The Evaluation of Indirect Enzyme-Linked Immunosorbent Assay Using Antigens Prepared from *Brucella abortus* RB51 and *Brucella canis* M- Variant Strains for Serologic Diagnosis of *Brucella ovis* Infection

Osman Yaşar TEL [1] 🖋   Sevil ERDENLİĞ GÜRBİLEK [1]   Oktay KESKİN [1]

[1] Harran Üniversitesi Veteriner Fakültesi, Mikrobiyoloji Anabilim Dalı, TR-63200 Şanlıurfa - TÜRKIYE

Abstract

The aim of this work was to investigate the possible usage of hot saline extract antigens (HSE) of *Brucella abortus* RB51(HSE-RB51) and *B. canis* M- variant strains (HSE-M-) in ELISA by comparing the results with those of obtained from commercial I-ELISA kit for the serological diagnosis of *B. ovis* infection. In this study, a total of 183 serum samples collected from different cities (Şanlıurfa, Mardin, Gaziantep, Diyarbakır) in Southeastern Anatolia Region of Turkey were tested by using three ELISAs, one of which is from a commercial source, Rose Bengal plate agglutination tests prepared by rough (R-RBPT) and smooth strains (S-RBPT). Recombinant protein A/G conjugated with horseradish peroxidase (A/G-HRPO) was used as conjugate in the in house ELISAs. Seropositivity rate was 11% for HSE-RB51-ELISA and 3.3% for both HSE-M- and commercial ELISA. The percentage of positive results was 7.6% for S-RBPT and 2.7% for R-RBPT. Only 2 serum samples were positive for all the tests except S-RBPT. Because similar results were obtained from the same serum samples by both commercial ELISA and HSE-M-ELISA, these results may suggest that HSE-M- antigen could be used in ELISA for serologic diagnosis of *B. ovis* infection in sheep. Since 7.6% of the serum samples were found as positive by only HSE-RB51-ELISA, it was assumed that this test could be less specific or more sensitive than other tests used in the study. Although R-RBPT is a screening test, it showed the lowest seropositivity in the study. This could be explained by less mucoid nature of its antigen than other test systems using natural rough species.

Keywords: *Brucella ovis, ELISA, Serology*

Brucella abortus RB51 ve *Brucella canis* M- Varyant Suşlarından Hazırlanan Antijenlerin Kullanıldığı Enzim Bağlı İmmünosorbent Testinin Koyunlarda *Brucella ovis* İnfeksiyonunun Serolojik Tanısında Kullanılabilirliğinin Araştırılması

Özet

Bu çalışmada, biri ticari *Brucella ovis* ELISA kiti olmak üzere 3 ELISA ve smooth ve rough suşlardan hazırlanan Rose Bengal pleyt aglütinasyon testleri (S-RBPT ve R-RBPT) ile karşılaştırmalı olarak test edilerek, *B. ovis* infeksiyonunun serolojik tanısında ELISA'nın kullanılabilirliğinin araştırılması amaçlandı. Çalışmada, Güneydoğu Anadolu Bölgesinin çeşitli illerinden (Şanlıurfa, Mardin, Gaziantep, Diyarbakır) toplanan toplam 183 koyun serumu test edildi. ELISA antijeni olarak *B. abortus* RB51 ve *B. canis* M- varyant suşlarının sıcak tuzlu suda ekstraksiyonları (STE-RB51, STE-M-) hazırlandı. Konjugat olarak horseradish peroksidaz ile işaretli rekombinant A/G proteini (A/G-HRPO) kullanıldı. Seropozitiflik yüzdesi RB51 antijenini kullanan ELISA için %11 iken, diğer iki ELISA için %3.3 olarak bulundu. S-RBPT ile serumların %7.6'sı ve R-RBPT ile %2.7'si pozitif bulundu. Sadece 2 serum R-RBPT ve 3 ELISA ile pozitif reaksiyon verdi. Ticari *B. ovis* ELISA kiti ile STE-M-ELISA ile aynı sayıda ve aynı serumlardan pozitif reaksiyon alınması STE-M- antijeninin *B. ovis* infeksiyonunun serolojik tanısında ELISA için iyi bir aday antijen olabileceğinin kanısına varıldı. Kullanılan serolojik testlerden sadece STE-RB51-ELISA ile pozitiflik yüzdesinin %7.6 olması testin özgüllüğünün diğer testlere göre daha düşük olabileceği kanısı uyandırdı. Ayrıca R-RBPT'nin bir tarama testi olmasına rağmen en düşük seropozitiflik oranı göstermesinin M-varyant suşunun daha az mukoid bir yapı içermesi nedeni ile çapraz reaksiyonları azaltması sonucu olabileceği düşünüldü.

