

DIAGNOSTIC PERFORMANCE OF ROSE BENGAL TEST, IGM/IGG ELISA, AND IS711-PCR FOR DETECTION OF HUMAN BRUCELLOSIS: A COMPARATIVE CROSS-SECTIONAL STUDY

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Running title: RBT, ELISA, and IS711-PCR in Brucellosis Diagnosis

Abstract:

Background: Human brucellosis is an important zoonotic disease with non-specific clinical manifestations that affect laboratory diagnosis. Reliable serological and molecular methods are needed to detect the disease. In this study, we assessed the performance of the Rose Bengal test (RBT), anti-Brucella IgM/IgG ELISA, and IS711-based PCR for the detection of human brucellosis.

Methods: A comparative cross-sectional study, we investigated 101 clinically suspected patients. We collected blood samples and divided them into serological and molecular samples. Our sample was initially tested with Rose Bengal and IgM/IgG ELISA, and DNA extracted from EDTA whole blood samples was then subjected to conventional PCR targeting the Brucella melitensis IS711 insertion sequence, producing a 585 bp amplicon. Statistical analysis was performed with Chi-square test and ROC curve to assess diagnostic performance.

Results: Rose Bengal test was the most positive (44.5%), followed by ELISA IgG (37.6%), ELISA IgM (28.7%), and PCR confirmed 23.8% of cases. The diagnostic methods had significant differences ($p < 0.05$). For PCR, AUC = 0.92 (ROC) and diagnosis was determined by sensitivity of 92% and specificity of 95%. ELISA IgM showed moderate success (AUC = 0.78), followed by ELISA IgG (AUC = 0.63), and the Rose Bengal test showed the lowest accuracy (AUC = 0.52).

Conclusion: IS711-PCR was significantly more accurate for human brucellosis, while the Rose Bengal and ELISA methods are good for rapid screening. Diagnostic reliability can be improved by serological tests paired with PCR.

Keywords: Brucellosis, Rose Bengal test, ELISA, PCR, IS711, Diagnostic accuracy.



Introduction

Human brucellosis is a zoonotic infection that is mainly spread by coming into contact with sick animals or eating unpasteurized dairy products. It is still common in many underdeveloped nations and has symptoms that are not distinctive, like fever, joint discomfort, weariness, and sweating. This makes laboratory tests necessary for diagnosis[1]. Laboratory diagnosis of brucellosis includes culture, serological assays, and molecular tests. While culture is the most accurate diagnostic tool, it is slow to grow, less sensitive in chronic cases, and prone to biosafety issues. Serological tests are of great use for screening due to their simplicity and rapid results, but the diagnostic accuracy of different diagnostic tools needs to be compared [2].

The Rose Bengal test (RBT) is a common screening technique for brucellosis screening as it is inexpensive, quick and easy to perform. However, cross-reactivity with other Cross-reactivity with other Gram-negative organisms can produce false-positive reactions, and the test performance may change depending on the stage of infection and the endemic level of the disease so RBT is recommended as a screening test, not a confirmatory method [3]. ELISA, or immunosorbent assay using is used to detect anti-Brucella IgM and IgG antibodies and for that reason gives higher sensitivity and the ability to differentiate acute from chronic infection. ELISA is often more accurate than agglutination tests but serological assays alone cannot necessarily distinguish active from past infections [4]. Molecular techniques, such as polymerase chain reaction (PCR), are highly sensitive for detecting Brucella DNA in clinical samples and can be identified even in seronegative cases. The IS711 insertion sequence is commonly used as a target because it is conserved and present in multiple copies of the Brucella genome [5]. In fact, recent studies have shown that the integration of serological tests like RBT and ELISA with PCR results in a higher overall diagnostic accuracy for brucellosis. Serological methods are very useful for screening and PCR is a confirmatory tool for detecting active infection and reducing misclassification [6].

PCR has high diagnostic accuracy and high predictive value but the testing system, the target genes and sample types could differ and we would like to see a standard study of serological and molecular assays. ELISA is more often used to detect more cases than agglutination, but PCR is more effective. The results suggest that combined screening and confirmatory strategies should be used to identify brucellosis in order to make diagnosis of brucellosis effective [8]. The recent studies have shown that PCR and serological assays may be informative in different stages of the infection. So, multi-assay diagnostic models have been proposed to increase detection and reduce false results in endemic areas [9]. With these limitations, it is important to compare RBT, IgM/IgG ELISA, and IS711-PCR to evaluate diagnostic performance and identify optimal methods for early detection of human brucellosis [10]. The objective of this study was to comparatively evaluate the diagnostic performance of the Rose Bengal test, IgM/IgG ELISA, and IS711-PCR for detection of human brucellosis among clinically suspected patients. The novelty of this study lies in integrating serological screening with molecular confirmation and assessing their combined diagnostic value to improve early and accurate detection in endemic settings.



