Brucellosis is a systemic disease caused by bacteria of the genus *Brucella* that affects humans and numerous animal species. It has been a great concern for many countries, especially those in the Middle East and Mediterranean regions, in terms of public and animal health. Human brucellosis infections can be unpredictable, with periods of chronicity, re-infection, and relapse. A wide spectrum of clinical manifestations makes clinical diagnosis difficult, and thus it is necessary to confirm brucellosis infection by thorough laboratory diagnosis (1,2).

Laboratory diagnosis of human brucellosis is based on the isolation of *Brucella* spp. from blood cultures and on the demonstration of the presence of specific antibodies through the use of serological tests. Although automated culture systems have reduced the growth time of *Brucella* genus bacteria to 1 week, conventional methods require nearly 4 times longer, and have a very low success rate (only 10–70%) (3). Laboratory diagnosis of brucellosis is frequently based on demonstration of the presence of serum antibodies, and various laboratory tests are used for this purpose. The Rose Bengal (RB) test is commonly used to screen for brucellosis infections, but it has been suggested that the results of RB should be verified by other tests (4). The standard agglutination test (SAT), which was developed by Wright and colleagues in 1897 in order to detect total antibodies, is the most frequently used test to diagnose brucellosis. If the SAT test yields negative results due to the presence of blocking antibodies, the Coombs’ test may be used instead.

Brucellosis can manifest as 3 different clinical types, which are classified according to the duration of symptoms: acute (initial 2 months), subacute (2–12 months), and chronic (>12 months). The disease may also be asymptomatic, subclinical, and focal, or present with complications, relapses, and re-infections. The 2-mercaptoethanol (2-ME) test can be used to predict the course of the disease (5). Specific antibody classes (IgM, IgG, and IgA) can be demonstrated by enzyme immunoassay (EIA) and immunochromatographic lateral flow assay. Although all these tests may be implemented to evaluate the different clinical forms and development stages of the disease, in most cases they may not yield definite results, and the stage of the disease may not be determined serologically (1).

In some viral and parasitic diseases, it is possible to determine whether the disease is acute or chronic, and at which stage the disease is in, through implementation of the IgG avidity test, which is based on maturing of antibody affinity. The avidity test is not used routinely as a marker of acute brucellosis; however, this test may be used to differentiate between recent infections and previous infection. In addition, some studies have revealed promising results showing that the avidity test can be further developed for the evaluation of brucellosis infections (6).

In this study, our aim was to compare serological tests (RB, SAT, EIA, 2-ME) that are routinely used in patients prediagnosed with different types of brucello-
sis, and to evaluate these results using the IgG avidity test.

MATERIALS AND METHODS

The present study was conducted on patients pre-diagnosed with brucellosis, and followed up by the İzmir Atatürk Education and Research Hospital and Manisa State Hospital Infectious Diseases Polyclinic in Turkey. A diagnosis form was created for each patient whose disease course was known, and these were classified as acute, subacute, or chronic. For all patients, blood sera were analyzed by RB and SAT using the Brucella S99 strain according to the recommendations of the manufacturer (Seromed, Istanbul, Turkey). Eligible patients were required to have a titer ≥ 1/160 in the SAT test to confirm the serological diagnosis of brucellosis, and sera from 92 patients meeting this criterion were included in the study. Coombs’ test was performed on the sera from 2 patients who tested negatively in the SAT test, in order to remove blocking antibodies and/or eliminate the prozone phenomenon. These sera were included in the study (7). All sera were subjected to the 2-ME test using 0.05 M 2-ME (8). A decreased titer in the 2-ME test compared to that of the SAT was considered positive in terms of IgG antibody presence. All these tests were evaluated by at least 2 microbiologists. The sera were then stored at −20°C in Eppendorf tubes for later testing in the EIA and IgG avidity tests. After all the patient samples were collected, the presence of anti-Brucella IgM, IgG, and IgA antibodies was determined using commercial EIA kits and equipment (Radim, Rome, Italy). Results were considered either negative or positive on the basis of the calculated cut-off values. When EIA test results conflicted with SAT results, both the SAT and EIA tests were repeated.

