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Molecular Detection Of Brucella Spp. And Assessment Of Some Immunological Parameters In Suspected Brucellosis Cases From Babylon Province

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Abstract

Background
Brucellosis remains a main source of disease in humans and animal husbandry worldwide.
Objective : Genetic analysis for the detection of Brucella spp. and study of immune responses in potential Brucellosis infections in Babylon province.
Materials : The study was conducted over a six-month period, from March 2024 to August 2024. A total of 150 patients, both male and female, aged between 15 and 55 years, visited private clinics in Babylon province. These patients were positive for rose Bengal test. Blood samples were collected from each patient, along with 20 samples from healthy individuals serving as the control group. After detecting Brucella spp. using PCR, several immunological markers associated with brucellosis were evaluated.
Results: The Rose Bengal test detected Brucella spp. in 53 (46.2%) of 150 blood samples from brucellosis patients. Results of study on recognizing Brucella bacteria using spesific Bcsp31 gene primer from blood samples. The primer combination utilized in this investigation amplified a (223bp) fragment from Brucella (22 from 53 blood samples tested positive for Rose Bengal). Study group mean Perforin and granzyme B protein differences (case and control). The study found significant increases in Perforin (19.52±3.88) and granzyme B (74.31±13.88) levels in Brucellosis patients compared to the control group (6.39±6.33) ($P \geq 0.05$). The study found that patients with Brucellosis had higher IL-12 levels (287.54±12.18ng/ml) compared to the control group (188.08±2.29ng/ml), with a significant difference ($P<0.05$).
Conclusion: In order to have a complete understanding of the immune response to Brucella, it was essential to have a solid understanding of the action of perforin and granzyme B, since these two proteins were played an important role in The process.

Keywords Brucella, Immunological markers, Brucellosis, PCR, Gene.

Introduction

Brucellosis the contagion of which is attributed to the "Brucella" genus poses a great threat to human health, and livestock, especially cattle, suffer huge losses through abortion and infertility [1]. In humans brucellosis can present itself as an acute illness, sub-acute or chronic depending on the degree of exposure to the bacteria, or the animal, or ingestion of raw milk or meat [2]. There are six classical Brucella species, each with a distinct host preference: B. abortus is mainly a disease of cattle; B. meitatensis affects sheep and goats, B. suis infects pigs; B. canis affects dogs; B. ovis affects only

sheep and goats; and B. neotomae is identified in the desert wood rat [3]. Later in the twenty-first century, new Brucella species was reported. B. ceti and B. pinnipedialis, were isolated from marine mammals. Other species include the Brucella pathogens including B. microti from voles and seals and B. Strains inopinata originating from a human breast implant infection have also been recognized to be members of the Brucella genus [4]. Moreover, new strains of Brucella have recently been identified in wild rodents in North Queensland, Australia and from still birth cases in non-human primates though they have not been still categorised under the Brucella genus officially [3]. By reason of the low infective dose and the ability to be spread in the droplet nuclei, Brucella species have been deemed as having bioterrorism potential [5] .
Nevertheless, even now, when new methods of molecular diagnostics are widely used, culture isolation is the most accurate method for brucellosis confirmation. Characterization of Brucella and particularly its biotyping can best be achieved using biochemical, serological and molecular techniques [6]. Typical biochemical characters are CO2 test, H2S test, dye tolerant test, and favourite enzyme test urease, oxidase, catalase and so forth. Also, the use of monospecific A and M antiserum in agglutination helps in the determination of isolate characteristics [7]. The common tests used in the diagnosis of human brucellosis include the Serum Tube Agglutination Test (STAT), Rose Bengal Plate Test (RBPT), 2-mercaptoethanol (2ME) test, Complement Fixation Test (CFT) and the enzyme-linked immunosorbent assay (ELISA). However, these tests are not competent in certain aspects; for example: there are background antibodies prevalent in the endemic areas and it cannot distinguish between acute and previous infections [8]. Many PCR-based tests have been designed depending on genus specificity or a high level of homology of the Brucella species including the 43kDa outer membrane protein-omp gene, BCSP 31, IS 6501/711, and 16S rRNA gene [9] .

