

Is the Status of CXCL9-CXCL10-CXCL11/CXCR3 axes Altered in Brucellosis Patients?

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Abstract

Aim and objectives: Brucellosis is common in most parts of the world between humans and domestic animals. The causative agent is bacteria from the Brucella family, which are intracellular pathogens. Despite, Brucella spp. Is able to infect humans, their ability to induce production of chemokines by lung cells is unknown. In this study, we showed an association between brucellosis and CXCL9, 10 and 11 chemokines

Methods: We have totally recruited 71 brucella patients who have referred to the either local health centers or hospitals of kerman province. Diagnosis of acute brucellosis was made based on a brief history of the disease, clinical examinations results of serological tests, antibody titration in serums was performed by bacterial wright method, agglutination and 2ME with antigen. Brucellosis patients CXCL9, CXCL10 and CXCL11 serum levels were detected by sandwich ELISA using paired chemokine-specific mAbs, according to the manufacturer's guidelines. Transcription levels of mRNA for the CXCR3 as the chemokine receptor for CXCL9, CXCL10 and CXCL11 was examined by QRT-PCR using primers.

Results: We have found that there was a positive correlation between the level of chemokines and their receptor. Here ELISA test showed that B. abortus induces expression of some IFN- γ induced CXCL9, 10 and 11 chemokines in brucellosis patients. We have also showed the increased expression of CXCR3 as their shared receptors in brucellosis patients with real time-PCR.

Conclusion: According to these findings there was a positive correlation between the level of chemokines and their receptor. We have found that there was a positive correlation between the level of chemokines and their receptor.

Introduction

Brucellosis is common in most parts of the world between humans and domestic animals (1, 2). Domestic animals such as goat, sheep, and camel are well known as reservoirs of the disease. Cattle may also serve as the host of Brucella in some cases. The most common case of Brucella abortus is in cattle, wild cattle, buffalo, camels, and Asian cattle. Canines are producing the disease in dogs, and these types of Brucella are possible transmitted to humans and relative produce the related types of Brucellosis. In Iran, brucellosis could also be transmitted to the people via uncontrolled blood transfusions, animal contacts dairy products and meat. Various types of brucellosis are intracellular organisms that present inside the reticuloendothelial system cells including macrophages and monocytes. The causative agent is bacteria from the Brucella family, which are intracellular pathogens (3, 4). The airways epithelial cells in addition to alveolar macrophages are of first cells that are exposed to inhaled types of microorganisms and are therefore ready to run an immediate immune response. In spite of, containing of anatomical barriers against processes of microbial invasion, the respiratory system epithelium is itself able to respond the arms of pathogens with a relative inflammatory response. The cytokines/chemokine network members are able to control infection and inflammation (5, 6). These types of epithelial responses might

possibly further be elevated by the stimulated actions of cytokines that are secreted by alveolar macrophages (7, 8). The respiratory epithelium generates reagents such as β -defensins, small antimicrobial peptides which normally present within fluid lining of the respiratory tract alongside with other antimicrobial components e.g. lysozyme and cathelicidins, in response to the infection. The respiratory epithelium is also able to actively respond to infections throughout generating several other inflammatory mediators, like chemokines, especially CCL20. Alternatively, the so called liver and activation-regulated chemokine (LARC), the macrophage inflammatory protein-3 (MIP-3) or Exodus-1 and CCL20 as a named chemoattractant for the maturing dendritic cells as well as effector/ memory T-cells and B-cells. Although, CCL20 and its cognate receptor, the CCR6 have been indicated to regulate in vivo the chemo attraction of the dendritic cells and lymphocytes in various tissues, including lungs (9, 10). Notably, the repertoire of CCR6 positive T cells and Th17 cells are specifically recruited by CCL20 (11), a fact that could be relevant to the immune responses against infectious agents. CCL20 and β -defensins, especially hBD2, showed that share many similarities and both share CCR6 as a common membrane receptor. While reacting CCL20 to CCR6 was known as the mediator at chemotactic responses of the immature dendritic cells to the CCL20, more recent studies demonstrated that β -defensins also exhibits chemotactic activity for CCR6 (12, 13). They both are able to act as chemoattractants for a wide variety of cell types involved in innate and adaptive immunity and can also stimulate various immune responses (including production of cytokines maturation of dendritic cells and etc.) (14, 15). In particular, hBD2 induces several cell types memory T cells, immature dendritic cells, mast cells and neutrophils chemo taxis processes (16–18). In other words, despite the fact that, CCL20 was initially described as a chemokine but more recent studies have revealed that these types of molecules are also able to display antimicrobial activities against both the gram positive and gram negative bacteria (19, 20). It has thus been proposed that the antimicrobial activity of CCL20 could possibly be due to the fact that this chemokine shares structural properties with β -defensins, including antiparallel β -pleated sheet core structure and charge distribution (19). The CCL20 and hBD2 expression have been demonstrated to be elevated in respiratory epithelial cells following exposure to infectious agents or antigens (21, 22) as well as in response to members of pro-inflammatory cytokines network (23, 24). The human brucellosis is mainly caused by *Brucella melitensis*, *B. suis* or *B. abortus*. The disease is as a zoonotic illness and affects over 500,000 people annually which is distributed, worldwide (25, 26). These bacteria, inhalation of infected aerosols is frequently involved in contagion. Chemokines are a subclass of cytokines with the property of pro migratory and chemotactic feature for blood leukocytes. They themselves are divided in four sub-divisions of C, CC, CX3C. The studied chemokines in the (CXCL9, 10 and 12) present study belong to the CXC subdivision. In spite of the importance of the respiratory route of the entrance of *Brucella* within the organism, these bacteria reaction with the pulmonary cells has rarely been well examined, however, previous studies have reported that *Brucella* species have the ability to infect and replicate within the lung epithelial cells of human, and this can stimulate these cell, to produce CCL20 (27–29). Rewardingly, due to the both their chemotactic and antimicrobial activities, CXC chemokines have also been purposed to play fundamental parts in innate immune response of pulmonary system to the inhaled pathogens and compelling evidences have also indicated the enhancement of chemokines in lung tissues both following and during infection. Because (30, 31), almost nothing is reported regarding

