A panel of eight tests in the serodiagnosis and immunological evaluation of acute brucellosis

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استعراض ثمانية اختبارات تستعمل في التشخيص المصلي والتقييم المناعي لداء البروسيلات الحاد

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خلاصة: استُعملت مجموعة من ثمانية اختبارات لدراسة 200 حالة من داء البروسيلات الحاد و200 من الأشخاص السليمين للبروسيلة كمجموعة شاهدة و200 من الأشخاص الذين تبدو عليهم علامات الصحة كمجموعة شاهدة ثانية. وكان أفضل الاختبارات هو اختبار وردية البنغال الذي استُعمل فيه كاشف مستورد (من بيومريه، فرنسا) وكاشفان محليان. وقد تم تحسين هذا الاختبار حتى يصلح للمعايرة بعد أن كان يستعمل لأغراض التحرّي. أما أفضل اختبارين يستعملان معاً فكانا اختبار التراص الأنبوبي مع اختبار مماثل لاختبار كومز. وتبيَّن أن اختبار الضد المتألق غير المباشر لم يكن يتميَّز عن غيره من الاختبارات. أما اختبار 2 – مركبتو إثانول واختبار بروتين C التفاعلي فكانا مفيدين في التحقق من نشاط المرض. ولقد وُجدت في الدم المحيطي في حالات داء البروسيلات الحاد أعداد عادية من الخلايا المكونة للزهيرات "E" وعدِلات غير فعالة في البلعمة.

ABSTRACT A panel of eight tests was used to study 200 cases of acute brucellosis, 200 patients negative for brucella as a control group and 200 apparently healthy individuals as a second control group. The best diagnostic test was the rose Bengal test using an imported reagent (BioMérieux, France) and 2 local reagents. This test was improved from being a screening test to be a titrable one. The best two tests used together were the tube agglutination test with Coomb-like test. The indirect fluorescent antibody test had no advantages over the use of other tests. The 2-mercaptoethanol test and C-reactive protein test were useful in checking the brucellosis activity. Normal numbers of E-rosette forming cells and inefficient neutrophils in phagocytosis were found in peripheral blood during acute brucellosis.

Ensemble de huit épreuves pour le sérodiagnostic et l'évaluation immunologique de la brucellose aiguë

RESUME Un ensemble de huit épreuves a été utilisé pour étudier 200 cas de brucellose aiguë, 200 patients négatifs pour Brucella comme groupe témoin et 200 personnes en bonne santé apparente comme second groupe témoin. La meilleure épreuve diagnostique était l'épreuve au rose Bengal utilisant un réactif importé (BioMérieux, France) et 2 réactifs locaux. Cette épreuve a été améliorée, pour transformer une épreuve de dépistage en une épreuve titrable. Les deux meilleures épreuves utilisées ensemble étaient l'épreuve d'agglutination en tube avec l'épreuve de type Coombs. L'immunofluorescence indirecte ne comportait aucun avantage par rapport à l'utilisation d'autres épreuves. Les tests du mercapto-2-éthanol et de la protéine c-réactive étaient utiles pour contrôler l'activité de la brucellose. Des nombres normaux de lymphocytes T formant des rosettes E avec les hématies de mouton et de neutrophiles inefficaces dans la phagocytose ont été trouvés dans le sang périphérique pendant la brucellose aiguë.

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Introduction

Brucellosis has a worldwide distribution with a high prevalence in the Mediterranean countries [*1,2*]. In Iraq, the disease is a significant health and economic problem [*2*]. The commonest species isolated from Iraqi patients in Ninevah Province have been reported as *Brucella abortus* (77.4%) and *B. melitensis* (19.4%) [*3*].

The diagnosis of brucellosis is based on the clinical features and the results of laboratory tests [*4*]. The isolation of *Brucella* species by culture is disappointing since it requires special media and several weeks of incubation, and is positive in only about half of acute cases [*4–6*]. Therefore, the laboratory diagnosis of brucellosis is usually based on serological tests. These tests are easy to perform and the results can be obtained within a short time [*7*].

Several laboratory tests are used for the study of brucellosis. Some tests are used for the serodiagnosis of brucellosis, and others are used for the study of its activity and the immune response of patients against the organism [*8*].