Anahtar sözcükler: *Brucella ovis, Seroloji, ELISA*

🖋 İletişim (Correspondence)
☎ +90 414 1383918
✉ oyasar@harran.edu.tr

INTRODUCTION

Brucella ovis causes a genital disease in sheep manifested by epididymitis in rams and placentitis in ewes producing reduced fertility in the flock. Clinical diagnosis is not sensitive enough because many other bacteria might cause same clinical picture and only about 50% of infected rams present epididymitis [1]. Bacterial isolation is not practical for detection of the disease in large numbers of animals and it also is not very sensitive because of the intermittent shedding of bacteria through semen by infected rams. Therefore indirect methods using serological testing are preferred for routine diagnosis. The most widely used serological tests are complement fixation (CFT), agar gel immunodiffusion (AGID), and indirect enzyme linked immunosorbent assay (I-ELISA). But only CFT is prescribed for international or intra-community trade. However, CFT has found limited application because of its complexity, incompability with anticomplementary, prozoning and hemolyzed sera. In addition, among chronically infected rams which show CFT negative results are not rare. On the other hand, AGID is a very labour intensive test and has low sample capacity [2-6]. Various I-ELISAs have been developed for detection of antibodies against *B. ovis* antigens with various results. According to literature data, most I-ELISAs appear more sensitive and less prone to problems than the CFT and the AGID [7-9].

Antigens used in the immunodiagnosis of *Brucella* infections consist of various somatic proteins and surface components. When rough *Brucella* cells are heat-extracted with saline (HS), they yield water-soluble antigenic extracts mainly composed of outer membrane proteins (OMPs) and rough lipopolysaccharides (R-LPS). Although OMP and R-LPS contain immunodominant epitopes, some cross reactivities have been described between *Brucella* OMPs and bacteria belong to the *Rhizobiaceae* [2,10-14].

Nielsen et al.[14] demonstrated that rough lipopoly-saccharide of *B. abortus* RB51 could be used as antigen for detection of antibodies against *B. ovis, B. canis* and *B. abortus* RB51 by ELISA. More recently, other authors have reported that since *B. ovis* shares antigenic components with *B. canis,* it seems that this strain could be used as antigen to detect antibodies to *B. ovis* with the same results [15]. Since *B. ovis* shares antigenic components with *B. canis*, it would seem that either strain could be used as antigen with the same results. However, the advantage of the *B. canis* (M-) strain variant is that it can be used to develop a satisfactory antigen for agglutination tests because of less mucoid structure of its cell wall [16].

In this study, we aimed to investigate the possible usage of hot saline extract antigens (HSE) of *B. abortus* RB51 (HSE-RB51) and *B. canis* M- variant strains (HSE-M-) in ELISA by comparing the results with those of obtained from a commercial I-ELISA kit for the serological diagnosis of *B. ovis* infection.

MATERIAL and METHODS

Serum Samples

The study included 183 sheep sera collected from different cities in Southeastern Anatolia Region of Turkey. Blood samples were collected in tubes without antico-agulant by jugular venopuncture and kept at RT for 24 h. Sera were separated and stored at -20ºC until testing for detection of *B. ovis* antibodies.

Bacteria Cultures and Antigen Preparation

The M- strain of *B. canis* (kindly provided by Dr. Carmicheal, Cornell University, NY, USA) and *B. abortus* RB51 (kindly provided by Pendik Veterinary Control Institute, İstanbul, Turkey) were cultured in tryptic soy agar supplemented 10% sterile calf serum at 37ºC and harvested during the logarithmic phase of growth. For the antigen preparation, a hot saline extract antigen (HSE) was obtained by following the method described by Barrouin-Melo et al.[16], with minor modifications. Briefly, bacterial cells were harvested with 20 ml steril phosphate buffered saline (PBS; 150mM NaCl, 2.5 mM KCl, 1.5 mM KH_2PO4, 9mM $Na_2HPO_4.12$ H_2O, pH 7.4) and inactivated by heat (1 h, 56ºC). Inactivated bacterial suspansions were washed three times by centrifugation (3.500xg, 10 min) in PBS. Finally the resulted pellets were then re-suspended in PBS and autoclaved at 121ºC for 20 min. The cells were then centrifuged at 12.000xg for 20 min, at 4ºC. The supernatants were collected and identified as HSE and stored in small aliquots at -20ºC until their use as the ELISA solid phase antigen.

Serological Tests

The serum samples were tested comparatively by three ELISAs, one of which is I-ELISA kit from a commercial source, and Rose Bengal plate agglutination tests prepared by rough (R-RBPT) and smooth strains (S-RBPT).

Rapid slide agglutination tests (S-RBPT/R-RBPT) were performed as described previously [17] using antigens prepared with *B. canis* M- and *B. abortus* S99.

