Diagnosis of Chronic Brucellosis with Interferon-Gamma Quantification

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Abstract

Background: Brucellosis can vary in severity and duration, requiring multiple tests for diagnosis. Its prevalence in Iraq complicates the interpretation of test results. Objective: This study aimed to develop a diagnostic tool leveraging variations in γ interferon to aid the immune system in recognizing illness. Method: A comparative study was conducted at Anbar Teaching Hospital from October 2022 to September 2023. 86 blood samples were collected from the outpatient infectious clinic, comprising 43 confirmed Rose Bengal-positive and 43 negative cases. Various diagnostic methods were employed, including specific IgM and IgG antibodies, culture, polymerase chain reaction (PCR), and gamma interferon targeting the brucella antigen. Results: Patients with positive results exhibited symptoms validated by PCR. The gamma interferon assay demonstrated 79.2% sensitivity and 100% specificity. PCR showed 89.3% sensitivity and 100% specificity, while brucella-specific IgG had 21.4% sensitivity and 100% specificity. Other laboratory studies, including Brucella-specific IgM, showed 100% sensitivity and specificity, with positive Brucella culture observed in only one case. Conclusion: Chronic brucellosis's sustained high levels of gamma interferon may offer better follow-up for patients with lingering symptoms than Brucella-specific IgG, especially after acute cases with low-grade fever.

Keywords: Chronic Brucellosis, Interferon Gamma, Sensitivity, Specificity.

Significance | This study showed the improvement of brucellosis diagnosis using interferon-gamma for better detection in chronic cases.

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1. Introduction

Brucellosis, caused by various species of the Gram-negative bacteria Brucella, is a significant zoonotic disease affecting both animals and humans (Corbel, 2006). Brucella comprises several species, including Brucella abortus, Brucella melitensis, Brucella suis, and Brucella canis, demonstrating distinct host preferences. Transmission of Brucella bacteria primarily occurs through direct contact with infected animals, consumption of contaminated dairy products, or inhalation of aerosols (Pappas et al., 2006). In humans, brucellosis manifests as flu-like symptoms, including fever, sweats, malaise, and joint pain (Corbel, 2006).

Due to its substantial public health impact, extensive research has been conducted on Brucella's taxonomy, pathogenesis, and epidemiology (Seleem et al., 2010). The ability of Brucella to infect a wide range of hosts poses significant economic losses in livestock industries and occupational risks to individuals in close contact with infected animals. Controlling and eradicating brucellosis require collaborative efforts between human medical and veterinary practitioners, especially considering the challenge posed by animals acting as carriers of the microorganism (Mengele et al., 2023).

Chronic brucellosis presents a complex and challenging clinical scenario, characterized by nonspecific symptoms such as recurrent fever, fatigue, joint pain, and malaise (Dios-Diaz et al., 2019). This

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form of brucellosis is particularly challenging to diagnose and manage due to overlapping clinical presentations with other chronic febrile illnesses. Laboratory diagnosis involves detecting specific antibodies, such as IgG and IgM, and evaluating cellular immune responses, including measuring interferon-gamma (IFN-γ) levels (Bosilkovski et al., 2012; Franco et al., 2007). Despite treatment with prolonged antibiotic therapy, challenges such as antibiotic resistance and treatment duration contribute to the complexity of managing chronic cases (Pappas et al., 2005).

Prevention strategies for chronic brucellosis include animal vaccination, raising public awareness about the risks associated with consuming unpasteurized dairy products, and contacting infected animals (Corbel, 2006). However, chronic brucellosis remains a clinically challenging condition, emphasizing the importance of timely and accurate diagnosis coupled with appropriate antibiotic therapy.

The diagnostic challenges of brucellosis are further compounded by the unreliability of laboratory investigations and nonspecific signs and symptoms (Díaz et al., 2011). Conventional diagnostic tools such as the Rose Bengal test have limitations, leading to false positive results and unreliable follow-up assessments. To address these challenges, the assessment of IFN-γ quantification has emerged as a promising method for detecting chronic brucellosis (Bosilkovski et al., 2012). IFN-γ, a key cytokine in the immune response against intracellular pathogens, has shown potential as a biomarker for Brucella infection. Research efforts are underway to evaluate its effectiveness in diagnostic assays and monitoring treatment outcomes (Casas et al., 2015; Cannas et al., 2017).

