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Original Article

Assessment of index value performance of enzyme immunoassay test in predicting the diagnosis of human brucellosis

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Abstract

Introduction: Clinical presentation of brucellosis is variable. Therefore, it must be confirmed with laboratory findings. Standard tube agglutination test (STAT) is commonly used for diagnosis of brucellosis. ELISA tests differentiate between IgM and IgG antibodies. However, there are evidences revealing that they do not have sufficient specificity. This study aimed to determine an ELISA optimal index value in the diagnosis of brucellosis.

Methodology: *Brucella* STAT and ELISA IgM/IgG tests of patients admitted to the hospital with signs and symptoms of brucellosis between January 2017 and December 2019 were evaluated in the Microbiology Laboratory.

Results: ELISA IgM and IgG serum median index value was significantly higher in STAT positive ($1 \geq 1:160$) group ($p < 0.001$ for both). By ROC analysis of 117 patients, when the IgM index value was determined to be 2.44, the sensitivity, specificity, positive and negative predictive values were 85.7%, 71.4%, 60%, and 90.9%, respectively, and when the IgG index 7.85 was determined, these values were 85.7%, 53.7%, 36.7%, and 92.3%, respectively was detected.

Conclusions: In this study, it was revealed that Vircell *Brucella* had a good clinical diagnostic performance for index value of 2.44 for IgM test kit and 7.95 for IgG test kit. If the diagnosis of brucellosis is correctly predicted with index values in *Brucella* IgM and IgG tests before STAT analysis, they can be used in the process of clinical decision. In addition to the results of *Brucella* ELISA, reporting index values and determining optimal index values for each laboratory can help the diagnosis of brucellosis.

Key words: Brucellosis; diagnosis; ELISA; sensitivity; specificity.

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Introduction

Brucellosis is one of the most important old zoonotic diseases known worldwide, with various names such as undulant fever or Mediterranean fever, and still a global public health problem. However, most of them have been brought under control [1-3]. Approximately 500,000 new human brucellosis cases are reported every year and it is an important health problem in many regions of the World, especially in Mediterranean countries of Europe, the Middle East, West and North Africa, and south and middle regions of the World [4,5]. According to the data of the General Directorate of Public Health, 9,818 brucellosis cases in a population of 71,517,100 people were reported in Turkey in 2008 (morbidity rate: 13.73 per 1,00,000) while the number of cases was 6,457 in a population of 80,810,525 in 2017 [morbidity rate: 7.99 per 1,00,000]. Only one mortal case was reported between 2008-2017 [6]. One of the most common sources of infection is unpasteurized milk, but transmission through contact with skin or mucous membranes or aspiration of infected particles is also possible [7]. In addition, accidental exposure to anti-brucellosis vaccines used in the practices of veterinary medicine is one of the primary transmission modes of the disease [8].

While *Brucella abortus*, *Brucella melitensis*, *Brucella suis* biotypes 1-4, and rarely *Brucella canis* account for the infections in humans, *Brucella melitensis* causes serious infection and accounts for the most of worldwide morbidity [9].

Clinical presentation of brucellosis is variable, but generally includes fever or fatigue. In some cases, it can

cause chronic symptoms that can affect a large number of systems and cause osteomyelitis, neurologic infections, orchitis and endocarditis as well as other symptoms [10-12]. Human brucellosis cannot only be diagnosed with clinical manifestations due to the great variety of clinical symptoms of this disease and it is essential to perform bacteriological and serological tests [5]. Serological diagnosis of brucellosis is mostly assessed with a specific titer in an agglutination test, appearance of a band in lateral flow test or a cut-off value in enzyme-linked immunosorbent assay (ELISA) [13]. It is reported that brucellosis serological tests have high sensitivity. However, their specificity is limited with antigenic cross-reactivity [14].

