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ORIGINAL ARTICLE

Evaluation of serological diagnostic tests for human Brucellosis in an endemic area

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ABSTRACT

Objectives: The clinical utility of complementary tests for brucellosis are not clear in many situation. This study aimed to evaluate value of these tests for brucellosis in an endemic area in Turkey.

Materials and methods: This study was performed at Çanakkale General Hospital in 2009. In a retrospective approach, records of the patients who evaluated for brucellosis were collected. During the study period, 236 people (131 symptomatic and 105 non-symptomatic) were evaluated for diagnosis of brucellosis. All of the samples from these patients were tested for Brucella antibody seropositivity by RB slide agglutination, standard serum agglutination, Brucella Coombs, BrucellaCapt, and ELISA IgG and IgM tests. Results: In total, 49 symptomatic patients were hospitalized and blood cultures were obtained. *Brucella spp*. were isolated from nine of them (18.4%).The BrucellaCapt test was found to be the most sensitive for Brucella (74.0%) and close behind it was the Coombs test (72.5%). The sensitivity for the RB test was 48.1%. The ELISA IgG test was found more sensitive for brucellosis than the ELISA IgM test was (65.6% and 49.6%, respectively). All examined tests were found about 100% specific for brucellosis but the RB test was found less specific than the others were (96.1%) Positive predictive value for all tests was about 1 but negative predictive values were only valuable for the Coombs and Brucella Capt test (0.744 and 0.755, respectively). The other serological tests were around and below 0.50, which was weak for negative results.

Conclusions: The ELISA IgG and IgM tests were no superior to the other tests. By assessment of receiver operating characteristics (ROC) analysis, the Brucella Coombs and BrucellaCapt tests were found to be the most valuable tests for serological diagnosis of brucellosis in endemic areas. The seronegative tests in the symptomatic patients should be evaluated and repeated in short time. *J Microbiol Infect Dis 2012; 2(2): 50-56*

Key words: Brucella, Rose Bengal, Standard Tube Agglutination (STA) test, Brucella Coombs, BrucellaCapt

Endemik bölgede insan Brusellozu için Serolojik tanı testlerinin değerlendirilmesi

ÖZET

Amaç: Bruselloz tanısında kullanılan serolojik testlerin klinik kullanılışlılığı çoğu durumlarda açık değildir. Bu çalışma Türkiye'nin endemik bir bölgesinde bu testlerin bruselloz için değerini araştırmayı amaç edindi.

Gereç ve yöntem: Bu çalışma Çanakkale Devlet Hastanesinde 2009 yılında yapıldı. Retrospektif bir yaklaşımla bruselloz için araştırılan hastaların kayıtları toplandı. Çalışma süresinde 236 kişi (131 semptomatik, 105 semptomatik olmayan) brusella tanısı için araştırıldı. Tüm hastalardan alınan örnekler Rose Bengal lam testi, STA testi, Brusella Coombs testi, BrucellaCapt ve brusella IgG ve IgM kitleri ile brusella antikor seropozitifliği için test edildi.

Bulgular: Bunların 49'u hastanede yatırıldı ve kan kültürleri alındı. Bunlardan dokuzunda Brucella spp. izole edildi (%18,4). Bruselloz için en duyarlı test BrucellaCapt testi idi (%74,0) ve onu çok yakın bir değerle Coombs testi (%72,5) takip ediyordu. RB testinin duyarlılığı %48,1 bulundu. ELISA IgG testi ELISA IgM testine göre daha duyarlı idi (sırasıyla, %65,6'e karşı %49, 6). Tüm çalışılan testler bruselloz için %100 özgül bulundu ancak RB testinin özgüllüğü %96,1 idi. Yapılan tüm brusella testlerinin pozitif prediktivitesi 1 olmakla beraber; negatif prediktivite sadece Coombs ve BrucellaCapt testi için anlamlı bulundu (sırasıyla 0,744 ve 0,755). Diğer serolojik testler 0,50 civarında veya daha aşağı idi ve bu değerler negatif sonuçlar için zayıftı.

