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Epidemiology and the Agreement Rate of Serological Tests in Human Brucellosis in North East of Iran

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Background: Brucellosis still remains a major health problem with different symptoms and various diagnostic methods. Diagnostic methods of brucellosis are usually based on detecting specific antibodies in the patient's serum. Nowadays, many serological tests are applied for the diagnosis of human brucellosis. Most routine tests are serum agglutination tests based on Wright and 2-Mercaptoethanol (2-ME).

Objectives: The aim of this study (cross sectional study) was to evaluate the prevalence of brucellosis and assess the degree of agreement among serum samples of suspected brucellosis serological tests routinely performed in Mashhad, Iran.

Patients and Methods: This study was conducted in Mashhad from August 2011 to September 2012. Sera (2 - 3 mL) were collected from 83 cases suspected of brucellosis among 594 patients. Ten serum samples were collected from healthy subjects as control sera. Rose Bengal test for initial screening and Wright and 2 ME as standard tests were conducted to determine antibody titers. Thereafter, IgG and IgM levels were determined by the Enzyme Linked Immunosorbent Assay (ELISA) method.

Results: Among 83 serum samples, Rose Bengal test was able to identify 20 (12%) positive specimens; the standard tube agglutination test was able to detect 30 (18%) positive samples, and the ELISA IgG and ELISA IgM were able to trace 42 (21%) and 13 (6.5%) positive samples, respectively. Ten control samples had negative results for the ELISA method. The results were calculated by the Kappa formula. The highest level of agreement was among 1 = KRB-SAT tests and the lowest level of agreement was among tests $K_{ELISA\ IgM\text{-}IgG} = 0.30$.

Conclusions: According to the results, brucellosis has remained endemic in this region. Most cases were detected by ELISA IgG. The highest kappa agreements were between tests KRB-SAT, $K_{RB\text{-}IgG}$ and $K_{SAT\text{-}IgG}$, while the lowest levels of agreement were between tests SAT-IgM and ELISA IgM-IgG. Considering that ELISA IgM results are covered by SAT and ELISA IgG test results, applications of this test do not seem necessary.

Keywords: 2-Mercaptoethanol; Enzyme-Linked Immunosorbent Assay; Agglutination; Brucella

1. Background

Brucellosis is a zoonotic infection and systemic disease in humans and a wide range of animals. The causative agents are the bacteria of the genus Brucella, which are spread throughout the world (1, 2). Brucellae are gram-negative and non-motile bacteria, capable of intracellular growth. Brucellosis involves a large scale of organs in humans, which are associated with diverse and non-specific clinical signs and lead to numerous complaints from patients, such as drowsiness and anorexia, malaise, excessive sweating, weight loss, back pain or arthritis and depression. Sometimes physical findings, especially fever and lymphadenopathy, mild hepatomegaly and splenomegaly are reported in patients and these nonspecific symptoms make clinical diagnosis difficult. Accurate diagnosis requires paraclinical approaches. Diagnostic methods of brucellosis are usually based on detecting specific antibodies in the patient's serum. Nowadays many serological tests are applied for the diagnosis of human brucellosis (3, 4), Most routine tests are serum agglutination tests based on Wright and 2-Mercaptoethanol (2-ME), which were developed in 1897 by Weight et al. (5) and nowadays they are still used as reference tests in clinical laboratories. Other tests, which were developed later, include Rose Bengal, complement fixation, indirect coombs and the Enzyme Linked Immunosorbent Assay (ELISA) (6). Rose Bengal Test (RBT) is a simple screening test used in endemic areas, which may also lead to false-positive results. Therefore, in these regions Serum Agglutination Tests (SAT) are used to reduce false-positive results caused by Rose Bengal (7, 8). Agglutination test titers in most patients with acute brucellosis showed 1/320 or more at the end of the second week of disease, where

in 20% of patients these titers remain significantly high after one year of treatment. Higher levels of agglutinin titer for Brucella have been reported in patients with *Francisella tularensis* and *Yersinia enterocolitica,* and in patients who have recently received a vaccination of cholera or have been tested by the Brucella skin test. The ELISA test is highly capable of tracking IgG and IgM cross-reacting antibodies (9).

2. Objectives

Since brucellosis has multiple phases (acute, sub-acute, chronic and disabling horoscope), searching for a reliable diagnostic method especially to monitor this ongoing disease is in progress. In this study the agreement between routine serological tests for brucellosis diagnosis (RB, SAT, ELISA) was measured and the results are reported.