Granzyme B is the member of the protease family while perforin is responsible for cell killing in the immune system. They are involved in the process of CTL's and NK cells to kill target cells including virus or bacterial infected or tumor cells [10]. Perforin is a protein which acts as a pore in CTL and NK cells granules. Of these, when these immune cells come across an infected or abnormal cell, they release Perforin that forms a hole in the membrane of the target cell [11]. Thus with the formation of pores the other cytotoxic molecules like the granzyme B is also allowed to enter into the target cell. Granzyme B is a serine protease which has been identified to be present in the granules of CTLs and NK cells. When perforin forms gaps on the outer wall of the target cell, granzyme B gets into the cell through that gap [10]. Within the cell, granzyme B engages apoptotic processes that bring about the orderly dismantling of the infected or malignant cell hence contributing to the extermination of the pathogen or the cancerous cell without overproduction of inflammation [12].

Perforin and granzyme B play a role in the immune system's defense against intracellular pathogens including the Brucella organism which causes brucellosis [13]. Macs can be infected with Brucella and Cytotoxic T cells and NK cells can recognize these infected macrophages and kills them using perforin and granzyme B thus controlling the spread of Brucella [14]. During brucellosis, cytokines such as IFN-γ are produced and cause activation of macrophage to increase its ability to eliminate Brucella within it. Therefore, if the sources of infection are not effectively controlled, local CTLs and NK cells may be activated to destroy infected cells by the perforin-granzyme ladder. It is used in the destruction of infected cells that harbor Brucella during intracellular infections such as brucellosis hence aiding the immune system [15]. The increase of the concentrations of perfotin and granzyme B may be related with an active cytotoxic response during the infection by Brucella, particularly in chronic or severe cases [16]. Several research indicate that Brucella interfere with immune responses such as the perforin-granzyme pathway, that may help the bacteria to establish a chronic infection in the host [17]. Aims of study were Molecular detection of Brucella spp. and evaluation of immunological changes (Perforin and granzyme B levels), IL-12 in patients suspected of brucellosis in Babylon province.

Materials and methods

Patients

The study was conducted over a six-month period, from March 2024 to August 2024. A total of 150 patients, both male and female, aged between 15 and 55 years, visited private clinics in Babylon province. These patients were positive for rose Bengal test.

Collection of blood samples
Blood samples were collected from each patient (150 case and 20 healthy individuals serving as the control group. Five ml of blood were obtained from each subject by vein puncture, it was pushed slowly into disposable tubes containing separating gel containing tubes was allowed to clot at room temperature for (30) minutes and then centrifuged at 3000×g for approximately (3) minutes. Then the sera were obtained and stored at (-20˚C) until analyses.

DNA extraction
Genomic DNA was extracted from the each blood samples according to instruction provided by manufacturer using Genomic DNA purification kit supplemented by (Geneaid, USA).

Detection of Bcsp31 specific gene primer from clinical isolates by RCR technique:
Polymerase Chain Reaction technique used for amplification of specific gene of Brucella spp. that suspected to be present in blood samples were shown in Table (1). [18] .

Table (1): Detection primer sequence with their amplicon size Base pair (bp) and their condition

Specific gene primer	Primer sequence (5′-3′)	Size (bp)	PCR condition
Bcsp31	F:5-TGGCTCGGTTGCCAATATCAA-3 R: 5-CGCGCTTGCCTTTCAGGTCTG-3	223	Step 1: 95ºC, 2 min. Step 2: 95ºC, 30 sec. Step 3: 57.6ºC decrease 0.5ºC per cycle, 30 sec. Step 4: 72ºC, 100.0 sec. Step 5: Repeat steps 2-4 14 more times Step 6: 95ºC, 30 sec. Step 7: 50.6ºC, 30 sec. Step 8: 72ºC, 100.0 sec. Step 9: Repeat steps 6-8 19 more times Step 10: 72ºC, 5 min. Step 11: 4ºC, forever

Determination of Perforin and granzyme B proteins level
The human Perforin and and granzyme B enzyme-linked immunosorbent assay kits were used in this study for quantitative determination of proteins concentration adult patient's serum samples and done according to company instruction (Sunlong/ China).