Sonuç: ELISA IgG ve IgM test sonuçları diğer testlere üstün bulunmadı. İstatistik ROC eğrisi analizine göre Brusella Coombs ve BrucellaCapt testi endemik sahada en değerli serolojik testler olarak bulundu. Semptomlu hastalarda serolojik testler negatif ise bu araştırılmalı ve kısa tekrarlanmalıdır.

Anahtar kelimeler: Brusella, Rose Bengal testi, Standart Tüp Agglütinasyon (STA) testi, Brusella Coombs, BrucellaCapt.

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INTRODUCTION

Brucellosis is a zoonotic disease caused by the bacteria genus Brucella and it is encountered in animals, such as sheep, cows, and goats, as well as humans. The onset of human brucellosis may be acute or insidious, and the clinical manifestations are protean. The disease is generalized and it may involve any organ or system of the body. Presenting complaints are numerous and nonspecific, including malaise, anorexia, fatigue, sweats, weight loss, back or joint pains, and depression. Objective physical findings are few, notably fever, mild lymphadenopathy, and, occasionally, hepatomegaly or splenomegaly.[1]

The clinical features are non-specific and can overlap with a wide spectrum of other infectious and non-infectious diseases. To reach a diagnosis, clinicians must use a wide range of non-specific routine hematological and biochemical tests in addition to Brucella-specific assays. The latter are microbiological (culture), serological (e.g., slide or tube agglutination, Coombs test, immunocapture-agglutination, BrucellaCapt test, immunochromatographic lateral flow, enzyme-linked immunosorbent assays, and the indirect fluorescent antibody test), and molecular (e.g., polymerase chain reaction (PCR) and real-time PCR). Each of these tests has advantages and limitations and, thus, requires careful interpretation.

The isolation of the organism from blood samples or other clinical specimens (e.g., spinal fluid, bone marrow, or tissue cultures) is the golden standard for brucellosis diagnosis. However, Brucella is a slow growing organism and depending on illness stage, it may not be cultivatable.[2] Therefore, diagnosis is often made by serological tests.

The Rose Bengal (RB) test is a simple screening test but may give false positive results in endemic areas. The standard serum agglutination test (SAT) is used in endemic areas for this reason.[3,4] This test can detect both IgM and IgG and, consequently, can be helpful in diagnosing acute, relapsing, and chronic infection. However, the SAT is relatively complicated, time consuming, and requires qualified personals to perform. For this reason, some immunochromatographic assays were developed for the detection of Brucella-specific IgG and IgM antibodies.[5]

A negative reaction in the SAT will not exclude active infection as the infection may be in the incubation period in which the patient has not yet produced detectable antibodies against the organism. The prozone phenomenon also results in a negative reaction; this can be prevented by using serial dilutions of the serum. A positive reaction with a given antigen may not be diagnostic, as the patient may exhibit a rise in heterologous agglutinins during the course of the illness. Such reactions are known as nonspecific anamnestic reactions because the patient has responded to an antigenic stimulus with production of nonspecific agglutinins. This makes serological diagnosis based on a single high antibody titer too uncertain, and only seroconversion with a fourfold or greater rise in titer on serial dilutions of sera should be accepted as an indication of a recent infection.

Most patients with acute brucellosis will have an agglutinin titer of 1/320 or greater by the end of the second week of illness. Even one year after treatment, 20% of patients will continue to have a significant Brucella agglutinin titer. High Brucella agglutinin titers have also been recorded in patients with *Francisella tularensis* and *Yersinia enterocolitica* infections and in patients who have recently had a cholera vaccination or been tested with a brucellergin skin test. They have also occasionally been recorded in abattoir workers.