the expression of these molecules in brucella infection or about their antimicrobial activity against this pathogen, the main goal of the present study was to address these issues.

Material And Methods

Patients and specimens

We have totally recruited 71 brucella patients who have referred to the either local health centers or hospitals of kerman province. Diagnosis of acute brucellosis was made based on a brief history of the disease, clinical examinations, results of serological tests, antibody titration in serums was performed by bacterial wright method, agglutination and 2ME with antigen and wright rate. The Elisa Juan behring device was used to read Elisa's results. Fifty (50) people of both sexes from the blood donors and students from kerman province of medical science who were normal without history of brucellosis or other types of bacterial infections control as participants in this study.

RNA isolation and preparation of cDNA

The expression of the CXCR3 mRNA was examined using quantitative real time PCR (qRT-PCR) for all of patients and controls. From the buffy coat of blood samples total RNA was isolated employing Invitrogen Trizol according to the instructions of the manufacturer. Both quality and purity of the isolated RNA were examined by running on agarose gel and electrophoresis as well as measurement of optical density (A260/A280 ratio) by Nano Drop 1000 Spectrophotometer (Wilmington, DE, USA), respectively. The genomic DNA was removed from the RNA preparations, throughout DNase I, RNase-free kit from Thermo (Thermo Scientific, USA), according to instructions to manufacturers. To achieve this, ; 1 µg RNA, 1 µl 10X Reaction Buffer with MgCl₂, 1 µl DNase, RNase-free, water nuclease-free up to 10 µl and was incubated at 37°C for 30 min and then 1 µL 50 mM EDTA was added and the incubation was prolonged at 65°C for 10 min. The processes of reverse transcription (RT) was undertaken using the Revert Aid First Strand complementary DNA (cDNA) Synthesis kit from Thermo (Thermo Scientific, USA). The RT reaction was made up 20µl and 11µl of solution that prepared in before staging for removal of genomic DNA, 1 µl Random Hexamer Primer, 4 µl 5x Reaction Buffer, 2 µl dNTP Mix (10 mM), 1 µl Ribo Lock RNase Inhibitor (20 U/µl), and 1 µl Revert Aid M-MuLV Reverse Transcriptase (200 U/µl) as a master mix. Adapted time scales and temperature profiles of RT reaction was as follow: incubation 5 min at 25 °C followed by 60 min at 42 °C. The reaction was terminated by heating at 70 °C for 5 min (13).

Isolation of PBMCs (peripheral blood mononuclear cells) by using LeucoSep tubes.

PBMCs were purified using LeucoSep tubes according to the instructions of the manufacturer (Greiner Bio-One). In brief, 3 ml of Ficoll-Paque was preloaded in a 14 ml LeucoSep tube by centrifugation for 30 s

at $1,000 \times g$. The heparinized whole-blood samples were diluted with equal volumes of PBS, and 6 ml of the diluted blood was added to a LeucoSep tube. The cell separation tubes were centrifuged for 15 min at $800 \times g$ without braking at room temperature. The cell suspension was collected, and the cells were washed twice in PBS (for 10 min at 640 and $470 \times g$, respectively, for the two successive washes) and resuspended in complete RPMI medium before counting (14, 31).

Cell yield/viability.

The both PBMCs yield and viability were determined using a NucleoCounter (ChemoMetec A/S, Allerød, Denmark). The NucleoCounter is able to detect nonviable cells using propidium iodide to stain cell nucleus and determines cellular viability of a blood sample employing total cell count and counting dead cells.