Infections with Brucella melitensis induce a cell-mediated immune reaction comparable to Mycobacterium tuberculosis, highlighting the importance of memory T-cell functionality for predicting chronicity (Aljanabi et al., 2020). The evaluation of IFN-γ quantification as a method for detecting chronic brucellosis represents a promising avenue for enhancing diagnostic accuracy. Ongoing research endeavors seek to refine and validate these assays, ultimately contributing to developing more reliable tools for the timely and accurate identification of chronic brucellosis cases.

An optimal approach involves evaluating the test's accuracy, reliability, and cost to assess the benefits of any diagnostic investigation. Comparative studies with other established investigations are essential, considering geographic variations in chronic brucellosis prevalence due to differences in exposure, healthcare infrastructure, and dietary habits. This study aims to develop a diagnostic tool that could enhance the immune system's ability to recognize illness by leveraging variations in IFN-γ.

2. Materials & Methods

2.1 Study Design

This case-control study was conducted at the Outpatient Infectious Clinic of Anbar Teaching Hospital, Iraq, to investigate the clinical presentation and diagnostic markers of brucellosis. Ethical approval was obtained from the Ethical Committee at Anbar University, Ministry of Higher Education in Iraq, under number 478 on December 30th, 2022, ensuring compliance with ethical standards.

Inclusion Criteria

A total of 86 out of 105 patients were enrolled in the study. Inclusion criteria comprised patients presenting with fever (37.8-38.5°C), headache, and joint discomfort lasting no more than two days, with a clinical diagnosis of Malta fever for at least the past six months. Patients with focal diseases, such as spondylitis, sacroiliitis, hip arthritis, knee arthritis, spleen abscess, hepatic abscess, or multifocal motor neuropathy, were included based on appropriate findings from physical examinations and various diagnostic studies, including bone scintigraphy, magnetic resonance studies, and electroneurograms. Participants exhibited white blood cell counts within the normal range or slightly elevated. All patients tested negative for Widal but positive for Rose Bengal as a screening test. An additional 43 participants without symptoms, indicators, or a history of brucellosis were included as controls and tested negative for all diagnostic measures.

Exclusion Criteria

Cases without a history of brucellosis, even with a positive Rose Bengal test, were excluded from the study. Patients with a positive Rose Bengal test but without a diagnosis of Malta fever were also excluded. Seven patients were further excluded due to incomplete investigations. All eligible volunteers provided written informed consent to participate in the study.

Sample Size Calculation

The sample size was calculated using the formula:

$$n = \frac{Z^2 \times \sigma^2}{d^2}$$

where n is the sample size, Z is the table-based normal distribution index at 5% type-one error ($P<0.05$), σ represents the variable variance, and d shows the accuracy of quantitative variable estimation. For this study, a type-one error α, Z, σ, and d were set to 0.05, 96.3, 7.38, and 0.99, respectively. After adjusting for a non-response rate of 10%, a final sample size of 90 was considered. Therefore, 105 participants were included in the data analysis.

2.2 Detection of Interferon-gamma

The Rose Bengal test was conducted to rapidly screen for Brucella infections. To detect interferon-gamma, 4 mL of the patient's whole blood was drawn and then transferred to three endotoxin-free heparinized tubes in the following manner: Positive control Mitogen tubes (1 mL), Negative or background patient tubes (2 mL), and Brucella Antigen stimulation tube (1 mL). The Brucella antigen stimulation tube was prepared by adding 50 µL of the