ELISA tests can give rapid results, differentiate between IgG and IgM antibodies, and provide a decrease in costs, and less training is required to interpret these tests [15]. It is believed that *Brucella* ELISA generally has higher sensitivity and specificity in determining antibodies specific to *Brucella* compared to the other serological tests [14,16]. However, there are also evidences revealing that ELISA tests do not have sufficient specificity to be used as diagnostic tools. Diagnostic performance of a test must be assessed by comparing its results with those obtained with the gold standard method [17,18].

Although bacteriological detection of the active microorganism in human brucellosis provides a final diagnosis, this is not always possible. The most commonly used serological test for confirmation of brucellosis is standard tube agglutination test (STAT). Sensitivity of ELISA tests is high in diagnosis and they have lower positive predictive values (PPVs) and higher false positivity rates in populations with low prevalence. In this study, it was aimed to determine *Brucella* IgM and IgG index values in the diagnosis of brucellosis, to compare test results with STAT and evaluate the diagnostic performance of ELISA test.

Methodology

This retrospective cross-sectional study included patients with suspected brucellosis based on clinical evaluation who had at least one positive serological test for the simultaneous STAT or ELISA. This study group consists of patients from central and rural parts of Ankara and from different provinces outside of Ankara. *Brucella* STAT/coombs test, ELISA IgM and IgG test results of these patients were evaluated in the Serology Laboratory of the Microbiology Department. Since most of the patients included in the study were outpatients, they did not have the result of any other gold standard diagnostic method such as blood culture. The demographic and diagnostic information of the patients were obtained from the hospital information system.

Ethical approval was obtained from Ethical Committee of the University of Health Sciences, Gulhane Training and Research Hospital (reference number: 2020/19/325).

Standard tube agglutination test (STAT)

Brucella abortus S.99 antigen (Seromed, Turkey) was used for STAT. The procedure was initiated from the primary dilution of 1:20 in sterile glass tubes to overcome a possible prozone phenomenon. The final dilution ranged from 1:20 to 1:1280 in accordance with the recommendations of the manufacturer. While 900 µL of saline solution was added to the first of seven sterile tubes 500 µL of solution was distributed to the others. To the first tube, 100 µL from the patient serum was added and mixed. A total of 500 µL was transferred from this tube to the second one and the same process was kept with the other tubes. Finally, 500 µL of fluid from the seventh tube was taken out. Thus, serial serum dilution was increased one more time in each tube. Then, 500 µL of bacterial suspension (*Brucella abortus* S.99 antibody) was added to all tubes. The tubes were incubated at 37 ºC for 24 hours and then, the samples were analyzed in terms of the presence of agglutinin particles. Positive titers were recorded according to the clarity of the fluid above and degree of the sediment. Agglutination tubes were read without shaking and assessed especially according to the turbidity degree of the fluid above. For STAT test, serum samples titrated 1:160 and above were evaluated as positive. Positive sera and normal saline were used as quality controls for tests with patient samples.

Brucella Coombs test

The tubes without agglutination were centrifuged at 2,000 rpm for 20 minutes, the antibody was settled and the fluid above was poured. After adding 0.5 cc saline solution to each tube, the tube was vortexed to mix the suspension. The tubes were centrifuged again, and this process was repeated 3 times. Then, 450 µL of saline solution and 50 µL of human antiglobulin (coombs) serum were added to each tube. The tubes were mixed and kept at 37 ºC for 16 hours and then, the agglutination was read. Titer of the tube with the last agglutination was accepted as positive value.

ELISA IgM and IgG

Brucella IgM and IgG tests in serum samples were performed using *Brucella* VirClia®IgG Monotest and

Brucella VirClia®IgM Monotest chemiluminescence immunoassay (CLIA) test kits (Vircell S.L, Granada, Spain) on VirClia EIA/CLIA device (Vircell S.L, Granada, Spain). In the CLIA method, relative luminescence units (RLU) in the wells were measured with the aid of a luminometer. The calibrator and negative control were used in each test run, and the test and kit were validated. The test results were assessed with index values: Antibody index = (sample RLU / calibrator RLU).

For *Brucella* IgM and IgG antibodies, the index value of < 0.9 was negative, between 0.9-1.1 was equivocal and > 1.1 was positive. Samples giving equivocal results were re-tested and/or a new sample was requested for confirmation.