Materials and Methods

Study design and period

A comparative cross-sectional investigation was carried out between March 2025 and December 2025 to assess the diagnostic accuracy of the Rose Bengal test (RBT), anti-Brucella IgM/IgG ELISA, and IS711-targeted PCR for identifying human brucellosis in clinically suspected individuals.

Study population and sample collection

A total of 101 patients who were clinically suspected to have brucellosis were included in the current study. Participants were recruited from Baqubah General Hospital and private internal medicine clinics. Most patients with brucellosis symptoms showed fever, sweating, arthralgia, fatigue, malaise and a history of coming into contact with animals or eating unpasteurized dairy products. All the subjects were aseptically transferred 5 mL of venous blood. Blood was divided into two sections: blood in plain tubes for screening of Rose Bengal and ELISA; and blood in EDTA tubes for PCR. Blood was spun at 3000 rpm for 10 min to obtain serum and stored at -20°C before analysis, and EDTA-anticoagulated whole blood samples were treated at -20°C until DNA extraction.

Rose Bengal Test

Serum specimens were examined using the Rose Bengal slide agglutination test (Linear Chemicals, Spain) following the recommended procedure. Briefly, equal amounts of serum and Rose Bengal antigen were placed on a clean glass slide and mixed thoroughly, then gently rotated for approximately 4 minutes at room temperature. The appearance of visible agglutination indicated a positive reaction, whereas a smooth homogeneous mixture without clumping was interpreted as a negative result.

ELISA assay

Anti-Brucella IgM and IgG antibodies were calculated by monitoring serum samples with a commercial ELISA kit (Vircell, Spain), as per the manufacturer guidelines. In short, serum samples, along with calibrators and controls, were introduced into antigen-coated microplate wells and incubated at 37°C for antigen–antibody interaction. Subsequent to incubation, the wells were washed to remove unbound material. Then enzyme conjugate was added and incubated at 37°C with further washing. A chromogenic substrate was transferred and the plates were maintained at room temperature in the dark. The reaction was terminated by adding stop solution, and optical density was measured at 450 nm with a microplate reader (Bio-Tek ELx800, USA). Interpretation of the results was done according to the cutoff value supplied by the manufacturer.

DNA extraction

Genomic DNA was obtained from EDTA-anticoagulated whole blood according to the recommended extraction protocol via commercial extraction kit (Geneaid Biotech Ltd.,



Taiwan). In short, 200 μ L of whole blood were treated with lysis buffer and proteinase K in order to give full cellular disruption. After incubation, absolute ethanol was added to allow DNA binding and the solution was transferred to a silica-based spin column. The wash buffers were then used to wash the column sequentially to remove impurities as well as residual proteins. Afterwards, purified DNA was eluted in 50 μ L elution buffer and stored at -20°C for further conventional PCR amplification.

PCR amplification

To perform the process of identifying the Brucella IS711 gene by amplifying it using a conventional PCR device, we used the primers designed for this study and shown in Table 1, which included the following: The reaction volume used was 25 microliters, consisting of 1 microliter of the forward and reverse primers, 12.5 microliters of master mix, and 5 microliters of extracted Brucella DNA, and the reaction was completed to 25 microliters with nuclease-free water. A specific program was developed for our study. We began with a pre-denaturation phase at 95°C for 5 minutes, using 30 PCR cycles. These cycles included a denaturation phase at 95°C for 30 seconds, followed by an annealing phase at 60°C for the same duration. These two steps were followed by an extension phase at 72°C . We used a 1.5% agarose gel to determine the band size of the bands obtained after electrophoresis. The band size was 585 base pairs, which matched the product of the primers designed for our study. UV light was used, confirming the accuracy of targeting this specific Brucella gene segment.