3. Patients and Methods

This study (cross sectional study) aimed to assess the level of agreement between serological tests that detect brucellosis. It was conducted at the Kenevics district of Mashhad, Iran from August 2011 to September 2012 on 83 serum samples gathered from patients (594 cases) with clinical symptoms of brucellosis. The majority of these patients were from rural areas. Rose Bengal test was carried out using an antigen kit from the Pasteur Institute of Iran. Next, serum agglutination tests based on Wright and 2-ME were carried out on samples and antibody titers were determined. Wright serum dilutions from 1/20 to 1/320 were prepared and 0.5 mL of *Brucella abortus* antigen solution (antigenic kit from Pasteur Institute of Iran) was added to these dilutions and incubated for 24 hours at 37°C. The last tube with agglutination was considered as positive. Wright samples with titers of 1/160 or higher and 2-ME samples with titers of 1/80 were considered and reported as positive. In many reference books, this titer is recommended as a positive (10). The 2ME test was conducted in the same way as the Wright test yet with one difference, which was that the antigenic solution was mixed with 2ME to break the chemical bonds of IgM. This test is able to remove the remaining IgM and IgG, and therefore this test is capable of differentiating between acute and chronic phases of the disease (11). The ELISA IgG and IgM tests were performed on all serum samples to differentiate acute and chronic phases of the disease (using the Vircell-Granada kit, SPAIN). The test was carried out using the sandwich method according to the manufacturer's instructions. Initially, antibodies in the serum of patients were bonded to antigens present in the wells, and after the washing step, unbounded antibodies were excluded. In the second stage anti-human globulins were added to Ag-Ab complexes formed in the previous step with the enzyme. After an additional washing step, the bonded conjugate would expand by means of adding a substrate solution. A blue solution was created that changed to yellow after the addition of the stop solution. The intensity of the generated color, which was proportional to the amount of antibodies, was read at a wavelength of 450 nm by the BioTek ELX800 plate reader. Values less than 9 U/mL were considered to be negative, between 9 - 11 U/mL indicated ambiguous results and values higher than 11 U/mL were considered as positive results. Ten serum samples from healthy individuals were used as the control of the ELISA test. Calculations were performed and the degree of agreement among tests was determined. Kappa agreement coefficients were used to conduct this evaluation. The statistical analysis was done by the Prism software, version 5.0.

4. Results

Amongst 594 patients with clinical symptoms of Bordellos from the Kenvics Heath Center, 83 cases were considered as positive and suspicious of Brucella by the 2-ME and Wright tests. Among these subjects (83 cases), 34 patients were male and 49 were female between the ages of 30 and 50 years. Most of the subjects lived in rural areas, and thus had a history of contact with animals or their products. Of all 83 serum samples, Rose Bengal test was able to detect 20 (12%) positive samples; the Standard Tube Agglutination test was able to identify 30 (18%) positive samples and ELISA IgG could detect 42 (21%) positive samples, mostly with high titers of IgG antibody (25 to 35). The ELISA IgM was able to trace 13 (6.5%) positive samples while the 10 control samples had negative ELISA results. The results were calculated using the Kappa formula (Tables 1 and 2).

Table 1. The Results Calculated Using the Kappa Formula

Kappa Statistic	Result
$K_{\text{ELISA IgM-IgG}}$	0.30
$K_{\text{SAT- IgG}}$	0.64
$K_{\text{RB- IgG}}$	0.64
$K_{\text{SAT- IgM}}$	0.54
$K_{\text{RB- IgM}}$	0.54
$K_{\text{RB-SAT}}$	1

Table 2. Interpretation of Kappa Results

Poor	Slight	Fair	Moderate	Substantial	Almost Perfect
0.0	20	40	60	80	1.0
Less than < 0 chance agreement	0.01 - 0.20 Slight agreement	0.21 - 0.40 Fair agreement	0.41 - 0.60 Moderate agreement	0.61 - 0.80 Substantial agreement	0.81 - 0.99 Almost perfect agreement

5. Discussion

According to the results, brucellosis is still endemic in this region. In the Kappa formula, six modes were considered for all tests and each of the two tests were compared with each other. The results were less than one;, the closeness of this value to one indicates the degree of agreement between the two tests. The results showed that the level of agreement between Rose Bengal test and Serum Agglutination Test (SAT) was one (k = 1), which confirms that there is reasonable consistency between the two tests. Rose Bengal test could detect 20 positive samples out of 83 suspicious sera whereas; tube Serum Agglutination Tests detected 30 positive sera. The disputes that occurred in identification of positive samples between Rose Bengal and SAT might be due to the *prozone phenomenon* in the Rose Bengal slide tests and subsequently observation of false negative results. Therefore, it is better to accomplish tube agglutination test along with Rose Bengal slide test. After that, the best compatibility was demonstrated between Rose Bengal tests-IgG and SAT-IgG (K = 0.64). The advantage of using the ELISA method over the agglutination tests is that it prevents the complexities of interpreting the results of the SAT obtained by blocking antibodies (12). In this study, it was shown that ELISA IgG Could be responsible instead of both Rose Bengal and serum agglutination tests, and was able to identify positive samples with high titers, which were negative in the two aformentioned tests. these samples were also negative using Coombs'-Wright test and these tests do not have enough accuracy in screening chronic brucellosis (13). These results were consistent with the study of Rajai et al. who detected more positive cases using ELISA IgG, than that detected by SAT (14). In most studies due to the high sensitivity of this test it is referred to as the diagnostic test of choice. Results of the ELISA IgG could be due to the absence of active disease or occupational disease. However, this test might be solely useful for assessing response to treatments and its application along with the two other mentioned tests is of great value in detecting suspicious cases due to the high level of agreement between these tests. The lowest level of agreement was between SAT-IgM and ELISA IgM-IgG. According to the coverage of ELISA-IgM results by SAT and ELISA IgG tests the application of this test does not seem to be essential.

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Authors' Contributions

Study concept and design: Hadi Safdari and Hamid Sadeghian. Acquisition of data: Samaneh Saedi and Morteza Akhlaghi. Analysis and interpretation of data: Hadi Safdari and Hamid Sadeghian. Drafting of the manuscript: Saeed Mohammadi. Critical revision of the manuscript for important intellectual content: Ali Sadeghian. Administrative, technical and material support: Hadi Safdari and Hamid Sadeghian.

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