Patients with localized brucellosis may be afebrile and may not have significant levels of Brucella agglutinin titer. In these cases, the infection should be suspected on epidemiological grounds and by detection of calcified lymph nodes on X-ray, but the diagnosis should be confirmed by culture. The RB test is a screening test designed to detect agglutinins, whereas the SAT is a confirmatory test designed to measure the agglutinin quantitatively. Any positive result obtained with the RB test should be verified with the tube test. Agglutinins may be found in healthy individuals, and single sera with titers of less than 1/80 are of doubtful significance. False-positive results may occur with sera from patients infected with F. tularensis or vaccinated against *Vibrio cholerae*. It is not possible to differentiate between Brucella abortus and *Brucella melitensis* infections using this test.[6]

Since brucellosis can have several presentations and phases (acute, sub-acute, chronic,

relapsed, active, and inactive), the search for reliable, discriminatory diagnostic and prognostic markers, especially for monitoring disease evaluation, are ongoing. In this study we aimed to determine negative and positive predictivite values for these serological tests to help general practitioners working in endemic area.

MATERIALS AND METHODS

Canakkale province is located at north-west region of Turkey with about 450.000 populations. The most important income for city region is dairy products (especially cheese). An important part of the cheese production made by traditional methods in little dairy farm corporations. Canakkale State Hospital is the only hospital provides secondary care facility to region.

Patients and Clinical Specimens: Totally 131 adult patient admitted to Infectious Diseases Clinic of Canakkale State Hospital between Jan-December 2009 were evaluated in this study. Patients ranges between 19-68 years (mean: 41,71±12,49) and 74 female, 57 male. All patients had no history of brucellosis and onset of symptoms was less than 3 months.

The diagnosis of brucellosis was based on clinical findings (fever, sweating, muscle weakness, arthralgia, appetite loss and weight loss), positivity of blood cultures for Brucella or a Standard serum agglutination test titter of ≥ 1/160 and at least fourfold rise of titter in 15 days period. Differential diagnosis for tuberculosis, yersinosis, salmonellosis, tularemia, were all ruled out by serological tests for pathogens following standard microbiological procedures. Blood cultures were performed from hospitalized 43 acute brucellosis cases but only 9 isolate is cultivated. Twenty millimeters blood samples were obtained and extracted seras frozen at -20°C until processing for serological studies. Totally 105 sera samples from healthy individuals (blood donors) were taken and used as control group. This group consist of 43 female and 62 male, ranged between 18-59 years (mean: 40,34±9,82).

Serological and bacteriological methods

The Rose Bengal test was performed with commercial Brucella abortus antigen (Refik Saydam Laboratories, Ankara, Turkey), according to manufacturer's instructions. Titrations were made by serial twofold dilutions with saline solution. Standard serum agglutination test (SAT) was performed as described by Kerr et al.[7] Standard commercial Brucella abortus antigen was used (Refik Saydam Laboratories, Ankara, Turkey). Serum titrations was started from 1/40 titters and dilated as twofold titrations until 1/5160 by saline solution. Coombs test was performed as described by Kerr et al.[7] Titters was made as SAT and after centrifugation of seras, tubes were washed tree times by using 3% saline solution. After that one drop Coombs sera (Refik Saydam Laboratories, Ankara, Turkey) was added and inoculated at 37°C for 24 hours before samples read. The Brucellacapt test (Vircell SL, Granada, Spain) was performed as specified by the manufacturer. Brucella IgG and IgM ELISAs were performed using a commercial kit test (Vircell SL, Granada, Spain). The testing procedure was followed as manufacturer's instructions. Titters over 30U for IgG and over 20U for IgM were considered positive by the manufacturer. Blood cultures were processed in automated system (VERSA-TREK™, USA). If no growth was detected within five day, incubation was maintained for 30 days and blind subcultures were plated on Brucella agar (Beckton Dickinson, USA) after 7 days repeatedly. Subcultures were incubated at 37°C in 5-10% CO_2 atmosphere for three days. If growth appeared, the suspected colonies were identified by morphology, Gram staining, oxidase, catalase, urease tests and positive agglutination with specific antiserum. Bacteria serotyping was not performed.

Statistical analysis: Sensitivity, specificity and positive and negative predictive values were calculated. Statistical analysis was made by using SPSS10.0 for Windows statistical package programme.