Determination of IL-12 level
The Sandwich ELISA concept was used to generate this ELISA kit. Micro ELISA plates pre-coated with an antibody specific to Human IL-12 were included in each kit. A positive result may be obtained by mixing samples (or standards) with the appropriate antibody and adding them to wells on a micro ELISA plate.

Ethical Approval
A valid consent was achieved from each patient before their inclusion in the study.

Statistical analysis
Statistical analysis was carried out using SPSS version 26. Continuous variables were presented as (Means ± SD). Student t-test was used to compare means between two groups.

Results

The results of the Rose Bengal test were positive in 53 (46.2%) out of 150 blood samples taken from individuals exhibiting brucellosis symptoms, as indicated in Table (2), it was identified as Brucella spp.

Table (2): Rose Bengal Tests Brucella spp. positive

No. of blood samples	No. of rose Bengal test Brucella positive %
150	53(46.2%)

The results of the research that looked at the possibility of identifying Brucella bacteria using specific Bcsp31 gene primer from blood samples. Out of 53 blood samples that were tested using the positve Rose Bengal tests, only 22(54.3%) of the isolates were determined to be connected to Brucella positive, the primer pair used in this study succeeded in the amplification of a (223bp) fragment from Brucella spp.

Figure (1): Agarose gel electrophoresis (1.5%) of RCR amplified of Bcsp31 gene (223 bp) of Brucella spp. for (55) min at (70) volt L: ladder (DNA marker), (1, 2, 3, 4, 5, 6, 7, 8, to 22) Amplify of Bcsp31 gene was positive clinical isolates of Brucella spp.

The mean differences between Perforin and granzyme B proteins levels according to study groups (case and control groups) was shown in Table (3). The results showed that, there were significant increase in Perforin level (19.52±3.88) (P≥0.05) in patients with Brucellosis infection compared to control group (6.95±1.96), and there were significant increase in granzyme B (74.31±13.88) (P≥0.05) in patients with Brucellosis infection compared to control group (6.39±6.33).

Table (3): mean differences between Perforin and granzyme B proteins levels according to study groups including (patients with Brucellosis infection and control group).

Parameter/ protein	Patients with brucellosis infection	Control group	p-value
	Mean ± SD	Mean ± SD	
	No. 22	No. 20	
Perforin	19.52±3.88	6.95±1.96	**P≥0.05**
granzyme B	74.31±13.88	6.39±6.33	

* p value ≤ 0.05 was significant

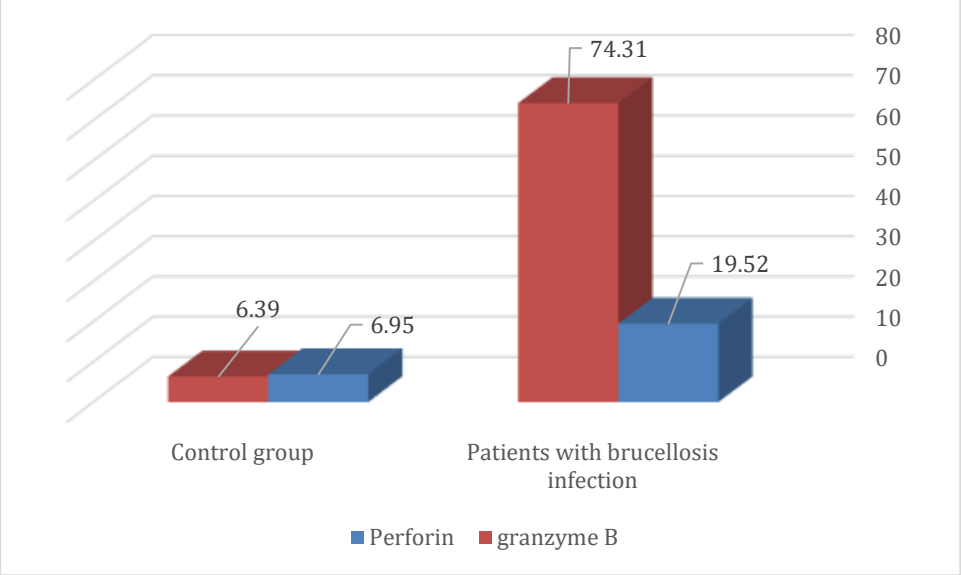

Figure (2): mean differences between Perforin and granzyme B proteins levels according to study groups including (patients with Brucellosis infection and control group).

