)

CTLA-4: Cytotoxic T-Lymphocyte Associated Protein 4

Btp1/TcpB: TIR domain-containing protein.

Ethic Approval and Consent to participate

Written consent was obtained from all participants, and all volunteers participated in the study. This study was approved by the Research Ethics Committee of Kermanshah University of Medical Sciences (KUMS.REC.1394.518.)

Competing interest

The authors declare that they have no competing interest.

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Authors Contributions

Jalilian S. and Gorgin Karaji A designed the study. Saeed Jalilian, Ali Reza Janbakhsh, Amirhooshang Alvandi, Farhad Salari, and Ali Gorgin Karaji collected all data. Saeed Jalilian and Ali Gorgin Karaji drafted the manuscript, and all authors commented on and approved the final draft of the manuscript.

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Table 1. **Complaints of patients with brucellosis**

Complaint	Acute %	Sub-Acute %
Joint pain	97	100
Sweating	80	77
Fever	80	63
Shiver	71	47
Weight loss	66	67
Chronic fatigue	51	57
Headache	43	40
Splenomegaly	51	13
Sacroiliitis	29	20
Hepatomegaly	6	0
Arthritis	0	7
Orchitis	3	0

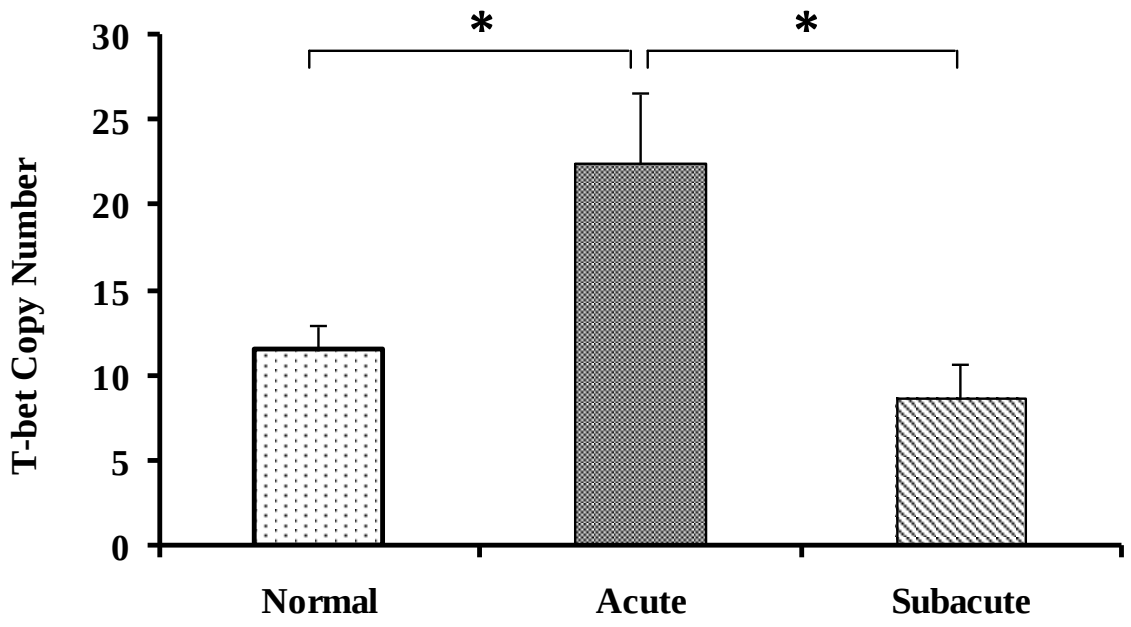

Fig 1. T-bet copy number values in acute and subacute brucellosis and healthy controls. Patients with acute brucellosis had higher T-bet copy numbers than healthy controls and patients with subacute brucellosis (P< 0.05). Plot graphs display copy number mean values ± standard error, acute vs. normal (22.43±4.23 vs. 11.5±1.37) and acute vs. sub-acute (22.43±4.23 vs. 8.74±1.87).