Quantitative-real-time-polymerase-chain-reaction (QRT-PCR)

Transcription levels of mRNA for the CXCR3 as the chemokine receptor for CXCL9, CXCL10 and CXCL11 was examined by QRT-PCR using previously described primers [table 1]. As stated earlier, further isolation PBMCs were twice rinsed with phosphate-buffered saline and their total RNA content was isolated by TRIzol reagent (Invitrogen, Life Technologies). The total RNA (2 μ g) was then reversely transcribed by standard reagents (Invitrogen). The complementary DNA (cDNA) corresponding to 200 ng RNA was amplified in a PCR containing 0.1 mM of CXCR3 specific intron spanning primers (forward primer: 5'-CTCTGCTGGACCCCTATCA-3'; reverse primer: 5'-GTCTCAGACCAGGATGAATC-3', product: 255 bp) with an internal control for equal amounts of cDNA of a glyceraldehyde- 3-phosphate dehydrogenase (GAPDH)-specific intron spanning primer pair (5'-CCAGCCGAGCCACATCGCTC-3'; 5'-ATGAGCCCCAGCCTTCTCCAT-3'), which yielded a 360- bp amplified product. Amplification was performed using 25 to 40 cycles with denaturation at 94°C for 30s, primer annealing at 60°C for 30s, and extension at 72°C for 1 min. PCR products were subjected to electrophoresis on a 1% agarose gel and visualized by SYBR Safe staining (Invitrogen) (15).

Measurement of serum CXC chemokines by ELISA

Brucellosis patients CXCL9, CXCL10 and CXCL11 serum levels were detected by sandwich ELISA (R&D, Minneapolis or Peprotech respectively) using paired chemokine-specific mAbs, according to the manufacturer's guidelines.

Following clinical examinations by the infectious disease specialists, intravenous blood samples were collected from patients and serum samples were then isolated from the patients and harvested samples have been kept at -80°C for further chemokines (CXCL1, CXCL9, CXCL10 and CXCL12) measurement.

Statistical Analysis:

Following of the collection the desired data, statistical evaluations on the mentioned variables with SPSS computer software (version18) of tukey test were used to analyze the findings.

Results

The wright rate based CXC chemokines serum level

As our findings showed that the wright rate in %95 of the brucellosis patients ranged from 1/60 to 1/1280 and the 2ME test titer in %97 of these patients ranged from 1/80 to 1/320. Our findings indicated that cxc chemokines CXCL9, CXCL10 and CXCL11 have been enhanced in acute brucellosis patients, when compared with control, the mean serum measures of CXCL9 were 164 ± 63.585 pg/mL, 360 ± 30 pg/mL, 259.33 ± 25.7 pg/mL in patients with the wright rate of 1/80, 1/160 and 1/320, respectively and were 122.01 ± 101.94 pg/mL in control ($p < 0.05$, Fig-1). We have also showed that the serum levels of CXCL10 were 205.4 ± 80.72 pg/mL, 584.45 ± 185.40 pg/mL, 508.4 ± 222.12 pg/mL in acute brucellosis patients with the wright rate of 1/80, 1/160 and 1/320, respectively and 348.88 ± 69.4 pg/mL in control subjects. ($p < 0.05$, Fig-2)

These findings have also revealed that the serum concentrations of the CXCL11 were 1674.5 ± 540.16 pg/mL, 222.27 ± 788.5 pg/mL, 1691 ± 541 pg/mL in acute brucellosis patients with the wright rate of 1/80, 1/160 and 1/320, respectively while it was 142.4 ± 27.7 pg/mL and in control subjects ($P < 0.05$, fig-3)

The 2ME-based CXC chemokines serum levels

Results of the present investigation have demonstrated that the serum levels of the CXCL9 were 141 ± 66.48 pg/mL, 131.6 ± 25.7 pg/mL, 237.3 ± 220.6 pg/mL in acute brucellosis patients with 2ME rate of 1/80, 1/160 and 1/320, respectively in compare to control subjects (22.32 ± 107.8) ($P < 0.05$, fig-4).

We have found that the serum levels of the CXCL10 were 204.35 ± 90.7 pg/mL, 9584.4 ± 185.4 pg/mL, 517 ± 229 pg/mL in acute brucellosis patients respectively in compare to control subjects (350.16 ± 69.7). ($P < 0.05$, fig 4). Finding of this study showed that the serum levels of CXCL11 in brucellosis patients were 1795.36 ± 565.88 pg/mL, 2269.4 ± 81.334 pg/mL, 1699.4 ± 548.9 pg/mL respectively when compared to control subjects 142.84 ± 28.7 . ($P < 0.05$, fig-4).

Analysis of our real time-PCR data

Statistical analysis of our real time-PCR data showed that the mRNA expression of CXCR3 was significantly increased in acute brucellosis patients with 2ME rate of 1/80, 1/160 and 1/320,

respectively in compare to control subjects($P < 0.05$) (Fig. 5) and the expression level of CXCR3 gene increased in was significantly increased in in acute brucellosis patients with wright rate of 1/80,1/160 and 1/320, respectively in compare to control subjects ($P < 0.05$) (Fig. 5).