The investigation into IL-12 level determination revealed that, as illustrated in Table (4) and Figure (3), the control group had the lowest average IL-12 level (288.08±2.29ng/ml), while patients with Brucellosis had the highest (287.54±12.18ng/ml). This difference was statistically significant (P<0.05).

Table (4): Determination of IL-12

Parameter	Sample	N	Mean ± S.E	P. value
IL12 (pg/ml)	Patients	22	287.54±12.18	0.0001
	Control	20	188.08±2.29	

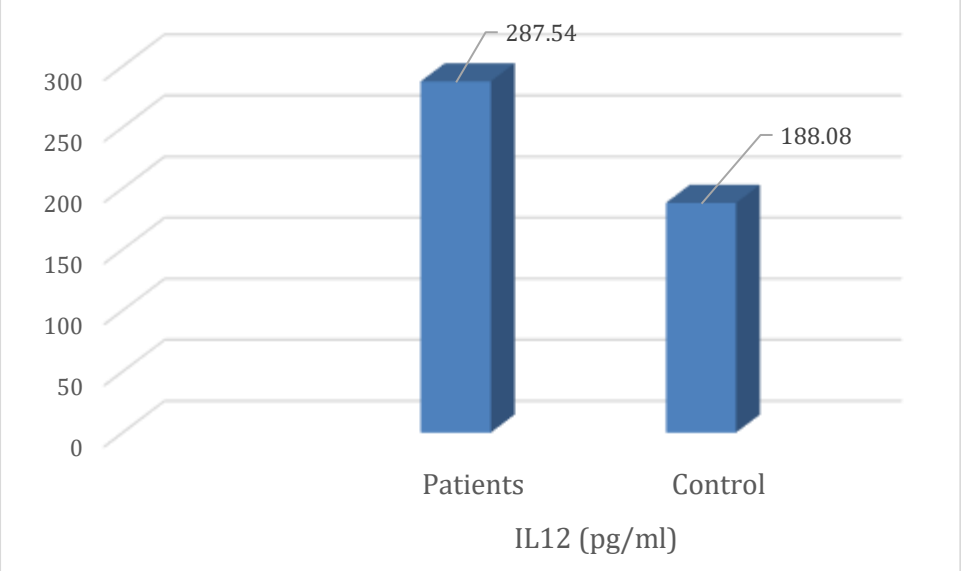

Figure (3): Determination of IL-12

Discussion

Serological testing are crucial for the diagnosis of human brucellosis since the illness may present in a broad range of ways [19]. The following symptoms were recorded during the collection of these samples: fever, perspiration, aches, fatigue, pain in the joints and back, arthritis, soreness in the spine,