Statistical Analysis

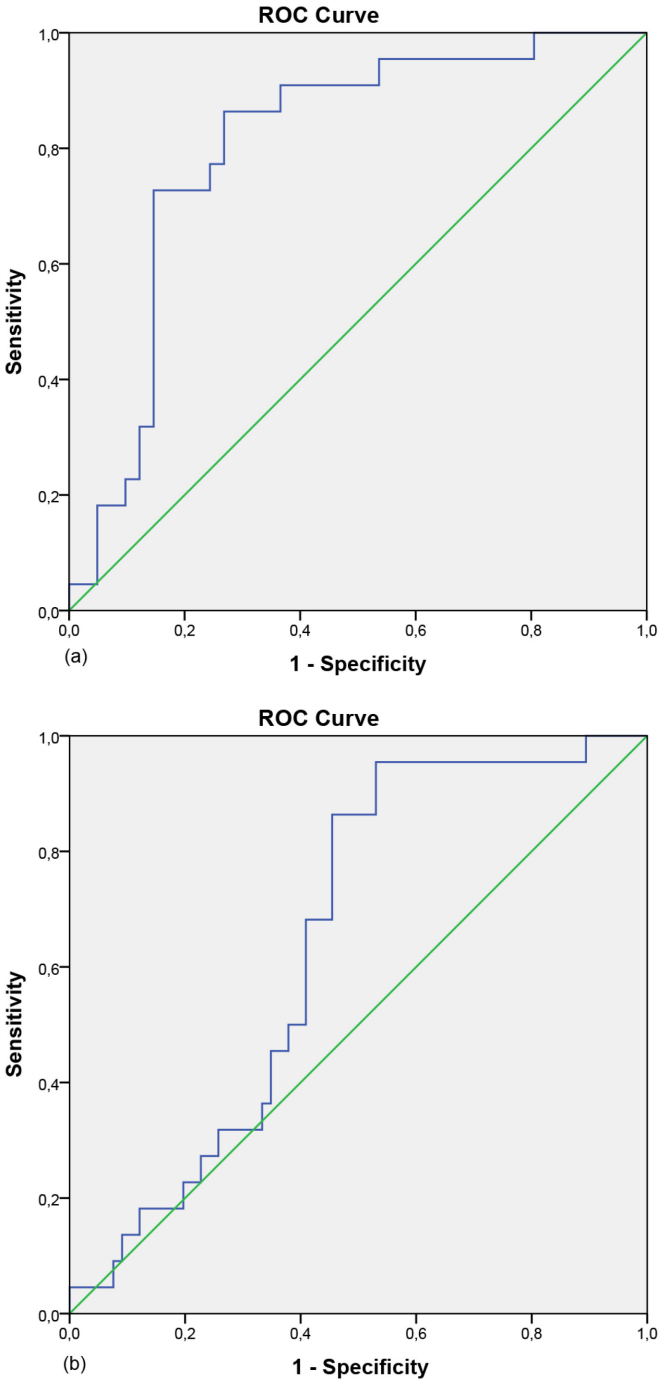
Data were analyzed using SPSS 22 (SPSS Inc, Chicago, IL, USA) software program. Visual methods (histogram and probability plots) and Kolmogorov-Smirnov test were used to determine whether the variables were normally distributed or not. Variables were compared using Mann-Whitney U test. Pearson's Chi-Square or Fisher's Exact test was used for qualitative variables. STAT was accepted as the reference diagnostic method for confirmation of brucellosis. The performance of *Brucella* ELISA IgM and/or IgG test in predicting brucellosis was evaluated by receiver operating characteristic (ROC) curve analysis. ROC curve analysis determined significant index values of the test and sensitivity, specificity, negative predictive value (NPV) and PPV were investigated; *p* values under 0.05 were accepted as statistically significant results.