Table 1. Primers used for amplification of Brucella IS711 gene

| Target gene/sequence | Primer | Sequence (5'-3') | Tm ($^{\circ}\text{C}$) | GC (%) | Product size (bp) |
|--------------------------|---------|----------------------|---------------------------|--------|-------------------|
| IS711 insertion sequence | Forward | GAAGCATATCTTCCGGGGCG | 61.0 | 60.0 | 585 |
| | Reverse | CCTTGACAGCTCCAGTCG | 60.1 | 60.0 | |

Statistical analysis

We analyzed the data using SPSS (version 26.0; IBM, USA) and GraphPad Prism (version 9.0; GraphPad Software, USA). Qualitative factors were presented as frequencies and percentages. The Chi-square test was used to find out how different diagnostic approaches were from each other. We used Cohen's kappa coefficient to see how well the tests agreed with each other. Receiver operating characteristic (ROC) curve analysis was utilized to assess diagnostic validity, encompassing sensitivity, specificity, and the area under the curve (AUC). A p-value of less than 0.05 was used to indicate statistical significance.

Ethical considerations

We obtained approval from the Ethics Committee at Tikrit University. We also obtained written informed consent from all participants in our study, in accordance with the Declaration of



Helsinki, ensuring that all ethical considerations were taken into account, with strict confidentiality and due regard for participants' privacy.

Results

3.1 Demographic distribution and clinical profile of the study group

The demographics and exposure-related characteristics of the study population are shown in Table 2. Most of the participants were from rural areas 81 (80.2%), whereas 20 (19.8%) were from urban areas. Males were more numerous in the study population (61 cases (60.4%)) and women (40 cases) were present (39.6%). According to the age distribution, the most patients were aged from 20 to 39 years (39, 38.6%) followed by 40 to 59 years (36, 35.6%), 17 patients (16.8%) having been aged ≥ 60 years and 9 patients (8.9%) younger than 20 years. For the occupation group, livestock breeders and farmers are the most active (34, 33.7%), followed by housewives (23, 22.8%), workers (18, 17.8%), employees (15, 14.9%), and students (11, 10.9%). We have seen that 58 (57.4%) of the participants had contact with animals, while 43 (42.6%) had no contact. 63 patients (62.4%) drank water that was unsafe, and 38 (37.6%) had access to safe water. As for education, 31 patients (30.7%) attended primary school, 28 (27.7%) went to secondary school, 22 (21.8%) were illiterate, and 20 (19.8%) completed university education. 69 patients (68.3%) documented intake of unpasteurized milk and dairy products, whereas 32 (31.7%) denied such exposure.

Table 2. Demographic and exposure-related characteristics of clinically suspected brucellosis patients (n = 101)

| Variable | Category | No. | % |
|-----------------------|--------------------------|-----|------|
| Residence | Rural | 81 | 80.2 |
| | Urban | 20 | 19.8 |
| Sex | Male | 61 | 60.4 |
| | Female | 40 | 39.6 |
| Age group (years) | <20 | 9 | 8.9 |
| | 20-39 | 39 | 38.6 |
| | 40-59 | 36 | 35.6 |
| | ≥ 60 | 17 | 16.8 |
| Occupation | Livestock breeder/farmer | 34 | 33.7 |
| | Housewife | 23 | 22.8 |
| | Worker | 18 | 17.8 |
| | Employee | 15 | 14.9 |
| | Student | 11 | 10.9 |
| Direct animal contact | Yes | 58 | 57.4 |



| | | | |
|---|------------------|----|------|
| | No | 43 | 42.6 |
| Drinking water source | Safe | 38 | 37.6 |
| | Unsafe | 63 | 62.4 |
| Educational level | Illiterate | 22 | 21.8 |
| | Primary school | 31 | 30.7 |
| | Secondary school | 28 | 27.7 |
| | University | 20 | 19.8 |
| Consumption of unpasteurized dairy products | Yes | 69 | 68.3 |
| | No | 32 | 31.7 |

3.2 PCR amplification of Brucella IS711 gene

PCR amplification of the IS711 insertion sequence of melitensis IS711 gene produced a specific amplicon of 585 bp. The amplified products were separated on 1.5% agarose gel electrophoresis and visualised under UV light. The clear bands corresponding to the expected size (585 bp) were clearly visible in the samples, indicating the presence of Brucella DNA in the clinical samples. Lane M is the 100 bp DNA ladder (100 to 1000 bp). The presence of a distinct band at around 585 bp confirmed the successful amplification of the IS711 target sequence (Figure 1).