The avidity test was performed on the basis of the 8 M urea denaturation method described by Hedmen and Rousseau (9). The IgG avidity test was performed on sera obtained from 78 patients who tested positive for IgG antibodies; the test was performed using the procedures, EIA anti-Brucella IgG kit, and equipment previously mentioned. Sera were diluted 1:100, and 1 pair from each was added to the well. After initial incubation and washing, 100 μl of 8 M urea was added to one of the serum pairs, and 100 μl of phosphate buffer solution was added to the other. Sera were then incubated for 5 min. The remaining steps were performed according to the manufacturer’s recommendations, and absorption of all the wells was measured at 450 nm. Avidity index percentage was calculated according to the following formula:

\[
\text{IgG avidity index (AI)} = \frac{\text{absorbance of urea-treated microwells}}{\text{absorbance of untreated microwells}} \times 100
\]

The AI cut-off value was set 40%, and the maturation time (the time required for evolution of low-avidity antibodies into high-avidity antibodies) was 6 months (1,5).

Data were analyzed using SPSS 15.0 software (SPSS, Chicago, Ill., USA). Pearson’s chi-square test was used to evaluate the avidity tests, and the McNemar test was used to evaluate the degree of agreement between the other serological tests. A P value ≤ 0.05 was considered statistically significant.

RESULTS

Of the patients in the study, 45 (48.9%) were males, while 47 (51.1%) were females. The average age of the study participants was 39.2 years, with patients ranging in age from 23 to 68 years. In the SAT, results revealed titters ranging from 1/160 to 1/1,280. Ninety-two patients were classified into 1 of 3 groups based on the duration of their symptoms, and the number of acute, subacute, and chronic patients was 42 (45.7%), 40 (43.5%), and 10 (10.9%), respectively. Distribution of tests according to the type of brucellosis is described in Tables 1 and 2.

The RB test was negative in 4 patients, and the agreement between the SAT and RB tests was calculated as 95.7%. In 3 (3.2%) of the patients, all EIA tests were negative. When EIA tests were evaluated according to the clinical types, a statistically significant correlation was found only between the presence of IgM antibodies and acute-stage brucellosis (\(P = 0.01\)). When EIA tests were also evaluated in combinations of 2, there was no statistically significant correlation between the combined antibody tests and the clinical groups.

In 53 (57.6%) patients, SAT titer was 1/160, which showed the highest ratio. The ratios of the other SAT titers, 1/320, 1/640, and 1/1,280, were 23.9, 8.7, and 9.5, respectively (Tables 2 and 3).

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6 months and 50 maturation time and AI cut-off value were evaluated at a high value. For 40 (51.3% of these 78 patients, a statistically significant correlation with the Sat test titres was obtained when the AI cut-off value was 40, 50, or 55.

We found 95.7% agreement between the RB and SAT tests, revealing that RB is a valuable, easy-to-use, and cost-effective screening test that can provide reliable results in a relatively short time. Although studies on this subject have yielded different results, those obtained by comparing the RB test with the SAT test have demonstrated results similar to ours (10). Some studies have also reported 100% sensitivity for the RB test (11,12). However, it has been shown that false negatives could be due to a decrease in the agglutination ability of antibodies at the low pH of the test medium (13). While the RB test yields results within minutes, a minimum of 24 h is needed for the SAT test. This increases up to 48 h when Coombs’ test needs to be used to clarify negative results. Unfortunately, evaluation of these tests involves a level of subjectivity, and this may cause inexperienced persons to report inaccurate results.