Results

Out of 117 patients (*Brucella* ELISA IgM and/or IgG positive) between the ages of 9 and 83 who were included in the study, 62% were male. Median age of the patients was 41 (IQR [Interquartile range]: 27-54.5) years. *Brucella* infection (≥ 1:160) was confirmed in 22 (19%) out of 117 patients with STAT. STAT titers were between 1:20 and 1:1280. Distribution of STAT titers in patients with brucellosis was showed in Table 1.

ELISA-IgM serum index median value was 4.73 (IQR: 2.75-5.88) in STAT positive group (≥ 1:160) and significantly higher compared to the brucellosis negative group (0.84, IQR: 0.16-1.53) ($p < 0.001$).

Figure 1. The receiver-operating characteristic (ROC) curve of ELISA IgM (a) and IgG (b) for prediction of brucellosis.



The area under the ROC curve (AUC) was 0.800 (95% CI: 0.686-0.915) ($p < 0.001$) (Figure 1a); The area under the ROC curve (AUC) was 0.647 (95% CI: 0.528-0.767) ($p = 0.04$) (Figure 1b).

Table 1. Distribution (in percentage) of titers of standard tube agglutination test (STAT) in brucellosis patients.

Titers	< 1:20	1:20	1:40	1:80	1:160*	1:320	1:640	1:1280
STAT (%)	32.5	38.5	8.5	1.7	9.4	3.4	4.3	1.7

* Titer values 1:160 and above were accepted as positive.

ELISA-IgG serum index median value was 11.82 (IQR: 8.69-16.71) in STAT positive group (STAT ≥ 1:160) and significantly higher compared to the STAT negative group (index median value: 3.40, IQR: 0.087-13.04) ($p < 0.001$).

Significant index value and performance of *Brucella* ELISA IgM and IgG test in predicting *Brucella* STAT results (≥ 1:160) for the diagnosis of brucellosis were assessed with ROC analysis.

For *Brucella* ELISA IgM, sensitivity, specificity, PPV and NPV were 85.7%, 71.4%, 60%, and 90.9%, respectively in index value of 2.44 with ROC analysis. The area under the ROC curve (AUC) was 0.800 (95% CI: 0.686-0.915) ($p < 0.001$) (Figure 1a).

For *Brucella* ELISA IgG, sensitivity, specificity, PPV and NPV were 85.7%, 53.7%, 36.7%, and 92.3%, respectively in index value of 7.85 with ROC analysis. The area under the ROC curve (AUC) was 0.647 (95% CI: 0.528-0.767) ($p = 0.04$) (Figure 1b).

Performance results of *Brucella* VirClia test kit in different index values were presented in Table 2.

Discussion

STAT is the most commonly used serological test in confirmation of human brucellosis. Seroconversion or detection of high antibody titers (≥ 1:160) can be accepted as diagnostic with a clinical presentation [19]. The use of serological tests to detect specific IgG and IgM antibodies with ELISA in diagnosis of brucellosis has been common in recent years [20]. The results of IgG and IgM antibodies must carefully be interpreted [17,21]. IgM antibodies specific to *Brucella* are produced in the first week after the onset of the disease and reach maximum level in the third month. Sometimes, it does not become negative for a long time or a few years. Presence of specific IgM is accepted as the indicator of acute or new infection. However, detection of IgM antibody in the absence of IgG can cause misdiagnosis of brucellosis [15,22,23]. In the presence of other diseases and additionally rheumatoid factor, IgM antibodies can be detected due to cross reactivity [24]. On the other hand, IgG antibodies are detected after the second month of the infection and reach the highest level after 6-8 weeks. They become negative a short time after recovery. IgG is an activation marker in brucellosis [22,23].

In this study, index values of specific IgG antibodies developing against *Brucella* were determined and reported as semi-quantitative values. IgG index median value of STAT positive (≥ 1:160) group was significantly higher compared to STAT negative group. High *Brucella* IgG index values were associated with *Brucella* STAT (≥ 1:160). This result was consistent with those in previous studies [25,26].