calibrated mixed antigen of Brucella abortus, melitensis, and suis. Mitogen tubes were pre-coated with mitogen to induce maximum T-cell activation, serving as a positive control. The patient's blood in the negative control tubes was left untreated to measure the minimal level of INF-γ already present. Dilutional trials of the specific Brucella antigen were conducted to obtain the best immune response, with the optimal trial involving dilution to a concentration of 20 pg/mL in 150 mL. After gentle mixing and immediate placement in an incubator at 37°C, the tubes were incubated for 1 hour, remixed, and reincubated for an additional 18-20 hours. The next day, centrifugation at 2000-3000 x g for 10 minutes separated the plasma from the red blood cells, and the collected plasma was labeled accordingly. Each tube's plasma was added to an ELISA plate well with 50 µL of diluent assay solution, followed by a 55-minute incubation period at 37°C. After gentle mixing, wells were washed with buffer solution, and 50 µL of horseradish peroxidase (HRP) was added, reincubating for 55 minutes. Following five more rinses with buffer solution, 100 µL of 3,3′,5,5′-Tetramethylbenzidine (TMB) was added, and the plate was set aside in darkness for fifteen minutes. Finally, 100 µL of a stop solution was added, and an ELISA reader (OD) was used to measure the concentration at 450 nm compared to 620 nm. Quality control measures were implemented throughout the process, including positive and negative control samples, to ensure the reliability of the test results.

2.3 Statistical analysis

Data analysis was performed using IBM SPSS-29 (IBM Statistical Packages for Social Sciences, version 29, Chicago, IL, USA). Results were presented using simple measures of frequency, percentage, mean, standard deviation, and range (minimum-maximum values). The significance of differences between means for quantitative data was assessed using Student's t-test for two independent means or ANOVA for more than two independent means. The significance of differences in percentages for qualitative data was tested using Pearson's Chi-square test (χ^2 -test) with Yate's correction or Fisher's Exact test when applicable. Statistical significance was considered when the p-value was equal to or less than 0.05.

3. Results

The study conducted at Anbar Teaching Hospital between October 2022 and September 2023 aimed to compare various diagnostic methods for brucellosis, utilizing 86 blood samples from the outpatient infectious clinic. Among these, 43 cases tested positive for Rose Bengal, while 43 tested negative. Laboratory assays included specific IgM and IgG antibodies, culture, polymerase chain reaction (PCR), and gamma interferon. Sensitivity and specificity analyses revealed notable results: gamma interferon demonstrated 79.2% sensitivity and 100% specificity, PCR showed 89.3% sensitivity and 100% specificity, while brucella-specific IgG exhibited 21.4% sensitivity and 100% specificity. Interestingly, Brucella-specific IgM showed 100% sensitivity and specificity, contrasting with a single positive culture. Chronic cases were associated with elevated interferon-gamma levels, suggesting potential for disease monitoring. However, challenges in antigen concentration adjustment were noted. Notably, Brucella-specific IgG's limited sensitivity in establishing chronicity underscores the importance of incorporating interferon-gamma stimulation tests for accurate assessment and follow-up. These findings align with existing literature, emphasizing the utility of gamma interferon in monitoring brucellosis progression and response to therapy, thereby guiding effective patient management strategies.

Table 1 presents the average percentage differences in IGRA test results among various clinical indicators. In cases with fever (24 in total), the average IGRA test result difference was 27.33%, suggesting that individuals with fever exhibited lower IGRA test results compared to those without fever. Similarly, for cases involving headaches (21 instances), the average difference was -26.44%. However, it's important to note that the P value, indicating the strength of the observed difference, was not particularly robust, standing at 0.723. The average difference for joint pain (9 cases) was 12.92%, with an associated P value of 0.081. Additionally, 24 cases with a positive Rose Bengal test displayed an average difference of -27.33%. A noteworthy finding emerges when IGRA test results are categorized based on specific thresholds. There is a statistically significant difference (P value = 0.007) between groups with IGRA test results falling below 0.300u and those registering at 0.300u and above. This indicates that IGRA test outcomes are distinct and significant when categorized based on these thresholds. Furthermore, categorizing IGRA test results as either positive or negative revealed substantial differences between these groups, with a highly significant P value of 0.0001. This underscores the relevance of this categorization in understanding variations in IGRA test outcomes. Results from both Enzyme-Linked Immunosorbent Assay (ELISA) IgG and Polymerase Chain Reaction (PCR) tests were presented similarly, elucidating the differences between positive and negative cases. However, it's crucial to note that not all observed differences reached statistically significant. In other words, while there were variations in test results, these differences were not universally significant across all comparisons. Finally, when examining PCR results, a significant difference was identified between cases testing positive and those testing negative, with a P value of 0.092. This showed that the PCR test outcomes also bear important distinctions based on the presence or absence of the condition under consideration.