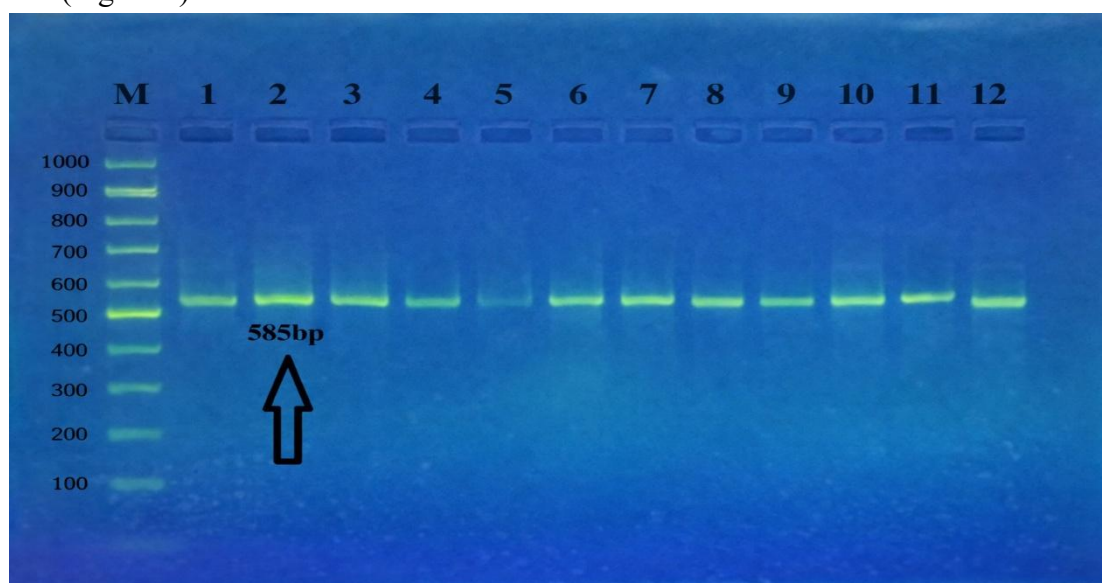


Figure 1. Agarose gel electrophoresis of PCR amplification targeting the *Brucella melitensis* IS711 gene. Lane M: DNA ladder (100–1000 bp). Lanes 1–20 represent clinical samples showing positive amplification at 585 bp.

3.3 Results of Rose Bengal, ELISA, and PCR



A total of 101 clinically suspected patients were tested using the Rose Bengal test, IgM/IgG ELISA, and PCR. The Rose Bengal test showed positivity in 45 cases (44.5%). ELISA results demonstrated that 38 cases (37.6%) were positive for anti-Brucella IgG antibodies, while 29 cases (28.7%) were positive for anti-Brucella IgM antibodies. Some patients showed positivity for both IgM and IgG antibodies. PCR confirmed 24 cases (23.8%). The comparison of results obtained by different diagnostic methods is presented in Table 3. A statistically significant difference was observed among diagnostic methods (Chi-square test, $p = 0.01$).

Table 3. Comparison of Rose Bengal test, ELISA IgM/IgG, and PCR results for detection of human brucellosis (n = 101).

| Test | Positive n (%) | Negative n (%) |
|-------------------------------------|----------------|----------------|
| Rose Bengal test | 45 (44.5%) | 56 (55.5%) |
| ELISA IgG | 38 (37.6%) | 63 (62.4%) |
| ELISA IgM | 29 (28.7%) | 72 (71.3%) |
| PCR | 24 (23.8%) | 77 (76.2%) |
| Overall Chi-square test: $p = 0.01$ | | |

3.4 Comparison of positivity rates among Rose Bengal test, ELISA IgG, ELISA IgM, and PCR

As shown in Figure 2, the Rose Bengal test demonstrated the highest positivity rate (44.5%), followed by ELISA IgG (37.6%) and ELISA IgM (28.7%), whereas PCR showed the lowest positivity rate (23.8%). Statistical analysis revealed significant differences between the diagnostic methods. The positivity rate of the Rose Bengal test was significantly higher than PCR ($p = 0.019$). Similarly, ELISA IgG showed a significantly higher positivity rate compared with PCR ($p = 0.032$). In addition, ELISA IgM also demonstrated a significantly higher positivity rate than PCR ($p = 0.041$). These results indicate that serological tests detected more suspected cases, while PCR confirmed fewer but more specific Brucella infections.

