Original Article

Brucellosis laboratory tests in Syria: what are their diagnostic efficacies in different clinical manifestations?

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Abstract

Introduction: Diagnosis of brucellosis in Syria is based on the presence of compatible symptoms in addition to positive agglutination results. This study investigated the potential of culture, ELISA and real-time PCR to support the diagnosis in different clinical manifestations of brucellosis.

Methodology: Peripheral blood samples from 34 suspected brucellosis patients and 42 probable chronic or relapsed brucellosis patients were tested by agglutination tests, culture, ELISA and real-time PCR.

Results: Among 34 samples collected from suspected cases, 18/34 (53%) were agglutination tests positive, 12/34 (35%) were culture positive, 12/34 (35%) were *Brucella* IgG positive, and 10/34 (29%) were real-time PCR positive. Three out of 34 patients were positive by real-time PCR but not by agglutination tests or culture. Among 42 samples obtained from probable chronic or relapsed patients, 27/42 (64%) were agglutination tests positive, 26/42 (62%) were *Brucella* IgG positive, 4/42 (10%) were culture positive, and 1/42 (2%) was real-time PCR positive.

Conclusion: To rule in or rule out the diagnosis of brucellosis, a combination of several tests should be applied. Agglutination tests should be performed first considering their high sensitivity. If the agglutination test is negative, real-time PCR, and/or ELISA, and/or culture should be performed. When relapse or chronic brucellosis are suspected, agglutination tests and/or ELISA are recommended.

Key words: brucellosis; agglutination tests; culture; real-time PCR; ELISA; Syria

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Introduction

Brucellosis remains the world's most common zoonotic disease with more than half a million new human cases reported annually [1]. Syria is ranked number one in the incidence of brucellosis worldwide, as 39,838 human cases were reported in 2007 [1,2]. The disease can mimic several illnesses such as typhoid, mononucleosis, leishmaniasis, and tuberculosis [3]. Thus laboratory tests are needed to confirm the diagnosis [4]. Diagnosis often relies on culture and agglutination tests. Culture, the gold standard method, is time-consuming, hazardous and insensitive. Therefore, the standard agglutination test (SAT, measures total IgM and IgG) is usually performed as a screening test, followed by Coombs test (which mainly measures IgG) to avoid false negative results [5]. Today, several diagnostic tests are increasingly proposed to improve the diagnosis, such as ELISA, which has a higher sensitivity compared to other serological methods [5], and quantitative molecular testing [6,7].

In Syria, agglutination tests are the primary method for diagnosis of brucellosis. Hence this prospective cross-sectional study was initiated to address the need for recommending additional tests to support the diagnosis of brucellosis in its different clinical manifestations.

Methodology

Peripheral blood samples were collected, in the period between May 2010 to December 2010, from subjects referred to the brucellosis laboratory of the Syrian Ministry of Health in Damascus as either suspected brucellosis (34 patients), or suspected relapse or chronic brucellosis (42 patients). Nine of the suspected brucellosis patients provided a follow-up sample after four to six months of clinical cure. Suspected brucellosis patients were not previously diagnosed with brucellosis but manifested clinical symptoms and reported potential exposure history. All probable relapsed or chronic patients had brucellosis in the past and had received proper

Table 1. Consistency and sensitivity comparison of dually combined agglutination tests, culture, ELISA IgG, and real-time PCR methods in confirmed brucellosis patients (n = 21)

Methods dual combinations	Consistency		Discrepancy		P-value**
	Positive*	Negative*	+/-*	-/+*	
Agglutination tests/Culture	12	3	6	0	0.010
Agglutination tests/ELISA IgG	11	2	7	1	0.030
Agglutination tests/ Real-time PCR	7	0	11	3	0.029
Real-time PCR/Culture	5	4	5	7	0.576
Real-time PCR/ ELISA IgG	5	4	5	7	0.576
Culture/ELISA IgG	9	6	3	3	1.000

* Consistent Positive: number of cases positive by the two methods, consistent negative: number of cases negative by the two methods, +/- discrepancy: number of cases positive by the first method and negative by the second method, -/+ discrepancy: number of cases positive by the first method and negative by the second method

** P-value associated with the student's t-test (used to determine the significance of differences between study tests in terms of sensitivity)

treatment. Patients with relapsing or chronic brucellosis were classified according to recurrence of symptoms after less than one year (relapsed patients) or after more than one year (chronic patients). Clinical diagnosis was made by the physicians in hospitals and health centers. The final diagnosis of acute, relapsed or chronic brucellosis was based on matched clinical picture together with positive results by agglutination tests ($\text{Titer} \geq 1:160$ by SAT or Coombs test), culture, real-time PCR, and ELISA IgG, or at least one of those applied tests, according to the World Health Organization and Centers for Disease Control diagnostic criteria [8,9]. All patients signed an informed consent, and approval of the ethical committee of Damascus University was obtained.