4. Discussion

Brucellosis, caused by Brucella bacteria, presents significant challenges in diagnosis and management, often characterized by

Table 1. Average percentage differences in IGRA test results among various clinical indicators. All data collected with the mean standard deviation.

Clinical signs		**IGRA test %Difference**	
		No	Mean ±SD
Fever	Yes	24	-27.331±31.434
	No	-	-
	P value		-
Headache	Yes	21	-26.444±30.393
	No	3	-33.539±45.383
	P value		0.723
Joint pain	Yes	9	-12.916±24.268
	No	15	-35.980±32.757
	P value		0.081
Rose Bengal	Positive	24	-27.331±31.434
	Negative	-	-
	P value		-
IGRA test results	<0.100u	3	-67.190±14.234
	0.100---	6	-42.134±30.432
	0.150---	6	-4.321±16.595
	0.200---	4	-1.107±2.516
	0.250---	3	-26.627±34.514
	=>0.300u	2	-45.669±35.062
	P value		0.007^
IGRA test final	Positive	19	-16.647±25.485
	Negative	5	-67.931±12.500
	P value		0.0001#
ELIZA IgG	<1.0u	1	3.430±
	1.0---	3	-26.311±35.735
	2.0---	4	-36.796±31.580
	3.0---	4	-25.753±39.046
	4.0---	-	-
	5.0---	8	-19.169±33.937
	6.0---	2	-40.819±21.520
	7.0---	1	-70.462±
	8.0---	-	-
	9.0---	-	-
	=>10.0u	1	-24.800±
	P value		0.821
ELIZA IgG	Positive (=>9.9995)	1	-24.80±
	Negative (<9.9995)	23	-27.44±32.14
	P value		0.937
PCR	Positive	22	-24.066±30.712
	Negative	2	-63.249±10.201
	P value		0.092

#Significant difference between two independent means using Students-t-test at 0.05 level.

^Significant difference among more than two independent means using ANOVA-test at 0.05 level.

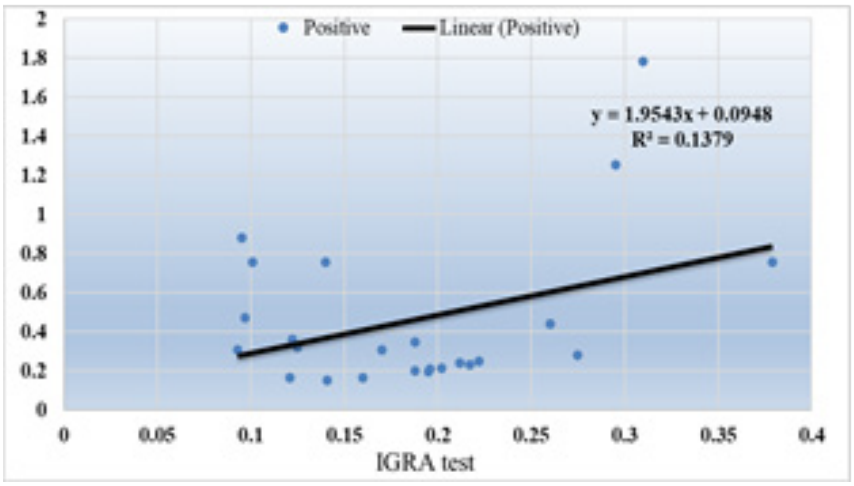

Figure 1. The Stimulation interferon γ to the mitogen value.