Evaluation of individual EIA tests in patient groups classified according to symptoms showed that only one immunoglobulin (i.e., IgG) can be used successfully, with a 100% agreement with the SAT test, during the chronic period. Although having a small number of patients classified as chronic (10 patients) reduces the strength of the study, similar results were found in a study by Mantecón et al., where 100% IgG was detected in 22 patients having a history of brucellosis (1). Sirmatel et al. found the percentage of IgG-positive patients to be 78.2% in a group composed of 92 chronic-stage patients (14). In regions where brucellosis is endemic, persistent IgG positivities may reduce the diagnostic value of this test when it is used individually. When evaluating the ratios of IgG-positive patients during the acute, subacute, and chronic periods of brucellosis (76.2, 90, and 100% respectively), it appears that IgG increases according to the duration of the disease. In the panel of EIA tests, IgM was the only immunoglobulin to show a statistically significant correlation with the clinical types of brucellosis (P = 0.01), suggesting that IgM can be used to diagnose acute brucellosis. The same result has been obtained in many studies on EIA. Klerk and Anderson reported that EIA-IgM is the “most sensitive test” in the serological diagnosis of brucellosis (15). Furthermore, Ariza et al. showed that a high level of specific IgM was present at the beginning stages of brucellosis, and that it decreased much more rapidly than IgG or IgA (16). The rapid decrease shown by Ariza et al. is also present in our results; number of EIA-IgM-positive patients during the acute, subacute, and chronic periods was found to be 32, 16, and 3, respectively. Since positivity is not an issue in all patients having brucellosis, it is not possible to exclude acute

Table 4. Comparison of 2-ME and IgM

<table>
<thead>
<tr>
<th></th>
<th>IgM</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Positive (%)</td>
<td>Negative (%)</td>
<td>Total (%)</td>
</tr>
<tr>
<td>Decreased titer in 2-ME test</td>
<td>Positive (%)</td>
<td>Negative (%)</td>
<td>Total (%)</td>
</tr>
<tr>
<td>Low</td>
<td>46 (90.2)</td>
<td>9 (22)</td>
<td>55 (59.8)</td>
</tr>
<tr>
<td>High</td>
<td>5 (9.8)</td>
<td>32 (78)</td>
<td>37 (40.2)</td>
</tr>
<tr>
<td>Total (%)</td>
<td>51 (55.4)</td>
<td>41 (44.6)</td>
<td>92 (100)</td>
</tr>
</tbody>
</table>

Table 5. Distribution of different AIs according to disease duration

<table>
<thead>
<tr>
<th></th>
<th>≤6 months (%)</th>
<th>&gt;6 months (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI 40% Low</td>
<td>27 (77.1)</td>
<td>8 (22.9)</td>
<td>35 (44.9)</td>
</tr>
<tr>
<td>High</td>
<td>30 (69.8)</td>
<td>13 (30.2)</td>
<td>43 (55.1)</td>
</tr>
<tr>
<td>AI 45% Low</td>
<td>39 (78)</td>
<td>12 (25)</td>
<td>51 (64.1)</td>
</tr>
<tr>
<td>High</td>
<td>18 (64.3)</td>
<td>10 (35.7)</td>
<td>28 (35.9)</td>
</tr>
<tr>
<td>AI 50% Low</td>
<td>24 (79.3)</td>
<td>7 (20.7)</td>
<td>31 (47.1)</td>
</tr>
<tr>
<td>High</td>
<td>10 (50)</td>
<td>6 (30)</td>
<td>16 (26.3)</td>
</tr>
<tr>
<td>AI 55% Low</td>
<td>20 (77.3)</td>
<td>5 (22.7)</td>
<td>25 (37.5)</td>
</tr>
<tr>
<td>High</td>
<td>6 (50)</td>
<td>6 (50)</td>
<td>12 (15.4)</td>
</tr>
</tbody>
</table>

We found 95.7% agreement between the RB and SAT tests, revealing that RB is a valuable, easy-to-use, and cost-effective screening test that can provide reliable results in a relatively short time. Although studies on this subject have yielded different results, those obtained by comparing the RB test with the SAT test have demonstrated results similar to ours (10). Some studies have also reported 100% sensitivity for the RB test (11,12). However, it has been shown that false negatives could be due to a decrease in the agglutination ability of antibodies at the low pH of the test medium (13). While the RB test yields results within minutes, a minimum of 24 h is needed for the SAT test. This increases up to 48 h when Coombs’ test needs to be used to clarify negative results. Unfortunately, evaluation of these tests involves a level of subjectivity, and this may cause inexperienced persons to report inaccurate results.