Serological methods

Results of agglutination tests and ELISA IgM tests were obtained from patients' clinical files. ELISA IgM test data were available only for patients with probable chronic or relapsed disease. ELISA IgG tests were performed using the DRG *Brucella* IgG ELISA kit (DRG, Marburg, Germany) according to the manufacturer's instructions.

Bacteriological methods

For blood culture tests, 5-10 ml of whole blood were inoculated immediately in BACTEC Plus Aerobic/F Medium (Becton Dickenson, New Jersey, USA). Blood cultures were processed using a BACTEC 9050 instrument (Becton Dickenson, New Jersey, USA), incubated for 21 days and subcultured weekly. Positive blood cultures were checked for growth by subculture on blood agar, *Brucella* agar, and MacConkey agar. Subcultures were incubated at 37°C for three days in 5% CO_2 atmosphere. If growth appeared, colonies were identified by special growth characteristics on subculture media, and by performance of Gram stain, oxidase test, catalase test and agglutination with specific antiserum.

DNA extraction and real-time PCR

DNA was extracted from serum (200 µl) with the QIAamp DNA blood Mini kit (Qiagen, Hilden, Germany). Real time PCR was performed on the LightCycler instrument (Roche Diagnostics, Penzberg, Germany) using LightMix Kit *Brucella* Genus (TIB Molbiol, Berlin, Germany) and FastStart DNA Master Hybridization Probe (Roche Diagnostics, Penzberg, Germany). The LighMix kit provided primers and probes for specific amplification and detection of a 207-bp fragment of the IS711 insertion sequence. An internal control, also included in the LightMix kit, was added to the reaction mix to monitor the real-time PCR reaction. Negative samples by real-time PCR were retested to increase assay sensitivity.

Statistical analysis

Data were analyzed using PASW Statistics version 18 (SPSS Inc, Chicago, IL, USA). Statistical analysis included assessment of correlation between variables using kendel tau coefficients, comparison of means using Student's t-test, and calculation of sensitivities, specificities, positive predictive value

Table 2. Clinical performance characteristics of *Brucella* agglutination tests, culture, ELISA and real-time PCR

Diagnostic Test	Sensitivity (%)*	Specificity (%)*	PPV (%)*	NPV (%)*	P value (correlation)**
Suspected brucellosis patients (n=34)					
Agglutination tests	86%	100%	100%	81%	0.000
Culture	57%	100%	100%	59%	0.000
ELISA IgG	57%	100%	100%	59%	0.000
Real-time PCR	48%	100%	100%	54%	0.002
Suspected relapse or chronic brucellosis patients (n= 42)					
Agglutination tests	90%	100%	100%	80%	0.000
Culture	13%	100%	100%	32%	0.094
Real time PCR	3%	100%	100%	29%	0.264
ELISA IgG	87%	100%	100%	75%	0.000
ELISA IgM	87%	50%	81%	60%	0.006

* Sensitivity, specificity, PPV (positive predictive value), and NPV (negative predictive value) were calculated against final diagnosis (which was based on clinical suspicion of brucellosis combined with positive result by agglutination tests, real-time PCR, culture and ELISA IgG or at least one of the applied tests)
** P-value associated with the Kendall's tau-b (used to study the correlation of study tests results with final diagnosis)

(PPV) and negative predictive value (NPV). A p-value < 0,5 was considered significant.