RESULTS

Totally 131 patient sera and 105 control sera were evaluated by RB slide agglutination test, SAT, Brucella Coombs test, BrucellaCapt and ELISA IgG and IgM. For RB test titters higher than 1/80 accepted as seropositive as many textbooks advised. 1,6,8 In patient group 45,03% (59/131) of sera was found seropositive and 54,96% (72/131) was found seronegative. Seropositivity for control group was found 3,80% (4/105) and seronegativity was found as 96,19% (101/105) respectively.

For SAT and Coombs titters, cut-off point is accepted as 1/160 as many textbooks advised. 1,6,8 In patient group seropositivity was found as 37,40% (49/131) and seronegativity was 62,59% (82/131). Seropositivity for control group was found as 0,95% (1/105) and seronegativity was 99,05% (104/105).

For Coombs tests seropositivity for patient group was found as 72,51% (95/131) and seronegativity was 27,49% (36/131). In control group no seropositivity was found, all the sera were seronegative.

When BrucellaCapt evaluated, most of authors advises 1/320 titter for cut-off value.[6,8] Seropositivity for patient group was found as 74,04% (97/131) and seronegativity was 25,96% (34/131). If the cut-off titter accepted as 1/160 seropositivity would be found as 80,91% (106/131). By this method no seropositivity found in control group and all sera samples were seronegative (Table 1).

Totally 236 sera samples were evaluated for Brucella seropositivity by ELISA IgG and IgM assays. Therefore all samples evaluated for both IgG and IgM seropositivity too. In patient group seropositivity rate was found as 65,64% (86/131) with IgG kit and 49,61% (65/131) with IgM kit. Seronegativitiy rate was found as 34,36% (45/131) with IgG kit and 50,38% (66/131) with IgM kit. In control group seropositivity was only 0,95% (1/105) with IgG and no seropositivity was found with IgM kit in that group. When both results evaluated for IgG+IgM, in patient group seropositivity was found as 34,35% (45/131) and seronegativity was 65,65% (86/131) eventually (Table 2).

Statistically in six test (RB, SAT, Coombs, B.Capt, ELISA IgG and IgM) p values were performed by Chi Square test with SPSS 10.0 and seropositivity differences were found meaningful between patient and control groups (p≤ 0,05).

Table 1. Distribution of serological test results from sera of symptomatic patients and controls.

Titers	No. of patient sera (n=131)				No. of control sera (n=105)			
	R.Bengal	SAT	Coombs	B.Capt	R.Bengal	SAT	Coombs	B.Capt
0	117	40	19	13	91	99	102	103
1/40	56	22	5	7	12	2	2	1
1/80	34	20	12	5	2	3	1	1
1/160	20	17	10	9	2	1	0	0
1/320	5	18	23	25	0	0	0	0
1/640	4	9	23	16	0	0	0	0
1/1280	0	2	21	14	0	0	0	0
1/2560	0	3	16	15	0	0	0	0
1/5120	0	0	2	27	0	0	0	0

Table 2. Results of serum tested for brucellosis by ELISA IgG and IgM commercial assay

Group	IgG ELISA		IgM ELISA		Combined (IgG+M)	
	Positive	Negative	Positive	Negative	Positive	Negative
Patient	86	45	65	66	45	86
Control	1	104	0	105	0	105

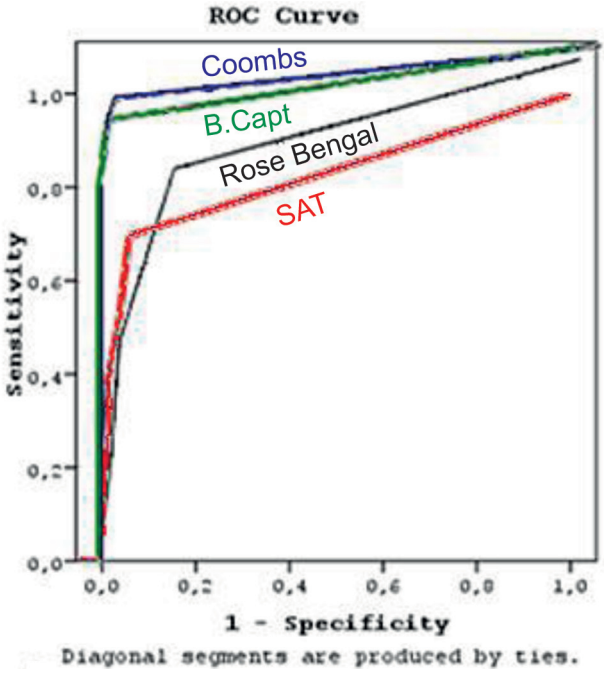
Table 3. The sensitivity, specificity and positive predictive (PPV) and negative predictive values (NPV)