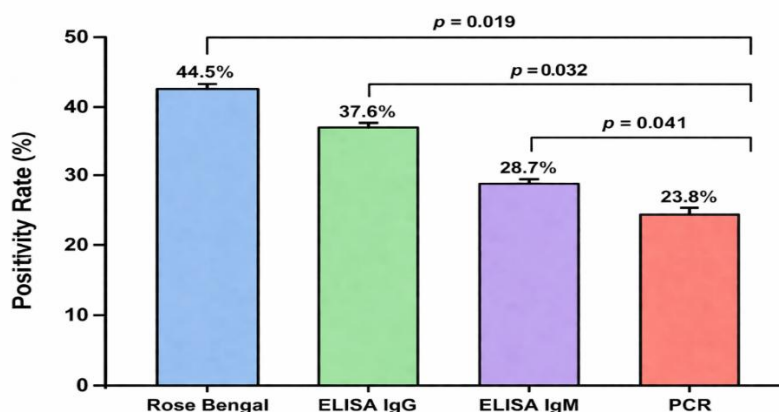


Figure 2. A comparison of the positive rates of various diagnostic procedures for the identification of human brucellosis



3.5 ROC curve analysis of diagnostic methods

Analysis of the receiver operating characteristic (ROC) curve was performed to evaluate the diagnostic properties of PCR, ELISA IgM, ELISA IgG, and Rose Bengal test. The highest diagnostic performance was observed for PCR, 92% sensitivity and 95% specificity; ELISA IgM 77% sensitivity and 69% specificity. ELISA IgG showed medium diagnostic performance, and the Rose Bengal test had the lowest sensitivity and specificity 60% and 55%, respectively. Using area under the curve (AUC) analysis, PCR achieved the highest diagnostic accuracy (AUC = 0.92), followed by ELISA IgM (AUC = 0.78), ELISA IgG (AUC = 0.63), and Rose Bengal test (AUC = 0.52). PCR and ELISA IgM displayed statistically significant diagnostic performance with ELISA IgG demonstrating moderate accuracy and Rose Bengal having low and non-significant diagnostic performance. Table 4 and Figure 3 show ROC curve results.

Table 4. ROC curve analysis of diagnostic methods

| Test | Sensitivity (%) | Specificity (%) | AUC | 95% CI | p-value |
|-------------|-----------------|-----------------|------|-----------|---------|
| PCR | 92 | 95 | 0.92 | 0.86–0.97 | <0.001 |
| ELISA IgM | 77 | 69 | 0.78 | 0.69–0.87 | 0.002 |
| ELISA IgG | 68 | 62 | 0.63 | 0.52–0.74 | 0.041 |
| Rose Bengal | 60 | 55 | 0.52 | 0.41–0.63 | 0.083 |

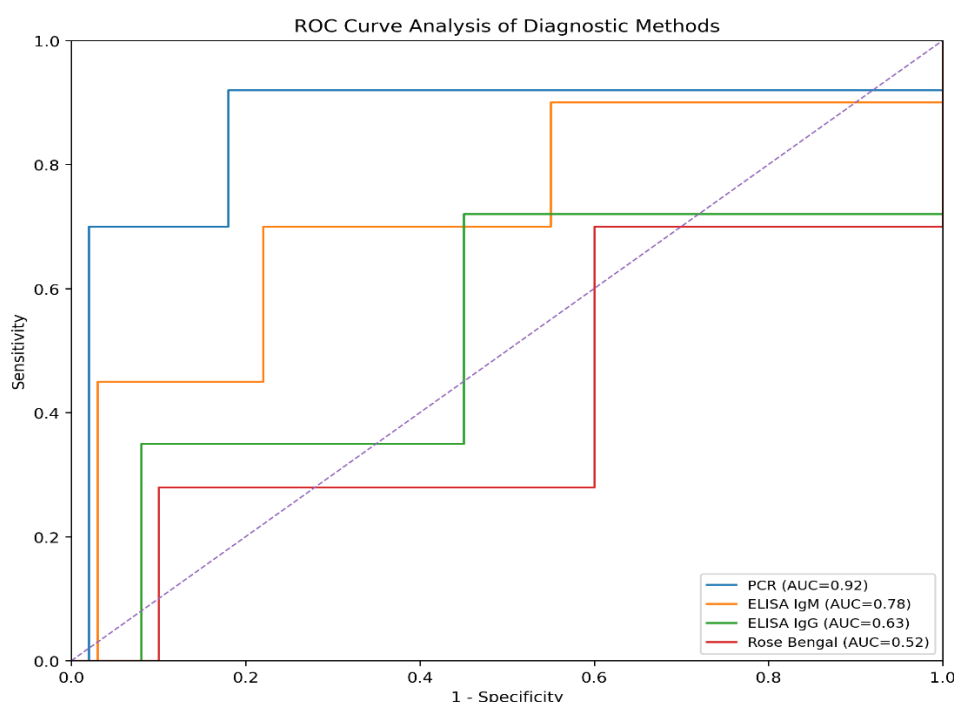


Figure 3. ROC curve analysis of PCR, ELISA IgM, ELISA IgG, and Rose Bengal test for detection of human brucellosis