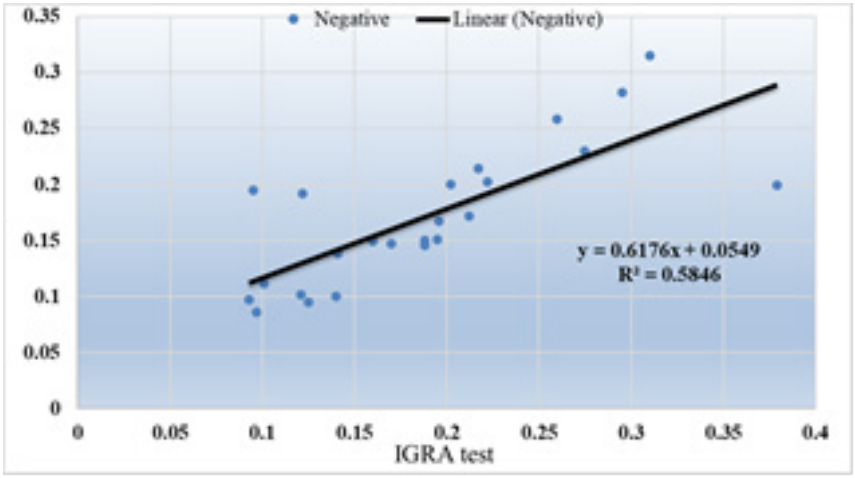

Figure 2. The Stimulation interferon γ to the patients' value before stimulation.

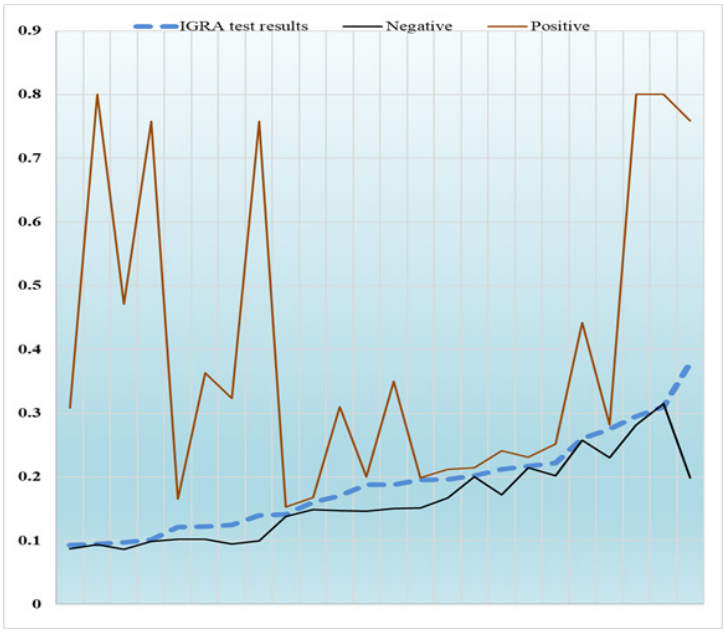

Figure 3. The plotting of all three interferon γ values (before, after, and mitogen)

fever and debilitating pain. The Rose Bengal test, a common diagnostic tool, detects antibodies against Brucella's S-lipopolysaccharide (S-LPS), although shared antigens among different bacteria compromise its accuracy (Díaz et al., 2011). To address this, PCR positivity for Brucella or Brucella-specific IgG antibodies were considered reliable markers (Mohseni et al., 2017). Notably, chronic brucellosis cases demonstrated a strong association with interferon-gamma stimulation tests, suggesting a prolonged immune response (Xu et al., 2019; Ghaznavi-Rad et al., 2017). Unexpectedly, Interferon-Gamma Release Assay (IGRA) yielded more positive results than Brucella-specific IgG, challenging established perceptions (Ghaznavi-Rad et al., 2017). This complexity underscores the need for further investigation into immune dynamics, particularly the roles of memory B-lymphocytes and T-cells beyond the traditional 120-day period post-infection (Martinez-Lopez et al., 2015). However, optimizing antigen concentrations remains challenging, necessitating precise trials to modify antigen concentrations effectively (Ghaznavi-Rad et al., 2017). Moreover, the persistence of high interferon-gamma levels after stimulation highlights its potential in disease monitoring (Durward et al., 2012). While PCR offers sensitivity in detecting Brucella, Brucella-specific IgG aids in understanding disease progression, emphasizing the importance of combining diagnostic tools for accurate assessment (Dasari et al., 2013). Future research may explore demographic factors influencing chronic brucellosis development, offering insights into tailored management approaches.