Our study had three aims:

- To evaluate the sensitivity and specificity of the rose Bengal agglutination test (RBAT), tube agglutination test (TAT) (Wright test), indirect immunofluorescent antibody test (IFAT) and Coomb-like test (CLT) in the laboratory diagnosis of acute brucellosis;
- To evaluate the activity of the *Brucella* species using the 2-mercaptoethanol test (2MET) and C-reactive protein (CRP) measurements;
- To study the cellular immunity of patients with acute brucellosis by counting peripheral T-cells using E-rosette formation (ERF) and by finding the phagocytic efficiency of neutrophils by the nitroblue tetrazolium test.

Materials and methods

Patients

A total of 200 patients with clinical and serological evidence of acute brucellosis (positive for rose Bengal screening test) were recruited from teaching hospitals, consultation clinics and health centres in Mosul, Iraq. The age of these patients ranged from 15 years to 75 years (mean = 33 years).

Two types of control group were used. The first was composed of 200 patients with clinical suspicion of brucellosis, selected on the basis of negative for brucellosis by the tube agglutination test, which is the standard method of serological diagnosis of brucellosis [*9*]. The age of these patients ranged from 15 years to 70 years (mean = 34 years). These patients were recruited from the same places mentioned above. The second control group comprised 200 apparently healthy individuals randomly selected from textile and soft-drink factories. The age of these individuals ranged from 17 years to 60 years (mean = 31 years).

Laboratory tests

Rose Bengal agglutination test

We used two types of reagent: brucella slide-test kit reagent for *B. abortus* (Bio-Mérieux Company, France) and two local reagents for *B. abortus* and *B. melitensis* (Iraqi Institute of Sera and Vaccines, Baghdad).

RBAT was performed by two methods: first, by a rapid slide screening method as described by Diaz et al. [*10*]; second, by a rapid slide titration method in which equal volumes of the brucella reagents and serum, serially diluted in normal saline, were mixed. The minimum antibody titres which could give positive results by this method

have been suggested to be 1/80 [*11*]. Therefore, serum dilutions of one-half, one-quarter and one-eighth would give titres of 1/160, 1/320 and 1/640 respectively [*10,11*]. These titres were also confirmed by the tube agglutination test (see following section).

Tube agglutination test (Wright test)
In TAT, a formalin- and heat-killed *B. abortus* suspension (Diagnostics Pasteur, France) was used. The test was carried out according to the manufacturer's instructions and as described by Cox [*11*].

Indirect fluorescent antibody test
The fluorescent-conjugated anti-human serum and slides coated with fixed brucella antigens were prepared and kindly provided by the Central Laboratory, Baghdad. The test was carried out as described by Edwards et al. [*12*]. Positive scores were from 1^+ to 4^+.

Coomb-like test
CLT was performed on the sera which showed no agglutination by TAT. The procedures as described by Diaz et al. [*10*] and Edwards et al. [*12*] were used.

2-mercaptoethanol test
The method used was that described by Diaz et al. [*10*]. It was similar to TAT, but the sera were treated with 2-mercaptoethanol (0.05 m/L, i.e. 14 mL 2-mercaptoethanol in 1 L of phosphate buffer saline) for 30–60 minutes at 37 °C prior to use [*13*].

C-reactive protein test
The latex reagent used to detect the serum concentration of CRP was purchased from Omega Diagnostics, United Kingdom. The method used was that described by the manufacturers. It was performed by mixing equal volumes of latex reagents with undiluted sera of patients. This test can detect CRP in a concentration of 6 mg/L or more.

E-rosette formation
ERF was carried out for 86 patients with brucellosis and for 100 individuals from the other two control groups. The lymphocytes were separated from the blood using Ficol–Hypaque (Pharmacia Fine Chemical, Uppsala, Sweden). The method of separation used was that described by Boyun [*14*], and the ERF technique applied was that described by Jondol et al. [*15*]. We counted 200 lymphocytes and the percentages of E-rosette-forming cells were found.

Nitroblue tetrazolium test
Nitroblue tetrazolium dye was obtained from BDH Biochemical Company, United Kingdom. The method described by Park was applied [*16*]. We counted 200 neutrophils and the percentages of neutrophils with dark blue formazine deposits were determined.

Results

Rose Bengal screening test

All patients positive with the rose Bengal reagent from BioMérieux Company were also positive with the Iraqi reagent of *B. abortus*, but only 91% were positive with the Iraqi reagent for *B. melitensis* (Table 1).