Discussion

Brucellosis is defined as a worldwide disease which is transmitted to human from the products of domestic animals such as sheep and goat milk. We have designed the current study to examine the serum concentrations of IFN- γ induced CXC chemokines along with their receptor (CXCR3) in brucellosis patients. There has been informed a lot of information regarding generation of antibodies against brucella within the body during brucellosis. With this regard, because the bacteria that cause brucellosis are basically intracellular, thus the cellular immunity plays a key part in destroying the intracellular bacteria and in turn improving the pathogen(30).

Several cellular safety based investigations in laboratory rodents have been performed and researchers reported that TNF (tumor necrosis factor alpha), IFN- γ and interleukin 12 play key parts in resistance to *Brucella abortus*, during brucellosis infection, as a profile of pre-inflammatory cytokines. Animal model studies showed that in brucellosis patients, the IFN- γ concentration tends to be sharply elevated and if patients failed to produce adequate amounts of IFN- γ the infection continuously stay in the patient's body (32, 33). As previously mentioned studies of cytokine, especially chemokine family production is very limited in brucellosis patients. Studies which was performed by some researchers have shown that IFN- γ and interleukin-12 levels in patients are higher than in normal subjects, however, they did not indicate in the report the clinical type (acute or chronic) of the studied patients (32). In accordance with the Ahmed K et al, we have also shown the elevated levels of IFN- γ -induced patients in response to both TNF- α and IFN- γ in several cell types and inflammatory disease (34). In addition to our group multiple other research teams also confirmed elevation of these chemokines in response to inflammation(34) and thus one possible mechanism which can explain the induced levels of these IFN- γ - induced mediators could possibly be related to the enhancement levels of inflammation-related mediators that are known to be well related to brucellosis in these patients. As an example, the so called CCL20 as a cc chemokine subfamily member has well been established to be produced following being infected with brucella infection by lung epithelial cells (19, 22, 35). CCL20 is elevated in the lungs by some types of various infections (22, 35). [In line with these findings, various studies have also reported that pulmonary derived CCL20 is able to both exert chemo active activity for lymphocytes and dendritic cells (36) as well as directly act against pathogens (19). Some other studies have claimed that in addition to CCL20, the pulmonary β -defenses are also able to act against infections, including *Brucella* spp. to infect hosts via inhalation. Here we have showed that the expression of some members of the cxc chemokine subfamily including IFN- γ -induced CXCL9,10,11 were elevated in serum samples which were obtained from Brucellosis patients, in parallel with the CXCR3 as their respective receptors. Although, there were not found a related investigation to do compare, but we have already shown the elevated measures of these chemokines in some other infectious diseases including, post-transfusion-transmitted hepatitis C virus infection, following hepatocyte isolation and in response to heat shock (34). As the brucellosis is

developed the titer of antibodies are reduced and becomes unmeasurable following 2-3 years. Continuous high level of IgG is of the causes of chronic infection and the disease recurrence. Brucellosis is frequently associated with extensive tissue necrosis and eventually abscess production. Tissue granulomatous within organs such as liver and spleen is also achieved via abortion of Ca. Therefore, brucella immunopathological features depends on the type of organism and thus the clinical symptoms, laboratory changes and thus immunology system and its related arms (cytokine and chemokines) are involved its pathogenesis. Results of the present study are novel and authors could not address a homologue set of data to compare with, but, there exist few articles that have referred to the upstream signals that are involved in production of these inflammatory mediators, following infection/inflammation as an example, CCL20 has well been found to be generated by lung epithelial cells in response to injury (22, 30, 31). CCL20 is elevated in the lungs by some types of infections (30, 31). In line with us various studies reported that pulmonary derived CCL20 is able to both exert chemotactic activity for lymphocytes and dendritic cells as well as directly act against pathogens (22). Again, investigations claimed that in addition to the β -defensins, CCL20 also defense against infections (including Brucella spp. that infect hosts) via inhalation. Studies reported that the elevate levels of cytokines mostly depends on the severity of the patient's symptoms. Nitric Oxide has also been slightly increased in patients, which in away may confirm that septic shock does not occur in brucellosis. Researchers have demonstrated that in addition to the PAF (Platelet activating factor), and IL-12 aboard spectrum of anti-inflammatory molecules such as interleukin 4, interleukin 10 and interleukin I and soluble TNF receptors are also enhanced in the blood of patients with severe toxic septicemia.

IL- 6 and IFN- γ as pro-inflammatory proteins, are also produced by many cell types in the body in response to cellular lesions and hence it appears that these are involved in running of the acute systemic response of the acute phase and serves as a members of this response.

Conclusion

According to these findings there was a positive correlation between the level of chemokines and their receptor. We have found that there was a positive correlation between the level of chemokines and their receptor.

Declarations

- Ethical Approval and Consent to participate:

The research and its tool were approved by Research Ethics Committee of Rafsanjan University of Medical Sciences, and ethical principles were observed during the

- Informed consent:

All subjects participating in the study and/or their respective legal representatives were informed about the objectives and signed the Free and Informed Consent Form.