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Table 3. SAT results according to titers and distribution of positive RB and EIA tests according to SAT titres

<table>
<thead>
<tr>
<th></th>
<th>1/160</th>
<th>1/320</th>
<th>1/640</th>
<th>1/1,280</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB</td>
<td>49 (92.5)</td>
<td>22 (100)</td>
<td>8 (100)</td>
<td>9 (100)</td>
<td>88 (95.7)</td>
</tr>
<tr>
<td>IgM</td>
<td>24 (47.1)</td>
<td>12 (54.5)</td>
<td>6 (75)</td>
<td>9 (100)</td>
<td>51 (55.4)</td>
</tr>
<tr>
<td>IgA</td>
<td>30 (56.6)</td>
<td>17 (77.3)</td>
<td>8 (100)</td>
<td>9 (100)</td>
<td>64 (69.6)</td>
</tr>
<tr>
<td>IgG</td>
<td>44 (83)</td>
<td>20 (90.9)</td>
<td>6 (75)</td>
<td>8 (88.9)</td>
<td>78 (84.8)</td>
</tr>
</tbody>
</table>

1: Ratios in SAT titers.
2: Ratios in total.

be 84.8% (Table 4). In the 2-ME test, 72 patients were found to be positive, despite decreased titers.

In the IgG avidity test, 35 (44.9%) of 78 EIA-IgG-positive patients had low AI values, while 43 (35.1%) had high AI values. Forty (51.3%) of these 78 patients were both EIA-IgG- and EIA-IgM-positive. When the IgG avidity maturation time was set at 6 months, and the AI cut-off value was 40%, there was no statistically significant correlation between cut-off value and maturation time (P = 0.465). When the AI cut-off value was set at 45, 50, or 55, P values were calculated to be 0.190, 0.035, and 0.050, respectively. Increasing maturation time by 1 month also did not reveal any statistically significant correlation. The lowest P value associated with these 2 parameters was obtained when the maturation time and AI cut-off value were evaluated at 6 months and 50%, respectively (Table 5).

**DISCUSSION**

In our study, RB test was found to be positive in 88 of the 92 patients whose SAT tests showed titers ≥ 1/160.
brucellosis only on the basis of negative results. EIA-IgG and IgA are not sufficient to make a diagnosis during the acute and subacute periods. These should be taken into consideration, along with the other tests, and should definitely be tested in cases where negative EIA-IgM results are found in acute period patients. EIA-IgM can be detected as early as the first week after brucellosis infection, while IgG and IgA only appear after a couple of weeks (17). Therefore, these specific immunoglobulins cannot be reliably used during certain disease phases. In fact, it is uncertain whether there is value in such classifications for brucellosis, since it is a disease that eventually evolves into a chronic state.

In 3 of the patients, all EIA tests were negative. Performance in EIA tests varies in different studies. Memish et al. found the sensitivities of SAT, EIA-IgG, and EIA-IgM to be 95.6, 45.6, and 79%, respectively (17), while Sisirak and Hukic reported sensitivities as low as 64.8% for EIA-IgM and 56.1% for EIA-IgG (11). In our country, Sirmatel et al. reported sensitivities of SAT, EIA-IgM, and EIA-IgG to be 83.7, 61.9, and 49.5%, respectively (14). However, some studies have reported very high sensitivities for EIA tests. For example, Araj et al. reported sensitivities of 91 and 100% for EIA-IgG and EIA-IgM, respectively (18). Similarly, in a study comparing blood cultures in various serological tests, Ciftci et al. calculated the sensitivities of RB, SAT, EIA-IgG, EIA-IgA, and EIA-IgM tests as 100, 94.3, 97.1, 94.1, and 71.4%, respectively (19). Our results have shown that EIA tests should be evaluated together, especially during the acute and subacute stages, and an evaluation using only a single immunoglobulin may cause false-negative results.