Results

Suspected brucellosis cases

The diagnosis of brucellosis was established according to the considered criteria in our study in 21/34 (62%) suspected brucellosis patients. The mean age of these 21 patients was 33 years (range 10-60 years). Twelve were males and 9 were females. All 21 patients acquired infection possibly by consuming unpasteurized milk or dairy products. The duration of symptoms before diagnosis of brucellosis was less than 2 months (19 acute patients); 2 to 12 months (one sub-acute patient); and more than one year (one chronic patient). As reported by the patients, the most common symptoms were fever, arthralgia, night sweats, nausea, fatigue, and headache. Fever was present in 18/21 (86%) patients. Arthralgia was also present in 18/21 (86%) patients. One patient had epididymo-orchitis.

Eighteen of 21 (86%) patients with established brucellosis infection were agglutination test positive, 12/21 (57%) were culture positive (all of whom were agglutination test positive), 10/21 (48%) were real-time PCR positive (7/10 were agglutination test positive and 5/10 were culture positive), and 12/21 (57%) were *Brucella* IgG positive (11/12 were agglutination test positive, 9/12 were culture positive and 5/12 were real-time PCR positive). All 21 confirmed brucellosis cases were positive by agglutination tests and/or real-time PCR (Table 1).

Out of 10 real-time PCR-positive patients, 8 had low mean bacterial DNA load (± SD); 73 ± 54 copy/ml (range, 7-144 copies/ml), 3 of which were initially negative but turned positive when the real-time PCR was repeated. The other two patients (2/10) had high bacterial DNA load (58,000 and 402,650 copies/ml).

The sensitivity of agglutination tests (86%) was significantly higher ($p < 0.05$) than the sensitivities of real-time PCR (48%), culture (57%), and ELISA IgG (57%). Real-time PCR, culture and ELISA IgG had statistically equal ($p > 0.05$) sensitivity (Table 1). There was significant correlation between all tests and final diagnosis ($p < 0.05$). Agglutination tests, culture, ELISA IgG and real-time PCR showed sensitivities of 86%, 57%, 57%, and 48%; specificities of 100%, 100%, 100% and 100%; PPV of 100%, 100%, 100%, and 100% and NPV of 81%, 59%, 59%, and 54%, respectively (Table 2). Follow-up samples at four to six months were negative by all tests except ELISA IgG (5/9 samples).

Table 3. Consistency and sensitivity comparison of dually combined agglutination tests, culture, ELISA IgG, and real-time PCR methods in confirmed chronic or relapsing brucellosis patients (n = 30)

Methods dual combinations	Consistency		Discrepancy		**P-value
	*Positive	*Negative	*+/-	*-/+	
Real-time PCR/Agglutination tests	1	3	0	26	0.000
Real-time PCR/IgM	1	4	0	25	0.000
Real-time PCR/ IgG	1	4	0	25	0.000
Culture/Agglutination tests	4	3	0	23	0.000
Culture/IgG	4	4	0	22	0.000
Culture/IgM	4	4	0	22	0.000
Real-time PCR/Culture	0	25	1	4	0.184
Agglutination tests/IgG	23	0	4	3	0.712
Agglutination tests/IgM	23	0	4	3	0.712
IgM/IgG	24	2	2	2	1.000

* Consistent Positive: number of cases positive by the two methods, consistent negative: number of cases negative by the two methods, +/- discrepancy: number of cases positive by the first method and negative by the second method, -/+ discrepancy: number of cases positive by the first method and negative by the second method
** P-value associated with the student's t-test (used to determine the significance of differences between study tests in terms of sensitivity)

Relapse or chronic brucellosis cases

Relapse or chronic disease was confirmed in 30/42 (71%) patients according to the considered diagnostic criteria in our study. The mean age of these 30 patients was 34 years (range between 4-73 years). Six were males and 24 were females. Fifteen out of 30 were defined to have relapsing brucellosis while the other 15 were defined to have chronic brucellosis. The patients' most common objective symptoms, as reported by the patients, were arthralgia, fatigue, mayalgia, sweating, headache, back pain, nervousness, fever, chills, depression, nausea, and insomnia.