Among the healthy control group, 6%, 7.5% and 2.5% were positive with the BioMérieux reagent, the Iraqi reagent for *B. abortus* and the Iraqi reagent for *B. melitensis* respectively. However, the patient control group showed positive results in 13.5%, 19.0% and 4.0% for the same reagents respectively.

The results obtained by the titration method in all the participants studied are summarized in Table 1, and their titres obtained by the three rose Bengal reagents are summarized in Table 2. The titres of patients varied between 1/80 and 1/2560, while that of the controls varied between 1/10 and 1/320.

Table 1 **Results of laboratory investigations in patients with brucellosis and two control groups**

Test	Brucellosis patients		Control patients		Healthy controls	
	Total	No. positive (%)	Total	No. positive (%)	Total	No. positive (%)
Rose Bengal screening test						
BioMérieux	200	200 (100)	200	27 (13.5)	200	12 (6.0)
Brucella abortus	200	200 (100)	200	38 (19.0)	200	15 (7.5)
B. melitensis	200	182 (91.0)	200	8 (4.0)	200	5 (2.5)
Rose Bengal titration test[a]						
BioMérieux	200	193 (96.5)	200	6 (3.0)	200	4 (2.0)
B. abortus	200	200 (100)	200	12 (6.0)	200	10 (5.0)
B. melitensis	200	182 (91.0)	200	1 (0.5)	200	1 (0.5)
Tube agglutination test	200	200 (100)	200	0	200	2 (1.0)
Coomb-like test	ND	ND	200	9 (4.5)	200	2 (1.0)
Indirect fluorescent test	48	44 (91.7)	47	5 (10.6)	47	3 (6.4)
2-mercaptoethanol test	200	159 (79.5)	ND	ND	ND	ND
C-reactive protein test	200	167 (83.5)	ND	ND	200	17 (8.5)

[a] *Titres of ≥ 1/160 were considered significant*
ND = not done

Table 2 **Anti-brucella antibody titres detected by different tests in patients and controls**

Test	Patients			Controls		
	Titre range	Log mean ± *s*	Geometric mean	Titre range	Log mean ± *s*	Geometric mean
Rose Bengal titration test						
BioMérieux	1/80–1/2560	2.78 ± 0.37	603.3	1/10–1/320	1.14 ± 0.31	13.8
Brucella abortus	1/160–1/2560	2.93 ± 0.31	851.0	1/10–1/320	1.20 ± 0.38	15.8
B. melitensis	1/80–1/2560	2.62 ± 0.39	415.8	1/10–1/320	1.06 ± 0.21	11.7
Tube agglutination test	1/160–1/2560	2.85 ± 0.36	717.5	1/10–1/320	1.10 ± 0.25	11.7
Coomb-like test	ND	ND	ND	1/10–1/640	1.23 ± 0.37	17.4
2-mercaptoethanol test	1/40–1/2560	2.44 ± 0.41	275.4	ND	ND	ND

s = standard deviation
ND = not done

The distribution by groups of titres estimated by the Biomerieux reagent were not statistically significantly different from those obtained by TAT ($\chi^2 = 5.1$, $P > 0.05$). Also, the McNemar test did not show any significant differences between the results obtained by these two tests, taking into consideration that titres ≥ 1/160 were considered to be positive ($\chi^2 = 0.235$, $P > 0.05$) (Table 3). Furthermore, the titres obtained using the Iraqi reagents for *B. abortus* and *B. melitensis* were significantly different from those obtained by TAT ($\chi^2 = 9.9$, $P < 0.025$; $\chi^2 = 30.23$, $P < 0.001$ respectively). They were even different from those obtained by the BioMérieux reagent ($\chi^2 = 25.43$, $P < 0.001$).