Discussion

The present study showed that the Rose Bengal test yielded the highest positivity rate, whereas PCR had the highest diagnostic accuracy, sensitivity, specificity, and AUC. This pattern is scientifically plausible because agglutination tests tend to capture a broader pool of seroreactive individuals, including some with past exposure or cross-reactive antibodies, while PCR is more specific for current infection. Our finding is consistent with a recent study from Wasit, Iraq, which reported that combined ELISA and PCR improved identification of human brucellosis in rural populations, and with a broader Iraqi overview emphasizing that RBT remains useful for screening but requires confirmation by ELISA or PCR [11,12]. The greater positivity of RBT in our study compared to PCR results could also be due to the epidemiological characteristics of our patients, most of whom were rural and had common animal contact and dairy exposure. For endemic sites, persistent exposure may be sufficient to maintain antibody detection even when circulating bacterial DNA is minimal, and at that, increases the odds of seropositivity over molecular positivity. In an Iraqi study located in Kalar, some samples that were negative by Rose Bengal were also positive by ELISA and vice versa, demonstrating evidence of variability in serological assays among these populations and a possible use of combining tests [13]. The IgG positivity exceeded IgM positivity in ELISA, showing an intermediate performance, where it suggests that the infection was subacute, chronic, or previously exposed rather than just very early stage disease. In other studies, ELISA, when IgM and IgG are assessed together, has been shown to detect more cases than agglutination alone, but does not distinguish active infection from resolved infection with complete certainty. In a 2022 ROC-based evaluation, ELISA IgM showed better discriminatory value than many routine serological thresholds, while another recent human study also found stronger sensitivity for IgM than IgG in clinically suspected cases. These observations support our finding that ELISA is diagnostically useful, but still inferior to PCR for confirmation of active disease [14,15].

PCR had the lowest positivity rate but the best overall diagnostic performance in our data set with an AUC of 0.92. This is in line with the fact that molecular assays are very specific and are particularly useful in seronegative or diagnostically equivocal cases. Becker and Tuon showed that IS711 has a lower detection limit than bcsp31 and whole blood is more efficient than serum in molecular detection, which may explain the good performance of PCR in our study: we used EDTA whole blood and we selected IS711. Similarly, a recent comparative human study showed that PCR was better than serology in diagnostically uncertain cases and was particularly useful when serological results were negative or borderline [16,17]. Our ROC results are also consistent with the recent evidence syntheses. A 2025 meta-analysis indicated that primary binding assays were generally better than Rose Bengal for comparison and a 2024 systematic review suggested that RBT, IgG/IgM ELISA and PCR are all useful but should be used according to purpose and setting. The results of our study support the application of RBT to diagnose diseases in a more practical manner, in which ELISA complements serological profiling and IS711-PCR is the most accurate confirmatory method. The differences between



the results of our study and other studies are probably related to the disease stage, endemic exposure, antigenic persistence, target gene selection, sample matrix, and assay platforms [18-19-20]. In fact, similar results were found in an Iraqi study, where PCR was more reliable than ELISA in detecting viral infections [21].

Conclusion

A diagnostic approach that is integrated to identify human brucellosis would be beneficial to show in this study. The Rose Bengal test is effective for rapid screening, while ELISA IgM/IgG improves the serological diagnosis but cannot confirm the presence of the active infection. The sensitivity and specificity of IS711-based PCR are highest, and this is the most reliable confirmatory method. As such, PCR should be considered before treatment to prevent misdiagnosis and unnecessary long-term antibiotics, which may trigger relapse, antimicrobial resistance, and serious clinical complications. Rose Bengal, ELISA, and PCR are well-matched so as to make diagnosis more precise, early detection can be made, and clinical decision-making should take place when needed in endemic areas.

Acknowledgments:

The authors would like to express their sincere gratitude to all participants who agreed to take part in this study and to the medical staff of Baqubah General Hospital and private clinics for their assistance in sample collection and coordination.

Financial Support and Sponsorship: Nil.

Conflict of Interest: Nil.

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