All patients in this study sought medical help due to fever, fearing a return of Malta fever, and the accompanying pain was too much to bear. While not all cases displayed an obvious headache or joint pain, the Rose Bengal test was positive in all chosen cases, indicating antibodies against the S-lipopolysaccharide (S-LPS), which Brucella might produce. Due to many different bacteria sharing the same S-LPS, the Rose Bengal test risks producing inaccurate results (Díaz et al., 2011). Only instances where PCR was positive for Brucella or IgG antibodies were positive for Brucella were considered genuine positives (Mohseni et al., 2017).

The findings of chronic brucellosis cases were significantly associated with the interferon-gamma stimulation test. Interferon gamma was reported to remain significantly higher for more than one-year post-therapy (Xu et al., 2019; Ghaznavi-Rad et al., 2017). IGRA's positive findings were more numerous than those of Brucella-specific IgG, unexpectedly suggesting that Brucella-specific IgG, the direct product of activated memory B-lymphocytes, might define acute-on-chronic cases. These cells behave as macrophages, causing clonal production of specific IgG antibodies against the bacterium. The presence of memory T-cells in the patient's blood, indicated by gamma interferon production, suggests that the patient has not yet finished the 120-day period after the first infection, indicating the continuation of their role after acute infection (Ghaznavi-Rad et al., 2017).

The findings of this study present a thought-provoking perspective on the prevalence of positive results in Interferon-Gamma Release Assay (IGRA) compared to Brucella-specific IgG, challenging conventional notions. Surprisingly, IGRA's dominance in positive findings contradicts expectations and raises questions about the role of Brucella-specific IgG, traditionally viewed as a direct product of activated memory B-lymphocytes. This unexpected prominence of IGRA may indicate a more complex immune response, possibly defining acute-on-chronic cases, as suggested by Ghaznavi-Rad et al. (2017).

However, it's essential to note that differing observations exist in the literature regarding the utility of Brucella-specific IgG in defining stages of Brucella infection. Some studies align with the present findings, suggesting that Brucella-specific IgG might play a role in acute-on-chronic cases (Ghaznavi-Rad et al., 2017). On the other hand, conflicting evidence exists, with studies proposing Brucella-specific IgG as a reliable marker for chronic brucellosis (Hasanjani Roushan et al., 2007). The proposed role of activated memory B-lymphocytes behaving as macrophages and contributing to clonal production of specific IgG antibodies against Brucella aligns with some studies highlighting the multifaceted functions of memory B-cells (Belachew et al., 2018). However, others may argue that the specific dynamics of Brucella infection may warrant further investigation into the interplay between memory B-lymphocytes and macrophage-like behavior.

Moreover, the presence of memory T-cells beyond the traditional 120-day period challenges established notions regarding the temporal dynamics of the immune response. Some studies corroborate this extended presence of memory T-cells, emphasizing their crucial role in sustained immune activity (Martinez-Lopez et al., 2015). Conversely, other research may argue for a more time-bound response, questioning the significance of prolonged T-cell activity in the post-acute phase of Brucella infection.

Finding the sweet spot for standard dilution and antigen concentration was the most challenging part of this study. Incubating patients' blood with Brucella-specific antigen at 37°C for 18 hours yielded favorable results, but they weren't significantly greater than those from interferon-gamma patients without stimulation. Figures 1, 2 and 3 make this point evident; the r2 value shows a 58% association between the non-stimulation values and the favorable stimulation outcomes. This might mean that the patient needs a more precise trial to modify the antigen concentrations, or it could mean that their IFN-γ levels are already high because of activated T-cells and their function in causing fever, indicating chronicity. In Iran, Ghaznavi-Rad et al. compared IFN-γ levels between acute and chronic cases simultaneously, finding

significantly higher levels in acute disease compared to chronic disease (Ghaznavi-Rad et al., 2017).