- Consent for publication:

All subjects participating in the study a signed the Free and Informed Consent for publication.

- Availability of data and materials:

Database will be made available if required.

- Competing interests:

The authors declare no conflict of interest.

- Funding:

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- Authors' contributions

- Study Design: Gholamhossein Hassanshahi, Mojgan Noroozi Karimabad, Farzaneh Hassanshahi

- Data Collection: Mojgan Noroozi Karimabad

- Statistical Analysis: Mojgan Noroozi Karimabad, Gholamhossein Hassanshahi

- Data Interpretation: Farzaneh Hassanshahi

- Manuscript Preparation: Gholamhossein Hassanshahi, Mojgan Noroozi Karimabad, Farzaneh Hassanshahi

- Literature Search: Farzaneh Hassanshahi

- Funds Collection: Mojgan Noroozi Karimabad

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Figures

Fig. 1

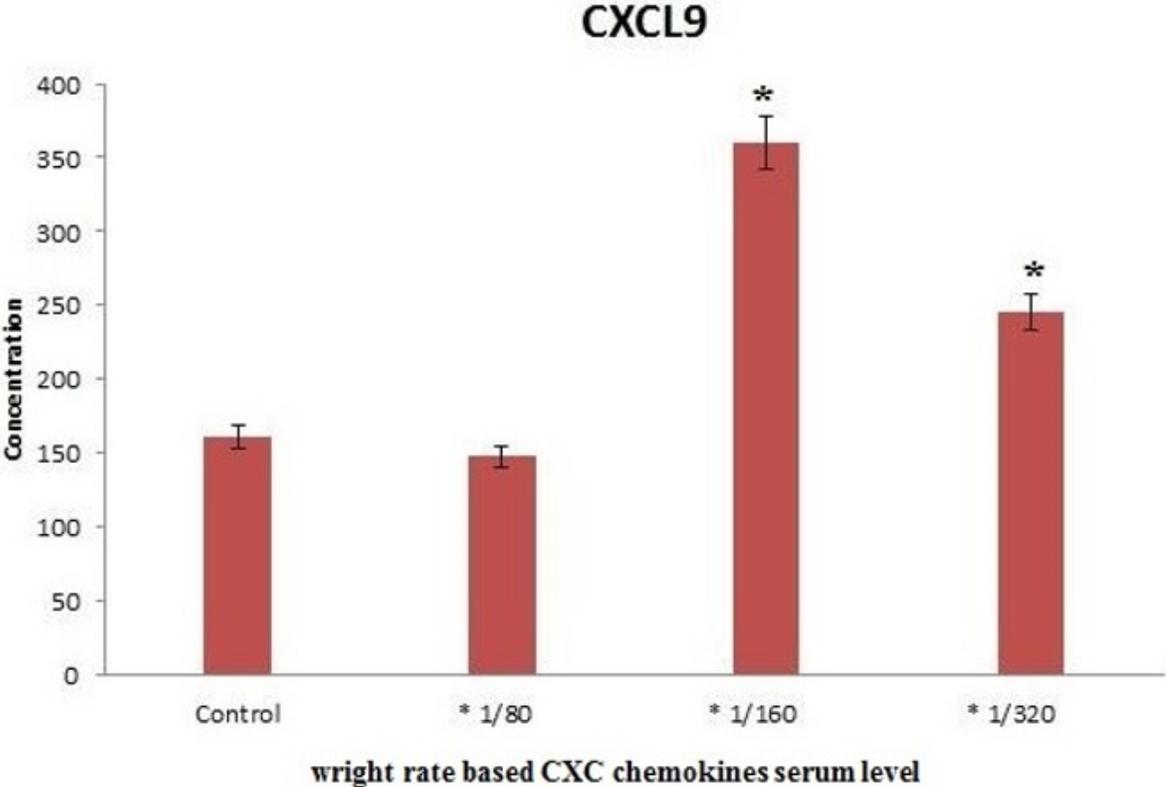


Figure 1

Demonstrates that the mean serum measures of CXCL9 in patients with the wright rate of 1/80 , 1/160 and 1/320 , in compare to control subjects (P<0.05).