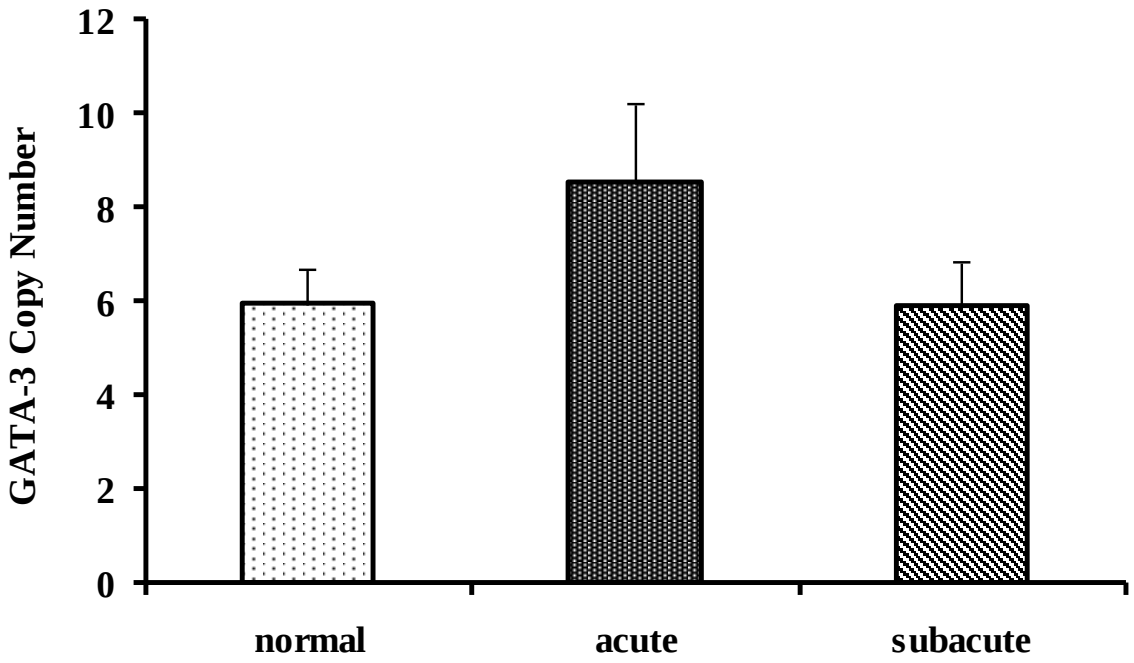

Fig 2. GATA-3 copy number values in acute and subacute brucellosis patients and healthy controls. There were no statistically significant differences between acute, subacute, and healthy controls regarding GATA-3 copy number values. The plot graphs display GATA-3 copy number mean values ± standard error: normal (5.95±0.74), acute (8.51±1.71), and sub-acute (5.89±0.97).

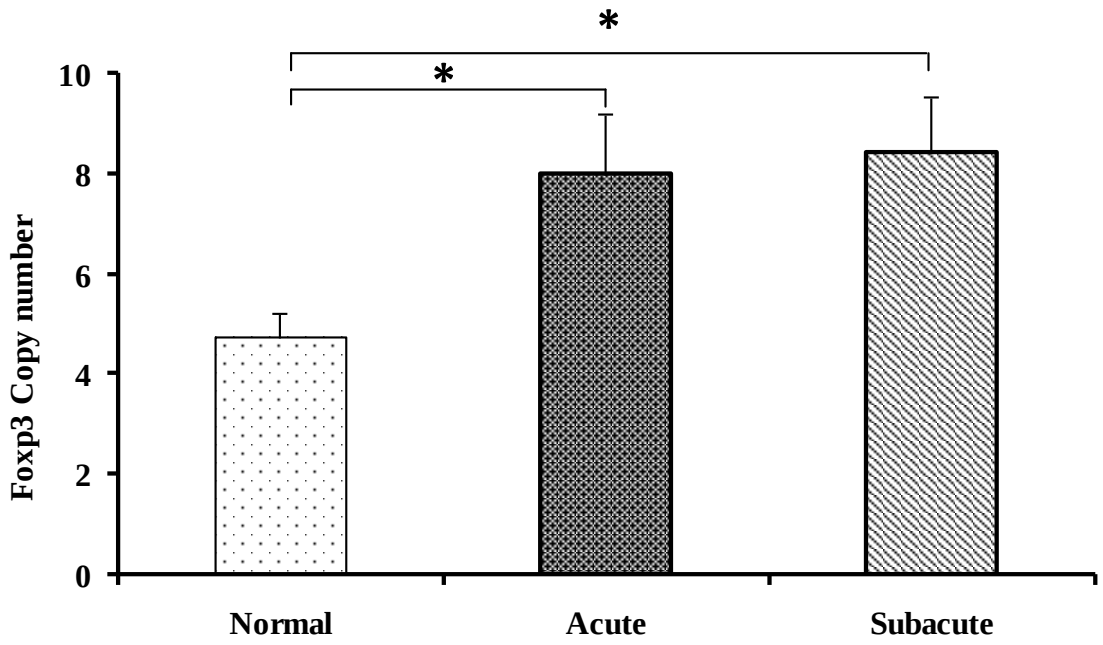

Fig 3. Foxp3 copy number values in acute and subacute brucellosis patients and healthy controls. Patients with acute or subacute brucellosis expressed more Foxp3copy number than healthy controls. Statistical significance is indicated by * (P< 0.05). Plot graphs display the copy number mean values ± standard error, acute vs. normal: (7.99±1.21 vs. 4.76±0.46) and subacute vs. normal: (8.42±1.09 vs. 4.76±0.46).