Although no statistically significant correlation has been found between EIA and SAT titers, nearly all patients having a SAT titer of 1/1,280 had positive results in EIA; in fact, only 1 patient was found to be negative in the EIA-IgG test. The number of IgA-positive patients increased roughly in parallel to the SAT titer values, with the $P$ value close to the significance threshold ($P = 0.07$). Since IgA titers increase with increased antigenic stimulation, patients having 1/1,280 and 1/640 titers were found to have a greater number of antibodies. Disagreement between IgM and IgG in the first 2 titers could be due to the delay in switching from IgM to IgG caused by antigenic stimulation. White demonstrated that such a delay was possible (20). The presence of IgM was revealed when comparing the decrease in titers of the SAT test and the 2-ME test, when the latter was performed in sera with total antibody activity shown. This decrease was observed in 55 (59.8%) patients. When compared with results from the EIA-IgM test, agreement was found to be 84.8%. This suggests that the 2-ME test can be reliably used to determine the presence of IgM. Buchanan and Faber reported that a negative 2-ME test is “strong evidence” of chronic brucellosis (21). In a study evaluating 50 sera by flow assay, Zeytinoglu et al. found a similar agreement between the EIA-IgM and 2-ME tests, with a ratio of 88% (22).

According to the results of the present study, 6 months avidity maturation time and 50% AI were found to be the best cut-off values for the avidity test. Under these conditions, AI values from 45–50% may be within the grey zone. Further studies investigating other parameters that may affect the avidity test should be performed with varied sets of parameters under controlled conditions, in order to determine the most appropriate criteria for application of this test. The other 2 parameters that can be changed in this test are the urea concentration and treatment time. Based on previous studies, we choose to use 8 M urea applied for 5 min. These conditions may vary according to the specific avidity test to be performed. However, in the diagnosis of brucellosis, these conditions have not yet been standardized. In some studies, successful results have been reported for the diagnosis of Brucella based on IgG avidity test. In a study of 188 patients, Kutlu et al. found a statistical correlation between AI and maturation time when the cut-off values for these parameters were 40% and 6 months, respectively. Furthermore, when maturation time was set at 6 months, Gutierrez et al. reported the sensitivity and specificity of the IgG avidity test to be 70.5 and 97.7%, respectively (6). Montecón et al. found significantly high Brucella IgG avidity in a group having a history of brucellosis, and reported that the IgG avidity test could differentiate between current and prior infections (1).

In conclusion, from a broad perspective, no perfect test is available for the laboratory diagnosis of brucellosis. The RB test remains an important and appropriate screening test. Furthermore, we believe that the SAT test, which is cost-effective, easy-to-use, and has high sensitivity and specificity, is the most appropriate test, especially in regions endemic for brucellosis.

The ability to measure 3 specific antibodies by EIA makes this an effective test that can be used to successfully diagnose brucellosis. This is especially important, since it may be possible to use these laboratory tests to confirm the clinical stage of the disease once the presence of IgM is apparent during the acute stage. Likewise, there may be similar value in the presence of IgG during the chronic stage. To differentiate between these 2 antibodies, the 2-ME test is a cheap and easy-to-use alternative that yields sufficient results. Additional studies aimed at further developing and standardizing the IgG avidity test may significantly improve the clinic diagnosis of brucellosis.

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Conflict of interest None to declare.

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185–195 (text in Turkish with English summary).