Fig. 2

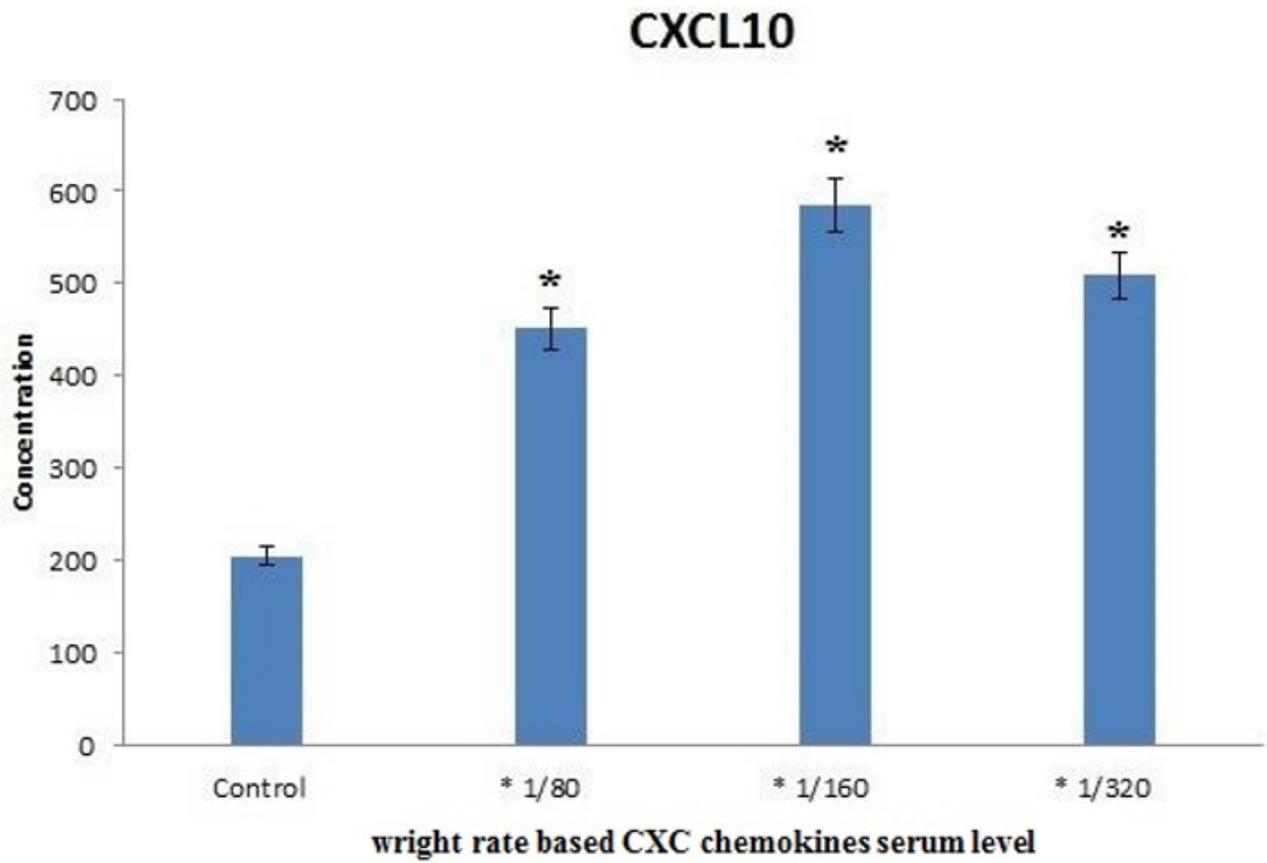


Figure 2

Demonstrates that the mean serum measures of CXCL10 in patients with the wright rate of 1/80 , 1/160 and 1/320 , in compare to control subjects (P < 0.05)