Out of the 30 chronic or relapsed patients, 26 (87%) were *Brucella* IgG positive, 26/30 (87%) were *Brucella* IgM positive, 24/30 (80%) were IgG and IgM positive, 27 (90%) were agglutination tests positive (21 were IgG and IgM positive, 2 were only IgG positive, 2 were only IgM positive), 4/30 (13%) were culture positive and 1/30 (3%) was real-time PCR positive (55 copies/ml). Out of 12/42 undiagnosed patients, 6 were IgM positive. All 30 confirmed chronic or relapsed brucellosis cases were positive by agglutination tests and/or ELISA. The sensitivity of the agglutination tests (90%) was similar ($p > 0.05$) to the sensitivities of ELISA IgM (87%) and IgG (87%). The sensitivities of agglutination tests, IgM, and IgG were significantly higher ($p < 0.05$) than the sensitivities of culture (13%) and real-time PCR (3%). The sensitivities of culture and real-time PCR were similar (Table 3). Agglutination tests, real-time PCR, culture, IgM and IgG showed sensitivities of 90%, 3%, 13%, 87%, and 87%; specificities of 100%, 100%, 100%, 50%, and 100%; PPV of 100%, 100%, 100%, 81%, and 100%; NPV of 80%, 29%, 32%, 60%, and 75%, respectively. Agglutination tests and ELISA showed significant correlation ($p < 0.05$) with the final diagnosis, whereas real-time PCR and culture showed no significant correlation ($p > 0.05$) with the final diagnosis (Table 3).

Discussion

In this study, the sensitivity of agglutination tests was higher than the sensitivities of culture, real-time PCR and ELISA IgG in suspected brucellosis. This observation favors adoption of agglutination tests as a first-line method in the diagnosis of brucellosis, considering their high sensitivity, limited cost and simplicity. The fact that three cases were undiagnosed by performance of agglutination tests alone emphasizes the need for further laboratory testing for patients with negative agglutination results, especially in cases of high clinical indication and persistence of symptoms. This recommendation is in agreement with those of other studies' findings [6]. Real-time PCR, culture and ELISA IgG were statistically similar in sensitivity, which suggests that applying these tests for patients with uncertain negative agglutination test results should be sought according to other considerations such cost and time.

The false negative agglutination test results obtained for three patients could be attributed to non-sufficient antibody production as all of them had

positive titers below the cut-off value, and this could be due to chronic disease (one patient), or to performance of agglutination tests in the early days of infection (two patients).

The observed low sensitivity of real-time PCR test (48%) in our study might be attributed to the use of very small-volume samples from patients with low numbers of circulating bacteria [10]. This explanation is further supported by the observation that 9 of 11 total real-time PCR positive samples had low bacterial load, and that 3 initially negative samples yielded positive results upon repetition of real-time PCR.

The sensitivity of blood culture in our study was 57%. Similar results (59%) were obtained by others following the same cultural procedures that were used in our study [11]. Un-successful isolation of *Brucella* in 9/21 samples could be due to the influence of the infective species (as the culture of *B. abortus*, which is prevalent in Syria [12], is usually less successful than culture of *B. melitensis* [13]), or to sampling from afebrile patients [13], as samples were obtained in the morning (temperature usually rises in the evening in brucellosis patients) during the working hours of the brucellosis laboratory.

Brucella specific IgG were not detected in 9/21 patients, which was probably due to delayed appearance of IgG. Our results appeared to be consistent with those of others [14].

Real-time PCR and culture were of limited value in the diagnosis of patients with suspected relapse and chronic brucellosis. This might be due to the absence of bacteremia [15]. Reduction in sensitivity of real-time PCR between acute and chronic patients was also noticed by other investigators [16], who reported a positivity of 56% in acute patients versus 17% in chronic patients. Our results demonstrated that ELISA and agglutination tests were the most sensitive in the diagnosis of relapse and chronic disease. Both IgM and IgG were detected in a significant number of patients (24 of 42, 57%), and this was of value as the combination of IgM and IgG allows proper serological diagnosis at any stage of illness. Elevation of both IgM and IgG in 24 out of 29 (82.7%) probable chronic brucellosis patients was also reported in one study [17]. The presence of IgM alone in six samples might not be indicative of current brucellosis, and could be due to persistence of IgM antibodies after treatment [18].

In conclusion, a combination of several tests should be applied to establish or rule out diagnosis of brucellosis. Agglutination tests are recommended as a first choice in suspected brucellosis. If a negative result is obtained, then testing by real-time PCR, and/or ELISA, and/or culture, should be conducted. In the case of relapsed and chronic brucellosis, agglutination tests and/or ELISA are recommended for proper diagnosis.

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