IFN-γ induces moderate fever, flu-like symptoms, headaches, and arthralgia, leaving individuals feeling as if they haven't fully recovered from brucellosis. This aligns with the findings of Durward M. et al., who highlighted the microorganism's ability to evade cytotoxic T-cells, causing continuous low-grade inflammation (Durward et al., 2012).

This study illustrates a marked continuous high level of INF-γ after stimulation, albeit lower than mitogen-positive findings. This may aid in accurately identifying a delayed cellular immune response, contributing to disease monitoring.

In tuberculosis (TB), the IFN-γ immune test fails to distinguish between latent and active tuberculosis. However, in brucellosis, determining chronicity is the primary concern. With only nine out of thirty individuals testing positive for Brucella-specific PCR and one testing negative for IGRA, it appears that IgG was insufficient in establishing chronicity in this study (Dasari et al., 2013).

The sensitivity for PCR, IGRA, and Brucella-specific IgG in this study was 89%, 79.2%, and 21.4%, respectively. While PCR data may reveal the presence of live or dead bacteria, it doesn't provide insights into how these bacteria contribute to the illness's pathogenesis or overall disease progression. For a general understanding of the illness's development or regression, the most effective tool for follow-up is Brucella-specific IgG. This requires two estimated values at least one week apart for accurate disease interpretation. To assess the illness's pathophysiology and provide a logical explanation for major complaints, symptoms, and signs, an immunogen stimulation test (IGRA) is essential to determine T-cell functioning. This aligns with the findings of Rodriguez-Zapata M et al. and Baldwin et al., emphasizing continuous high levels of IFN-γ in untreated brucellosis cases, which start to decline with therapy, making them valuable tools for case follow-up (Rodriguez et al., 2010: Baldwin et al., 2002).

Certain demographic characteristics may be associated with chronic brucellosis. Future research could develop into the specific demographic factors, such as age, gender, occupation, or geographical location, that may influence the likelihood of developing chronic brucellosis.

5. Conclusion

In conclusion, the diagnostic challenges of chronic brucellosis underscore the need for innovative approaches to identify and manage this debilitating condition accurately. While valuable, traditional diagnostic methods, such as Brucella-specific IgG testing, may not fully capture the complexity of immune responses in chronic cases. Our study sheds light on the potential of interferon-gamma stimulation tests, highlighting their prominence in detecting chronic brucellosis cases compared to conventional methods. The unexpected dominance of Interferon-Gamma Release Assay (IGRA) over Brucella-specific IgG challenges established paradigms and calls for a reevaluation of diagnostic strategies.

Moreover, the persistence of memory T-cells beyond the traditional 120-day period raises intriguing questions about the temporal dynamics of the immune response to Brucella infection. Further exploration of memory T-cell functionality and its role in defining chronicity could enhance our understanding of brucellosis pathogenesis and improve diagnostic accuracy.

Optimizing antigen concentrations and standard dilution protocols presents a significant challenge in diagnosing chronic brucellosis. Our study showed the importance of meticulous trial modifications to achieve optimal immune responses, paving the way for more effective diagnostic assays.

Future research should explore the demographic factors influencing chronic brucellosis development, offering insights into tailored management approaches. Collaborative efforts between medical and veterinary practitioners are crucial in controlling and eradicating brucellosis, considering its significant public health impact and economic burden.

Our study demonstrated a thought-provoking perspective on the diagnostic challenges of chronic brucellosis and highlights the potential of interferon-gamma stimulation tests as a promising diagnostic tool. By leveraging insights from immune dynamics, we can advance diagnostic strategies and ultimately improve patient outcomes in managing chronic brucellosis.

Author contribution

S.Y.A. conceived and designed the study, contributed to data analysis and interpretation, drafted the manuscript, collected, processed, and analyzed data, conducted laboratory experiments, and critically reviewed the manuscript. Y.M.A. supervised the work, contributed to the result interpretation, and critically reviewed the manuscript. S.Q.T.A.Q. contributed to study conception, design, and data analysis, providing intellectual input during manuscript preparation.

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Competing financial interests

The authors have no conflict of interest.

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