Fig. 3

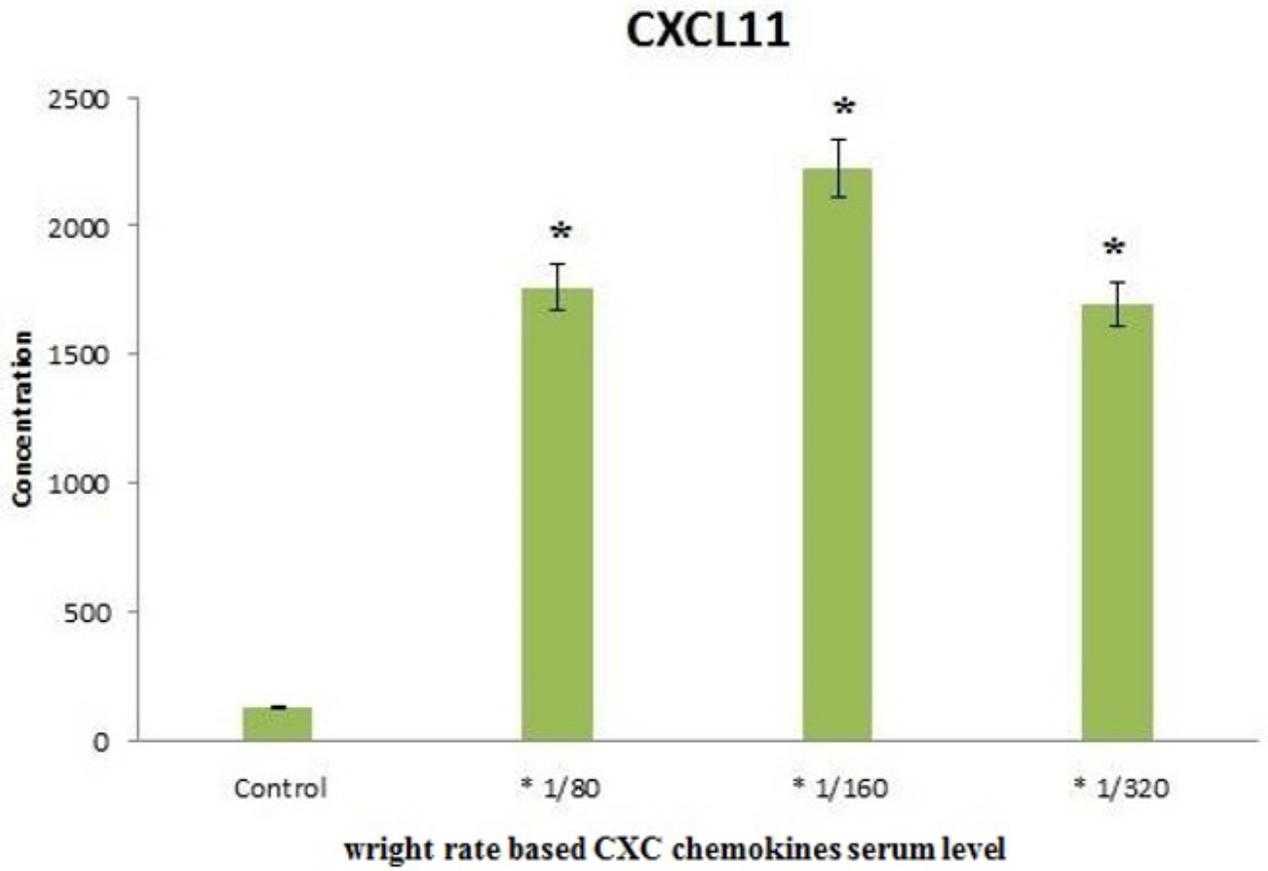


Figure 3

Demonstrates that the mean serum measures of CXCL11 in patients with the wright rate of 1/80 , 1/160 and 1/320 , in compare to control subjects (P < 0.05)

Fig. 4

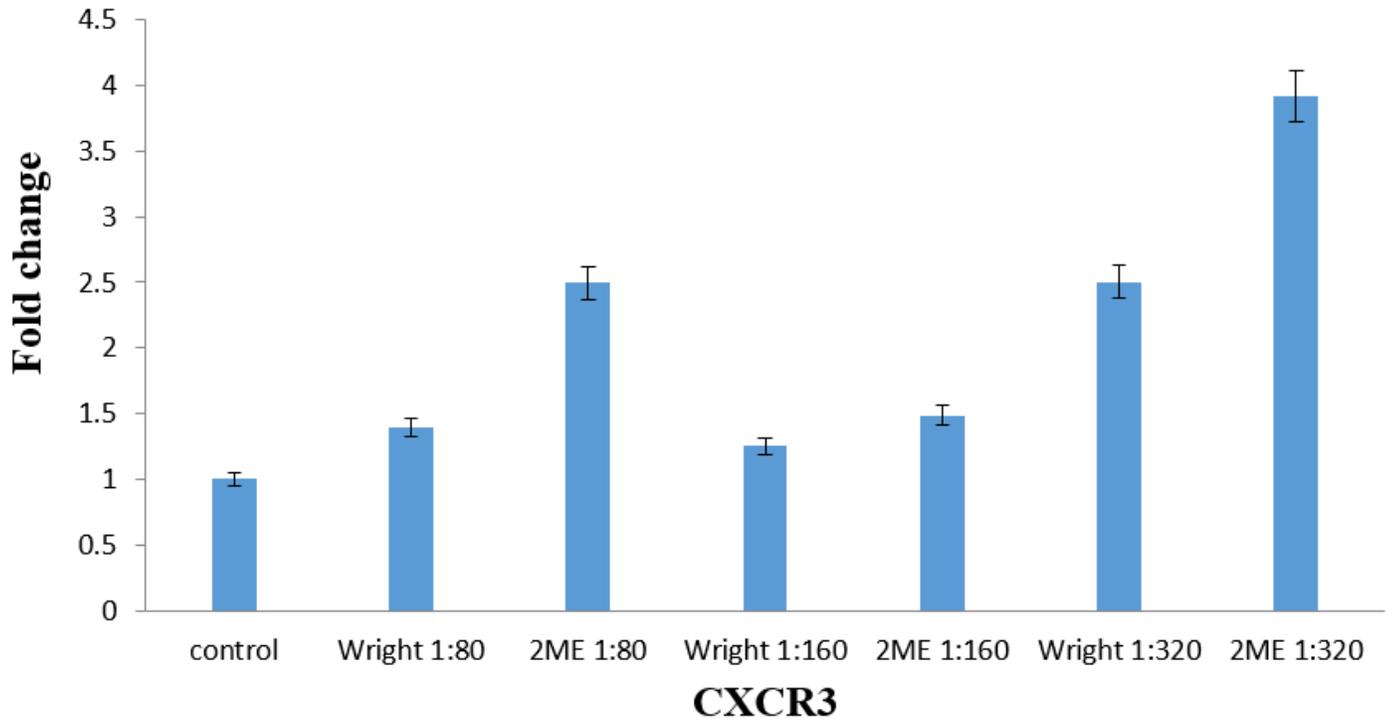


Figure 4

Demonstrates that the serum levels of the CXCL9,10,11 in acute brucellosis patients with 2ME rate of 1/80,1/160 and 1/320 in compare to control subjects (P < 0.05)