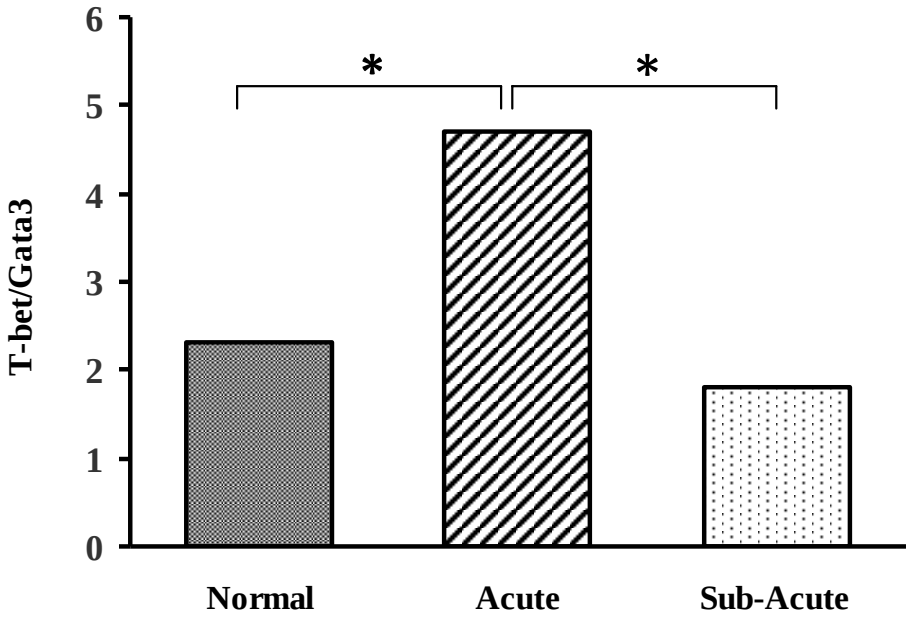

Fig 4. Patients with acute brucellosis showed high T-bet/GATA-3 ratio. The T-bet/GATA-3 ratio in patients with acute brucellosis was higher than that in patients with subacute brucellosis and in healthy controls. Statistical significance is indicated by * ($P < 0.05$). Acute (4.72) vs. normal (2.32) and sub-acute (1.8).

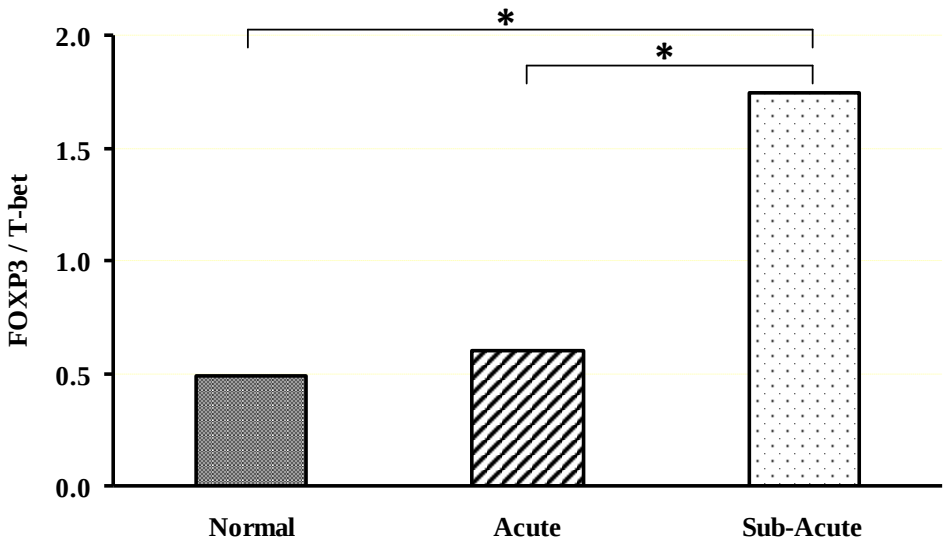

Fig 5. Subacute Brucellosis patients showed high FoxP3/T-bet ratio. The ratio of FoxP3/T-bet in subacute brucellosis patients was higher than that in acute brucellosis patients and healthy controls. Statistical significance is indicated by * ($P < 0.05$). Subacute (1.75) vs. acute (0.6) and normal group (0.48).