Table 3 **McNemar test between tube agglutination test and BioMérieux rose Bengal titration test**

BioMérieux rose Bengal titration test	Tube agglutination test	
	Positive	Negative
Positive	195	10
Negative	7	388

$\chi^2 = 0.235$, P > 0.05 (not statistically significant)

Table 4 **Evaluation of the different tests in comparison with the standard tube agglutination test**

Test	Sensitivity (%)	Specificity (%)	Accuracy (%)	False positive (%)	False negative (%)	PPV (%)	NPV (%)
Brucella abortus	100	87.0	91.5	20.0	–	79.8	100
Rose Bengal screening test							
BioMérieux	100	91.0	94.0	15.5	–	84.5	100
Brucella abortus	100	87.0	91.5	20.0	–	79.8	100
B. melitensis	91.0	97.0	95.0	5.6	4.40	94.3	95.5
Rose Bengal titration test							
BioMérieux	96.5	97.5	97.2	4.9	1.77	95.1	98.2
B. abortus	100	93.97	96.0	10.6	–	89.4	100
B. melitensis	91.0	99.7	96.6	0.5	4.50	99.7	95.4
Indirect fluorescent test	92.0	93.5	93.0	11.5	4.44	88.5	95.5
C-reactive protein (CRP) test	82.67	70.0	74.3	41.6	11.14	58.4	88.8
Rose Bengal screening test							
B. abortus + *B. melitensis*	100	87.0	91.3	20.5	–	79.5	100
BioMérieux + CRP	98.0	68.0	78.0	39.0	1.45	60.9	98.5
Rose Bengal titration test							
BioMérieux + IFAT	100	91.0	94.0	13.3	–	86.6	100
BioMérieux + IFAT + CLT	99.0	94.2	95.8	10.4	0.53	89.6	99.5
Tube agglutination test + CLT	100	97.2	98.0	5.2	–	94.8	100

These results were obtained from the subjects tested in Table 1. The missing values in Table 1 were not required according to the statistical formula of the tests
IFAT = indirect fluorescent antibody test
CLT = Coomb-like test
PPV = positive predictive value
NPV = negative predictive value

Tube agglutination test

The titres of patients and controls are summarized in Table 2. The titres of the patients varied between 1/160 and 1/2560, while those of the controls varied between 1/10 and 1/320.

Indirect fluorescent antibody test

IFAT was carried out for 48 patients with acute brucellosis and 94 individuals of the control group. Positive results were seen in 91.7% of the patients and 8/94 (8.5%) of the control (patients and healthy controls) (62.5% of them were also TAT- and/or CLT-positive) (Table 1). Furthermore, 12/17 (70.6%) patients with 1^+ or 2^+ IFAT-positive scores had TAT titres of 1/60 to 1/320, and 22/31 (70.9%) of patients with 3^+ or 4^+ IFAT-positive scores had TAT titres of 1/640 to 1/2560.

Coomb-like test

The titres estimated by CLT in both of the control groups varied between 1/10 and 1/640 (Table 2). Only 4.5% of the patient controls and 1.0% of the healthy controls were CLT-positive.

2-mercaptoethanol test

The titres estimated by 2MET varied between 1/40 and 1/2560 (Table 2). The titres obtained by 2MET compared with those obtained by TAT were lower in 76.5% of the patients, equal in 23.0% and higher in 0.5%. There was a significant difference between the titres recorded by these two tests ($\chi^2 = 73.9$, $P < 0.001$). The correlation between the titres obtained by 2MET and the duration of brucellosis was not significant ($r = 0.112$, $P > 0.05$).

Latex agglutination test for CRP

The CRP test was carried out on 200 patients with acute brucellosis and 200 healthy control individuals. A positive level ($\geq$ 6 mg/L) was detected in 83.5% of the patients and in only 8.5% of the controls, which was significantly different ($P < 0.001$) (Table 1). Significant correlation between the results of the CRP test and the duration of the disease was seen ($r = 0.149$, $P < 0.025$).

E-rosette formation

E-rosette cells in the peripheral blood of the patients with brucellosis varied between 40% and 84% (mean = 60.4 ± 11.7%) and in healthy controls it varied between 48% and 82% (mean = 59.9 ± 7.2%). There was no statistical difference between these two groups ($t = 0.33$, $P > 0.05$).

Nitroblue tetrazolium test

Positive cells varied between 2% and 31% (mean = 8.5 ± 5.2%) in 145 patients, and between 2% and 14% (mean = 5.6 ± 3.1%) in 75 controls, a significant difference ($t = 3.8$, $P < 0.05$).

Sensitivity and specificity of tests

The sensitivity, specificity, accuracy, positive and negative predictive values, and the percentages of false positive and false negative results of all the tests used in our study for the diagnosis of brucellosis are summarized in Table 4. The comparison was made with standard TAT. The rose Bengal test (screening and titration), particularly with the BioMérieux reagent, offered the best diagnostic results.