headache, nausea, vomiting, diarrhea, loss of appetite, weight loss, constipation, abdominal pain, cough, rash, splenomegaly, and hepatomegaly. The doctors who oversaw the collection of these samples were qualified medical professionals. Several factors contributed to the low number of Brucella DNA extracted from the blood [20]. The patient's antibiotic treatment was a major one. Other factors included the patient's immune system's effectiveness in reducing bacterial numbers, the constant flow of blood, and the richness and support of the culture media, which increased the chances of bacterial growth relative to the blood [21]. Conventional methods prove unworthy for the diagnosis of infectious diseases brought by fastidious or slow-growing micro-organisms including Brucella and therefore, molecular approaches like PCR appear more suitable for this purpose[22]. It was investigated in this work, PCR as a diagnostic tool for the identification of Brucella species in serums from human beings and calmly looked at the epidemiology of brucellosis [23] .It was successful in testing for brucellosis by using specific gene primers and 22 samples from 150 blood samples were positive to brucellosis [24]. In a similar manner, it compared the sensitivity and specificity of the bcsp31 gene target in PCR assays. PCR assays based on the bcsp31 gene and its primers were evaluated for their sensitivity and possibility of detection of Brucella DNA in human peripheral blood or serum [25]. In the current study, whole blood was not used for PCR assay while serum samples were preferred because the literature indicates that serum has relatively lesser inhibitors and better DNA extraction than whole blood had been found. PCR appears to offer several advantages over conventional methods: it is easy to perform; it is rapid; and it was safe for laboratory staffs because the serum based PCR-assay will reduce to risk of handling the microorganism in the laboratory [20]. Therefore, the use of the Bcsp31, based PCR assays described here was a promising method for detection of the Brucella genus and also identifying Brucella spp in clinical samples [26]. In a novel approach to diagnosing the most infectious disease, PCR-based tests have been investigated for the quick identification and verification of Brucella. Polymerase chain reaction (PCR) methods such as conventional, real-time, multiplex, and monoplex PCR are used. The findings corroborate those of the current investigation. In contrast, PCR was used by [27] (Liu et al., 2023) to diagnose brucellosis; they characterized it as highly specific, sensitive, and straightforward, suggesting that it has the potential to become a standard diagnostic tool for the disease [28]. Blood and milk, as well as fetal and maternal tissues acquired from infected animals, can be tested for Brucella using the polymerase chain reaction (PCR) method, which has been the subject of numerous studies since its description [29]. PCR has a high specificity of 90% and an extreme sensitivity of 98%. Perforin was a protein synthesized by cytotoxic T lymphocytes (CTLs), and natural killer (NK) cells which form a part of the immune system. There were various types, especially it was main purpose was to assist these immune cells eliminate infected and damaged cells including intracellular bacteria as well as viruses, and even cancer cells [30]. Perforin function was to penetrate the membranes of target cells (e. g., cells which harbor pathogens). This makes it possible for other cytotoxic molecules like granzyme B to gain entry into the said cell and make it to undergo apoptosis, thus culminating in the elimination of the infected or the abnormal cell [31]. Perforin was one of the critical components that the immune system uses to combating intracellular pathogens including Brucella. Brucellosis, caused by Brucella bacteria was of the intracellular type; that is; Brucella parasites lives and reproduces within host cells and predominantly in macrophages [32]. These intracellular parasites could only be fought with the help of cell-mediated cytotoxicity, for example, through the activity of CTL or NK cells. It could become elevated for the following reasons: Brucella bacteria could parasitize host cells and were found essentially within macrophages [16]. Subsequently CTL and NK cells respond to the immune system by synthesizing more perforin to target and destroy infected cells and to prevent the bacteria from seeding and proliferating. In response to Brucella infection cytokines including IFN-γ were secreted and promote activation of CTLs and NK cells. These activated cells synthesize perforin as part of their defense to neutralize Brucella infected cells [33]. Because of continual and frequent infections of brucellosis, the immune system might be constantly trying to destroy the infected cells thus making perforin to be continually or highly produced in the body as the body tries to control or destroy the diseases [30]. High level of perforin in brucellosis was a part of the overall response of the immune system that is usually proactive in the destruction of intracellular organisms. The enhanced levels of perforin enable the immune system to more effectively destroy and eradicate infected cells so as to help contain the spread of the bacteria [34]. Perforin was the molecule that plays the role in lysing the infected cells which are important in controlling the infection. The increase in perforin in

brucellosis represents an effort in immune-mediated clearance of infected cells especially where the disease was chronic or severe [35]. Perforin and granzyme B have recognizable roles in controlling intracellular replication of Brucella, nevertheless, in certain circumstances Brucella could avoid the impact of these immune mediators and results in chronic infections. This was seen in the increase in the levels of perforin in brucellosis, this was the body's way of targetting cells that have been infected with Brucella bacteria [16].

Conclusions

In order to have a complete understanding of the immune response to Brucella, it was essential to have a solid understanding of the action of perforin and granzyme B, since these two proteins play an important role in the process.

Conflict of Interest

The authors declare no conflicts of interest.

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Data Availability Statement

Additional data are available from the corresponding author upon reasonable request.

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