Fig. 5

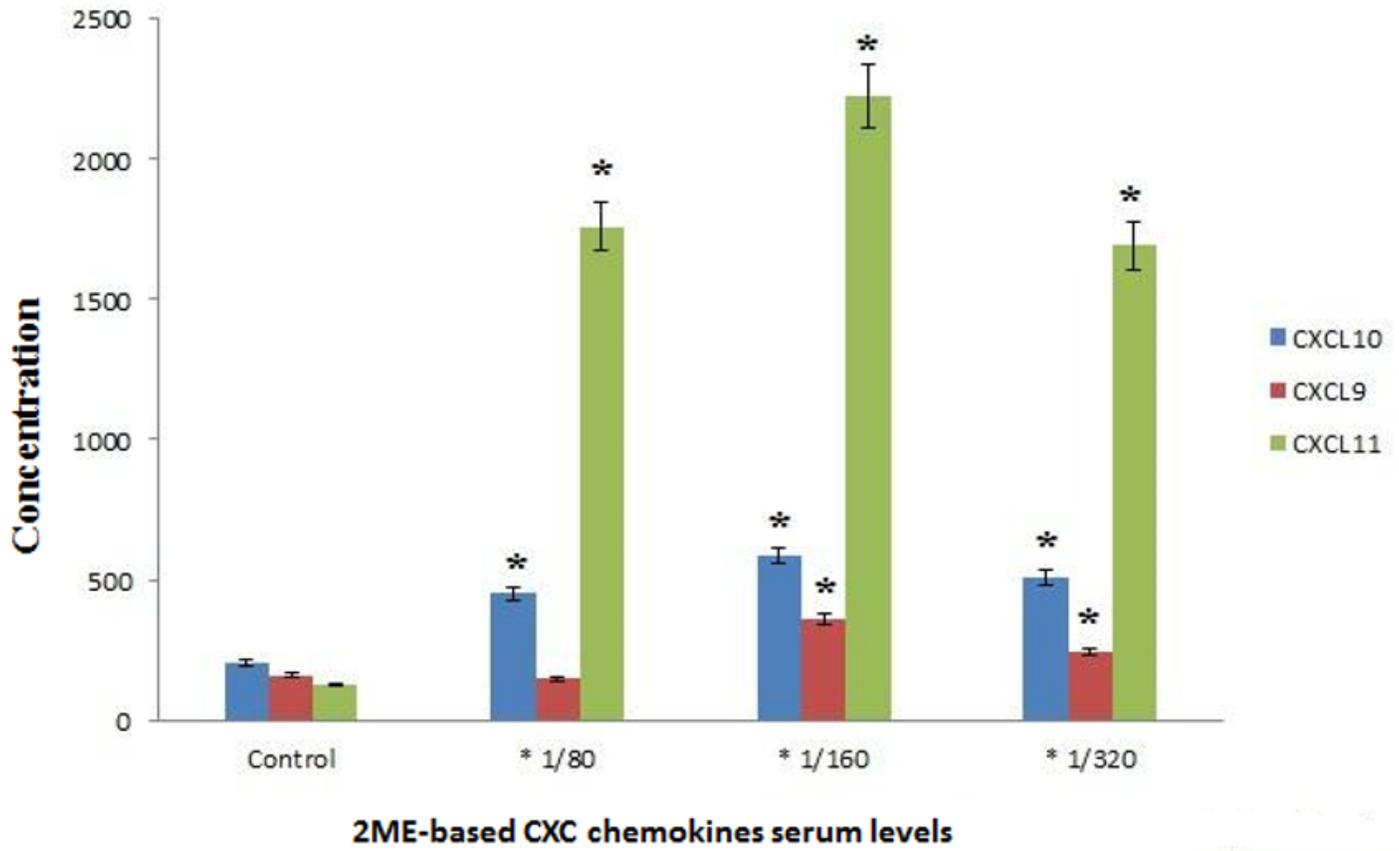


Figure 5

Demonstrates that that the mRNA expression of CXCR3 was significantly increased in in acute brucellosis patients with 2ME rate of 1/80,1/160 and 1/320, respectively in compare to control subjects(P < 0.05) and the expression level of CXCR3 gene increased in was significantly increased in in acute brucellosis patients with wright rate of 1/80,1/160 and 1/320, respectively in compare to control subjects (P < 0.05)