Although there are studies on prediction of sensitivity and specificity of ELISA cut-off value in diagnosis of brucellosis studies on prediction of STAT positivity are quite limited. Brucella IgM and IgG positivity is not always a marker of acute brucellosis and its negativity does not exclude the disease [20]. In clinical routine, it is interesting to define the best ELISA cut-off value before using *Brucella* STAT. In that way, false positive results with ELISA can be decreased. However, cut-off values can differ according to the kits and populations.

Dashti e*t al.* [25] investigated an optimal sample/cut-off value for ELISA with STAT test result in a brucellosis positive group and mean ELISA-IgG serum level in brucellosis positive group was 103.96 ± 11.08 IU/mL, and that level was significantly higher than the level of brucellosis negative group (69.10 ± 3.93 IU/mL). To differentiate brucellosis positive and negative groups, the area under ROC curve was 0.858 ($p < 0.001$). The highest sensitivity and specificity were detected with a cut-off value of 53 IU/mL for ELISA-IgG in diagnosis of acute brucellosis and that value was accepted as the best cut-off value. In that cut-off value, sensitivity, specificity, PPV, and NPV were 84.09%, 85.38%, 62.20%, and 94.90% respectively.

In a similar study performed in Iran, the optimal cut-off value for *Brucella* IgM and IgG was investigated with ROC curve analysis in study groups including 56 confirmed brucellosis cases and 126 controls. In order to differentiate between the cases and controls, the area under ROC curve was higher for IgG compared to IgM. According to the results of that study,

Table 2. Performance of the *Brucella* VirClia test according to index value.

Index points	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
***Brucella* IgM**				
1.27	95.2 (76.2-99.9)	21.4 (10.3-36.8)	37.7 (33.5-42.2)	90 (55-98.5)
2.44	85.7 (63.7-97)	71.4 (55.4-84.3)	60 (47.4-71.4)	90.9 (77.5-96.7)
3.9	66.7 (43-85.4)	85.7 (71.5-94.6)	70 (51.2-83.9)	83.7 (73.5-90.5)
***Brucella* IgG**				
1.91	95.2 (76.2-99.9)	11.9 (5.3-22.2)	25.3 (22.9-27.9)	88.9 (51.5-98.4)
7.85	85.7 (63.7-97)	53.7 (41-66)	36.7 (29.8-44.2)	92.3 (80.4-97.2)
12.15	52.4 (29.8-74.3)	62.7 (50-74.2)	30.6 (20.9-42.4)	80.8 (72.1-87.2)

ELISA IgG test was more reliable than ELISA IgM test in diagnosis of human brucellosis in Iran. Use of a cut-off value of 10 IU/mL and 50 IU/mL for ELISA IgG test had the highest sensitivity (92.9%) and specificity (100%) respectively [26].

In this study, the optimal index value providing the sum of maximum sensitivity and specificity for IgM test was 2.44 and AUC was 0.800 (95% CI: 0.686-0.915) with ROC curve analysis, while the optimal index value for IgG test was 7.85 and AUC was 0.647 (95% Cl: 0.528-0.767) with ROC curve analysis ($p < 0.001$ and $p = 0.04$, respectively). Results under optimum index value should be re-tested with another ELISA kit. Determining the index value for *Brucella* test kits can be clinically important to predict true *Brucella* infection.

The lack of performance comparison with ELISA and blood culture (BC) results might be considered as a limitation. However, all samples included to this study were from outpatients and BC samples were not obtained. Even though future studies might require BC confirmation, it is stated that prolonged infections may cause a culture-negative status. Furthermore, a previous study indicated poor correlation between STAT and BCs results [27]. Thus, we believe that evaluation of ELISA performance may be enhanced in combination with BCs and STAT results.

Conclusions

In this study, it was revealed that Vircell *Brucella* test kit had a good clinical diagnostic performance for index value of 2.44 for IgM test kit and 7.95 for IgG test kit. If the diagnosis of brucellosis is correctly predicted with index values in *Brucella* IgM and IgG tests before STAT analysis they can be used in the process of clinical decision. In addition to the results of *Brucella* ELISA, reporting index values and determining optimal index values for each laboratory can help the diagnosis of brucellosis.

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