Serol. test	Sensitivity (%)	Specificity (%)	PPV	NPV
R.Bengal	48,09	96,19	0,94	0,368
SAT	37,40	99,04	0,98	0,559
B.Coombs	72,51	100,0	1	0,744
Brucellacapt	74,04	100,0	1	0,755
ELISA IgG	65,64	99,04	0,988	0,697
ELISA IgM	49,61	100,0	1	0,614
ELISA IgG+M	34,35	100,0	1	0,549

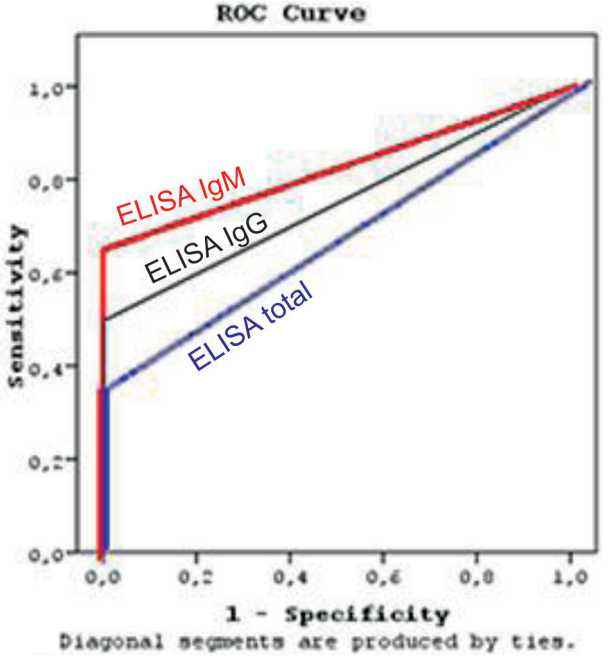
Table 4. Serological results for sera from Brucella sp. culture-positive patients.

Cases	Test results[α]					
	R.Bengal	SAT	B.Coombs	B.Capt	ELISA IgG	ELISA IgM
1	320	320	640	2560	Pos	Pos
2	640	2560	5120	5120	Pos	Pos
3	80	320	640	1280	Neg	Pos
4	160	640	640	2560	Pos	Pos
5	160	320	320	1280	Pos	Pos
6	80	160	320	640	Pos	Neg
7	320	640	1280	5120	Pos	Pos
8	640	1280	1280	5120	Pos	Pos
9	160	320	640	1280	Pos	Pos

[α] Results are shown as titers for Rose Bengal, STAT, Coombs and BrucellaCapt test.For ELISA results shown as positive (Pos) or negative (Neg).



Graphic 1. ROC curve graphs for evaluated serological tests



Graphic 2. ROC curve graphs for ELISA IgG, IgM and combined

The sensitivity, specificity, positive and negative predictive values (PPV and NPV) was calculated as seen on Table 3. All serological tests were found about 100% specific but sensitivity of tests variable. The most sensitive test was BrucellaCapt (74,04%) and Coombs test came after (72,51%). Third reliable test was found ELISA IgG (65,64%). Surprisingly SAT sensitivity was found less than Rose Bengal test (37,40% and 48,09% accordingly). Brucella ELISA IgG test was found more sensitive than SAT and RB tests (65,64%). ELISA IgM test sensitivity was found 49,61%. When we evaluate IgG and IgM test together, sensitivity was found only 34,35%. Positive predictive values for all tests were found about 1 and for seropositivity all tests were found reliable. On the other hand, NPV's were found very different from each other. The most reliable tests were found BrucellaCapt and Coombs test for negative results (NPV: 0,755 and 0,744 accordingly). The least reliable test for negative result was found Rose Bengal test (NPV: 0,368).

