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Investigation of *Brucella* antibodies in bovine sera by rose bengal plate test (RBPT), serum agglutination test (SAT), microagglutination test (MAT) and 2-mercaptoethanol-microagglutination (2-ME-MAT) test

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Summary: In this study, for the investigation of *Brucella* antibodies a total number of 524 serum samples were sent to the Department of Microbiology, Faculty of Veterinary Medicine, Ankara University. For the determination of most appropriate test for the investigation, first 100 samples were investigated by rose bengal plate test (RBPT), serum agglutination test (SAT), microagglutination test (MAT) and 2-mercaptoethanol-microagglutination test (2-ME-MAT). Following this pre-investigation study, 3 (3%) and 5 (5%) samples were found to be positive with RBPT and SAT, MAT, 2-ME-MAT (same results were achieved with each of the three tests) tests, respectively. No statistically significant difference was observed amongst the later three tests ($p>0.05$). Because it has a short evaluation time and since it was found to be economical concerning the used expenditure and reagents, MAT was preferred as the most appropriate test for the serological investigation. Following MAT tests of 524 serum samples 8 (1.5%) samples were found to be positive concerning *Brucella* antibodies. As a conclusion, MAT was determined to be a fast, reliable and an economic test, which can be easily used in routine diagnostic laboratories for serological diagnosis.

Key words: *Brucella*, microagglutination, serology, serum agglutination

Sığır serum örneklerinde *Brucella* antikorlarının belirlenmesinde rose bengal plate test (RBPT), serum aglutinasyon test (SAT), mikroaglutinasyon test (MAT) ve 2-merkaptoetanol-mikroagglutinasyon (2-ME-MAT) testlerinin karşılaştırılması

Özet: Bu çalışmada, özel bir sığırcılık işletmesinden Ankara Üniversitesi Veteriner Fakültesi Mikrobiyoloji Anabilim Dalı'na gönderilen toplam 524 serum örneğinin ilk 100 örneğinde *Brucella* antikorlarının belirlenmesi amacıyla, Rose Bengal Plate Test (RBPT), Serum Aglutinasyon Test (SAT), Mikroaglutinasyon Test (MAT) ve 2-merkaptoetanol-Mikroagglutinasyon (2-ME-MAT) Testi uygulandı. Yapılan ön çalışma sonucunda RBPT ile 3 (% 3), SAT, MAT ve 2-ME-MAT ile 5 (% 5) örnekte pozitiflik saptandı. Son üç test ile elde edilen titreler arasında istatistiksel bir farklılığın olmadığı ($p>0.05$) belirlendi. Kullanılan sarf malzemesi ve reaktifler açısından ekonomik olması ve değerlendirme süresinin kısa olması nedeniyle örneklerin değerlendirilmesinde MAT testi tercih edildi. MAT testi ile 524 örneğin incelenmesi sonucunda 8 (% 1.5) örnekte pozitiflik saptandı. Sonuç olarak, MAT'ın serolojik teşhis yapan rutin laboratuvarlarında hızlı, kolay, güvenilir ve ekonomik bir test olduğu belirlendi.

Anahtar sözcükler: *Brucella*, mikroaglutinasyon, seroloji, serum agglutinasyon.

Introduction

Brucellosis is an important infection and not only causes economical loss in animals but also spreads with milk and milk products. This is important because of public health. A national program for control of the disease, based upon vaccination of young animals with a living vaccine strain (sheep and goats with *B. melitensis* Rev1 and cattle with *B. abortus* S19) and a test and slaughter policy for infected herds, has been widely implemented in Turkey since 1984. Conventional serological tests, e.g., the serum agglutination test (SAT), the complement fixation test (CFT), and the rose bengal plate test (RBPT), are the standard tests used in the laboratories of both veterinary research institutes and veterinary faculties as the diagnostic measures to identify foci of infection (1, 2, 7, 14).

The laboratory diagnosis of brucellosis is made primarily by serological tests because the organism is isolated by cultural methods in no more than 20% of the cases (14). The routine brucella agglutination test is the most frequently used type of serological test for this purpose; however, it does not differentiate between active and inactive disease because it does not differentiate between between IgG and IgM agglutinins. The IgM titer can be eliminated by treating the serum specimen with 2-mercaptoethanol (2-ME), which breaks the disulfide bonds and depolymerizes the IgM. Depolymerized IgM does not contribute to agglutination;

therefore, the titer is due primarily to 2-ME-resistant antibody (IgG) (5).