Multiple tests in the diagnosis of brucellosis

A combination of two or more tests for the diagnosis of brucellosis was evaluated (Table 4). The use of TAT with CLT was found to offer the best sensitivity (100%) and a specificity of 97.2%, followed by RBT with IFAT, which had a sensitivity of 100% and specificity of 91.0%.

Discussion

We found that the *B. abortus* reagents used in the screening RBT had a better sensitivity and specificity than the *B. melitensis* reagents (Table 4). These results suggest that the use of both *B. abortus* and *B. melitensis* reagents does not provide advantages over the use of the *B. abortus* reagents alone. Comparable sensitivity and specificity were previously reported with *B. abortus* reagents [*6,9*]. Also, we found that the Iraqi reagents were reliable, particularly the *B. abortus* reagent, which could be used alone.

Furthermore, RBT was seen to be more than just a screening test but a titratable one also. We found that the titres obtained by RBT were comparable to those obtained by the standard TAT. Moreover, titration improved the specificity of all the three reagents used in the RBT (Table 4). Therefore, titratable RBT offers an easy, fast and reproducible test for the laboratory diag-nosis of brucellosis.

The overall titre obtained in 400 control individuals was 1/20. In order to estimate, as a cut-off value, the highest possible titre found in brucella-negative individuals, the significant titre for the diagnosis of brucellosis in Iraq is suggested to be more than the double this value (≥ 1/160). Similar suggestions for a significant titre have been reported from other countries [*1,17,18*].

It was found that IFAT did not provide significant advantages over the use of RBT in regard to sensitivity (92% versus 100%) and specificity (93.5% versus 91.0%). However, it has been reported that IFAT has a higher sensitivity and specificity than RBT [*2,19*]. This difference could be attributed to the nature of the antigens employed in these studies. However, our results are in agreement with those reported by Edwards et al. [*12*].

Only 11/400 (2.7%) of the cases negative for brucellosis by TAT in the two control groups were found positive by CLT (Table 1). Therefore, we concluded that the prozone phenomena could be overcome by CLT, and the sensitivity and specificity of TAT could be improved by the concomitant use of CLT.

It is known that 2-mercaptoethanol destroys the disulfide bonds linking the pentamer structure of IgM, rendering it inactive, while it has no effect on IgG [*13,20*]. This distinguishes active brucellosis from inactive brucellosis [*2*]. We found that 79.5% of our patients with brucellosis had positive 2MET. The 2MET-negative cases (20.5%) had antibody titres by TAT ranging from 1/160 to 1/320 and a duration of illness of less than 4 weeks. This could indicate the presence of insufficient quantities of IgG due to the short duration of the disease, or the inappropriate use of chemotherapy, which is known to decrease the IgG level [*13*].

CRP, although not specific for brucellosis, can be regarded as a valuable index of its activity and response to therapy [*22,23*]. Among 159 2MET-positive patients, 137 (86%) had positive CRP levels of ≥ 6 mg/L. The CRP level also correlated with the duration of brucellosis, similar to 2MET (r = 0.149, $P < 0.025$). It was found that the CRP level reached its peak in the early days of disease and then decreased with the duration of the illness, after which it remained at low levels. Because CRP is technically easier to conduct than 2MET, it is recommended for the follow-up of a patient.

Normal numbers of E-rosetted cells (mainly T-cells) [*24*] were found in most (83.7%) of the cases of acute brucellosis. The lower numbers found in the remaining 16.3% of the patients might be due to suppression of bone marrow by *Brucella* spp. or their products [*25*].

To our knowledge, there are no data on the use of nitroblue tetrazolium in the study of neutrophil function in acute brucellosis. Negative results (< 11%) were found in the majority (78.6%) of our cases with acute brucellosis. This may indicate a failure to produce superoxide anion from the respiratory burst occurring during phagocytosis. Thus, neutrophils do not appear to be effective in the defence against brucellosis.

In conclusion, the best single test for the diagnosis of acute brucellosis is titration RBT, and the best combined tests are TAT and CLT. To assess the activity of brucellosis, the 2MET and CRP test appear to be quite useful. Normal numbers of T-cells and ineffective neutrophils in phagocytosis were found in our patients with acute brucellosis.

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