Table 4 summarizes results for culture-positive patients. It has been found that all culture positive patients yielded seropositivity for brucellosis.

After than ROC analysis was performed for all six brucella serological tests (Graphic1- 2). As seen on graphic 1 the area under curve is most valuable for Coombs test (0,9251) so we found this test most reliable for brucellosis diagnosis. By the way BrucellaCapt test was found as powerful as Coombs test (area under curve: 0,9472). Surprisingly Rose Bengal test was found more reliable than SAT (area under curve: 0,8365 and 0,8263 eventually).

Graphic 2 shows about ELISA IgG, IgM and IgG+IgM results as ROC curve analysis. Of them IgG found most reliable test for detection of Brucella seropositivity (area under curve: 0,8234). Area under curve was 0,7480 for IgM and 0,6717 for IgG+IgM detection tests.

DISCUSSION

Brucellosis is an endemic disease for animal and human population in Turkey. In eastern part of country seroprevalance in human was found 11,9% by Rose Bengal test and 5,4% by SAT. [9] It has been found in another study that brucella seropositivity is 32,92% by SAT and 39,45% by ELISA in bovine sera samples from eastern part of Turkey.[10] In western part of Turkey which Canakkale province located at, brucella seroprevalance was found 4,8-8,5% by Rose Bengal test and 2,9-5,6% by SAT in non-symptomatic healthy population.[11,12]

Recently in a study from eastern part of Turkey diagnostic values for brucellosis was evaluated in symptomatic patient group.[13] In that study specificity for SAT, ELISA IgG, ELISA IgM was found as 100%, 95% and 75% eventually. Positive predictive value for SAT was found 100%, for ELISA IgG 96,3% and 90,9% for ELISA IgM. This values states that positive results are very powerful clues for diagnosis of Brucella serodiagnosis. On the contrary, a negative predictive value for SAT was found 90,9% for ELISA IgM 89,5% and for ELISA IgG 76,0% which means that negative serological tests can't exclude brucellosis diagnosis.

Symptomatic patient group Brucella IgG seropositivity was found 87,35% and IgM seropositivity 36,78% in a study performed in central-Anatolian part of Turkey.[14]

The sensitivities of Brucellosis tests were found 92% for SAT, 98% for ELISA IgG and 100% for ELISA IGM in another study from Kuwait.[15] Blood cultures have been found positive for only 53% of patients in that study.

In a study from Spain which is epidemic region for brucellosis, the sensitivity value for SAT was found as 65,8%, for Coombs test as 91,5% and for BrucellaCapt as 95,1% [16] In onether study from same region the sensitivity and specificity values for Rose Bengal, SAT, Coombs Test and Brucellacapt was found over 90% whereas IgM and IgG ELISAs have the lowest sensitivity (60% and 84%, respectively).[17]

In our study six test for evaluated for serological diagnosis of brucellosis. All tests were found valuable for positive results but only Brucella Coombs test and BrucellaCapt were found reliable for negative serological results. Brucella ELISA IgG and IgM tests were found no superior to others.

Accurate diagnosis of brucellosis in human may be very difficult in some cases. The 'gold standard', is the recovery of the causative agent from the host. Molecular biology as a diagnostic tool is advancing and will soon be at the point

of replacing actual bacterial isolation. Serological tests for the diagnosis of brucellosis have advanced considerably since their inception by Wright and Smith in 1897. The accuracy of modern assays has improved diagnosis resulting in more efficient control of the disease. However, the perfect test has still not been developed. We advise to make several tests for different functions of the immune response to make accurate diagnosis for brucellosis.

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