A method based upon measurement of agglutinating antibodies by a microplate agglutination test (MAT) has been reported (13). In a survey of bovine herds at the end of an eradication program, the MAT was found to be superior to the SAT in efficacy. Altogether, it was shown a suitable substitute for diagnosis of human, bovine, caprine, and ovine brucellosis (3).

In this study, for the investigation of *Brucella* antibodies a total number of 524 serum samples were sent to the Department of Microbiology, Faculty of Veterinary Medicine, Ankara University. For the determination of most appropriate test for the investigation, first 100 samples were investigated by RBPT, SAT, microagglutination test (MAT) and 2-mercaptoethanol-microagglutination test (2-ME-MAT) tests.

Material and Method

Material: A total of 524 serum samples obtained from 6 dairy herds of a commercial company with an abortion history sent to the Department of Microbiology, Faculty of Veterinary Medicine, Ankara University constit"uted the material of the study. Regarding to the anamnesis obtained from the local veterinarians of the company animals were vaccinated against brucellosis. First 100 sera were used to determine the most appropriate test for the investigation of the rest of the serum samples.

Negative and positive control sera: As negative control sera, sera from the herds without an abortion history and RBPT, SAT, MAT and 2-ME-MAT negative sera were used. As positive sera, bovine serum samples from positive sera archive of our laboratory which gave an (++) agglutination at a dilution of 1/80-1/640 was used.

Antigens: Antigens used in these tests were obtained from Pendik Veterinary Control and Research Institute.

Method: RBPT and SAT tests were performed following the instructions of the method reported by Alton et al. (1). A complete agglutination with the equal volume of test antigen was determined as positive result for RBPT. A 50% agglutination at a serum dilution of 1/80 or more was determined as a positive test result in the later 3 tests. MAT and 2-ME-MAT tests were modified from the method of Brown et al. (4). A working dilution of the antigen in a stained isotonic 0.5% phenol–saline solution which contained 0.02% safranin O (Sigma) was used. A primary serum dilution of 1:2.5 was further diluted in twofold steps in a 0.5% phenol–5% NaCl solution (4). Fifty microliters of both the antigen suspension and the serum sample dilutions per well were mixed together and incubated for 18 h at 37°C. Positive and negative sera were included as controls. To determine agglutination results, standard antigen suspensions simulating 100, 75, 50, and 25% agglutination levels were included. The MAT was performed in 96-well U-shaped microplates.

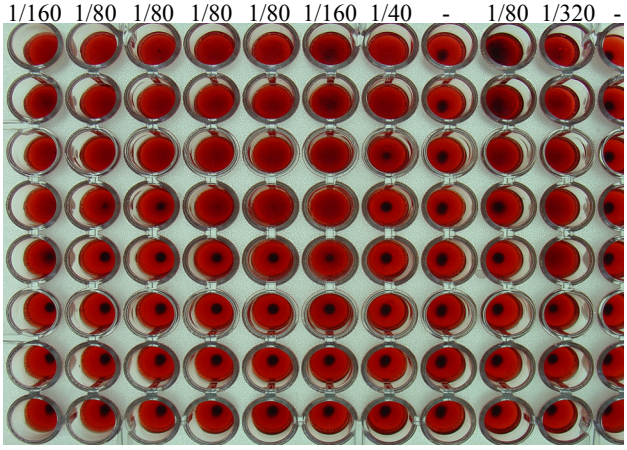

Figure 1. Microplate showing MAT results.
Şekil 1. Safranin O ile boyalı antijenin kullanıldığı MAT sonuçları.

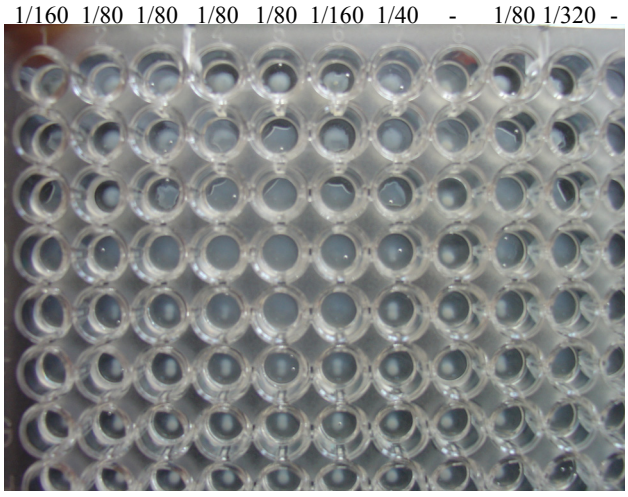

Figure 2. Microplate showing 2-ME-MAT results.
Şekil 2. 2-ME-MAT sonuçlarını gösteren mikropleyt.

Results

In this study, results obtained with the four tests with the first 100 sera as a pre-investigation study were given in table 1. Three (3%) and 5 (5%) samples were found to be positive with RBPT and SAT, MAT, 2-ME-MAT, respectively. Same results were achieved with each of the latter three tests (Table 1).

Table 1. Results obtained with the four tests with the first 100 sera.
Tablo 1. İlk 100 serum örneğinin incelendiği 4 testle elde edilen sonuçlar.

	RBPT	SAT	MAT	2-ME-MAT
Positive	3	5	5	5
Negative	97	95	95	95

Statistically the difference in the results between SAT and RBPT was found to be insignificant ($p>0.05$). Results of the statistical evaluation indicated that the sensitivity of RBPT according to SAT had been determined as 60% while the specificity had been 98%. So RBPT's ability of distinguishing infected animals was less than the ability of distinguishing the healthy ones. In herd scanning, SAT was found to be more useful than RBPT. No statistically significant difference was observed amongst the later three tests.

Following MAT tests of 524 serum samples 8 (1.5%) serum samples were found to be positive (Fig. 1 and Fig. 2).

Discussion and Conclusion

Brucellosis is diagnosed either by isolation of brucella organism in a culture, or by a combination of serological tests and clinical findings consistent with brucellosis. Isolation of the brucella organism is the definitive means of diagnosis, but in practice it is difficult due to the early tissue localization and the exacting culture requirements of the organism. In practice, sample cultures are positive in 10%-30% of brucellosis and the remainder is diagnosed serologically (1, 2, 14).

Although, RBPT is standardized, simple to perform, inexpensive and suitable to for screening individual animals, false negative reactions occur, mostly due to prozoning with this test (10, 11). Antibody resulting from *B. abortus* S19 vaccination and some cross reacting antibodies are detected by these tests and it is necessary to use other test(s) to confirm reactor animals as infected (1, 6, 8).

Since no single test provides 100% sensitivity and specificity, SAT still remains the test of choice in diagnosis. In the presence of appropriate signs and symptoms, a presumptive diagnosis of brucellosis is usually defined serologically as a standard tube agglutination titer of 1:40 or greater in unvaccinated and 1:80 or grater in vaccinated animals (2, 7, 8, 11). It is, however, time-consuming and costly for seroepidemiological studies, where a large number of samples have to be processed. Therefore, the microplate agglutination test (MAT) was developed as screening test and successfully utilized in population surveys (4). MAT is probably the ideal method for population surveys because it has the advantage of being more rapid, economical, and highly sensitive.

The use of SAT as a diagnostic tool in spite of its recognized limitations has led to controversy concerning its importance. Several laboratories still prefer to use this test because of its simplicity compared with CFT technique. However, the drawback of lowered sensitivity and specificity as well as the requirement of larger amounts of antigen for this method, and the non-suitability of automation to facilitate diagnosis makes the test unattractive. Instead, a MAT with qualities of improved sensitivity and specificity has been developed (3, 10, 12).

The MAT required less time to perform (20 min rather than 2 h with SAT) and to evaluate (2 min rather than 20 min) the test results. In the MAT less amount of antigen was used (0.1x rather than 1x) than the SAT. The MAT required less expenditure (U-bottomed 96 welled microplates and automatic pipettes rather than test tubes and glass pipettes) so required less costs than SAT. The MAT required less labor (disposable material) and time (no need for cleansing and regulation of pipettes and tubes).

The IgG antibody titer can be determined by treating the serum with 2-mercaptoethanol to inactivate the IgM brucella antibodies while leaving the IgG brucella antibodies intact (9). Due to the neutralizing effect of mercaptoethanol on IgM, 2ME-MAT inhibits the serological response to *Yersinia enterocolitica* O:9, as well as many other false positive serologic response producers (6). In this study, 2ME-MAT was used to compare and confirm the results obtained with SAT and MAT. A major objection to the use of the 2-ME test is that, 2-ME irritates the eyes and mucous membranes. It also has a very strong and offensive odor.

As a conclusion, although SAT is the most widely used laboratory test for the detection of brucella antibodies, The MAT should also be considered to be used in routine diagnostic laboratories for the serological diagnosis of brucellosis.

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