Indirect ELISA (I-ELISA): Commercial I-ELISA kit (Chekit *B. ovis*, Idexx, France) was used according to the manufacturer's instructions and 2 in house ELISAs (HSE-M-) and HSE-RB51) were performed in paralel on all test and control sera. The working dilutions of the horseradish peroxidase conjugated protein A/G (ImmunoPure, Pierce Lab), HSE-M- and HSE-RB51 antigen preparations and positive and control sera were determined previous check-board titrations to achieve the highest positive-to-negative ratio with the lowest background reading. The antigen diluted in 0.06 M sodium carbonate buffer (pH 9.6) was passively coated onto polystyrene plates (Nunc 269620, Denmark), 100 µl/well, incubated for overnight at 4ºC and then washed five times in 0.01 M phosphate

buffered saline containing 0.05% Tween 20, pH 7.2 (PBS/T). Control and test sera were added 1:100 in PBS/T, 100 μl/well, for 1 h at room temperature (RT). After five washes in PBS/T, protein A/G horseradish peroxidase conjugated was added, 100 μl/well, and incubated for 1 h at RT. Finally, after five washes in PBS/T, 100 μl of chromogenic substrate (4.0 mM hydrogen peroxide and 1.0 mM 2,2'-azino-bis (3- ethylbenz-thiazoline-6-sulfonic acid) diammonium salt in 0.05 M citrate buffer, pH 4.5) per well was added. The plates were shaken continuously on an orbital shaker for 15 min prior to reading at OD_{405} nm in a microplate reader (VERSAmax 3.13/B2573). Optimum antigen and conjugate working dilutions were established by making serial dilutions of both antigen and conjugate. Optimum dilution was considered as the one which gives the greatest differential between positive and negative sera.

Data Analysis

The triplicate mean optical density (OD_{405}) of the each positive, negative sera and test sera were calculated and the OD value of the test serum was substracted from the mean OD of negative sera. This figure was divided the difference between the mean OD of positive and negative sera and multiplied by 100. The results were expressed as a percent positivity value (%P). If the resulted figure was more than 50, the test serum was considered as positive.

RESULTS

Seropositivity rate was 11% for HSE-RB51 and 3.3% for both HSE-M- and commercial ELISA. The percentage of positive results was 7.6% for S-RBPT and 2.7% for R-RBPT (Table 1). Only 2 serum samples were positive for all the tests except S-RSAT. Three serum samples were positive by only R-RSAT. Two of the serum samples were found positive by only 3 ELISAs while 2 serum samples were positive to all tests except R-RSAT (Table 2).

Table 1. Test serum samples that were positive to at least one serological test employed
Tablo 1. Uygulanan serolojik testlerden en az birine pozitif sonuç veren test serum örnekleri

Serum No	Rapid Slide Agglutination Tests (S-RBPT/R-RBPT)		ELISAs		
	S-LPS	R-LPS	*B. ovis* (Idexx Commercial I-ELISA Kit) %P	*B. canis* M- (Home Made) %P	*B. abortus* RB51 (Home Made) %P
472	-	-	-	-	+ 69
468	-	-	-	-	+ 50
189	-	+	+ 61	+ 58	+ 51
474	-	-	-	-	+ 57
463	-	-	-	-	+ 91
420	-	-	-	-	+ 84
423	-	-	-	-	+ 68
493	-	-	-	-	+ 83
495	-	-	-	-	+ 54
15	-	-	-	-	+ 79
419	-	+	+ 72	+ 51	+ 88
461	-	-	-	-	+ 61
499	-	-	-	-	+ 71
460	-	-	-	-	+ 100
469	-	-	-	-	+ 59
478	-	-	-	-	+ 72
82	++	-	+ 65	+ 66	+ 52
446	+	-	+ 71	+ 100	+ 65
28, 98, 203, 355, 560, 600, 619	++	-	-	-	-
76, 135, 148, 580, 582,	+	-	-	-	-
471	-	-	+ 80	+ 67	+ 53
599	-	-	+ 78	+ 82	+ 61
108, 616	-	+	-	-	-
157	-	++	-	-	-
Total	14 (7.6%)	5 (2.7%)	6 (3.3%)	6 (3.3%)	20 (11%)

Table 2. *Comparison of serological results of RSAT and three ELISAs*
Tablo 2. *RBPT ve üç ELISA serolojik test sonuçlarının karşılaştırılması*

Number of Serum Samples	Rapid Slide Agglutination Test Using Smooth and Rough Strains		ELISA		
	S-LPS	R-LPS	*B. ovis* (Commercial, Idexx)	*B. canis* M-HSE Antigen	*B. abortus* RB51 HSE Antigen
14	-	-	-	-	+
12	+	-	-	-	-
2	-	+	+	+	+
3	-	+	-	-	-
2	-	-	+	+	+
2	+	-	+	+	+
148	-	-	-	-	-
Positivity rates	14 (7.6%)	5 (2.7%)	6 (3.3%)	6 (3.3%)	20 (11%)

DISCUSSION

Diagnosis of brucellosis based on clinical examination is not sensitive enough because similar symptoms are caused by other microorganisms or by trauma and almost half of the infected animals do not show clinical picture [6]. Bacteriological culture is not sensitive enough to detect all infected animals for practical reasons due to intermittent shedding of the agent by infected animals. Serological tests appear to be useful for routine diagnosis and control of the disease. The most widely used serological tests are CFT, AGID and ELISA. But only CFT is officially accepted test for international trade. However, CFT has some drawbacks like complexity, incompatibility with anticomplementary and hemolyzed sera, prozone phenomenon and occasional false reactions. On the other hand, AGID is a very labour intensive test and has low number of test capacity [2-4,6,18,19]. Of the serological methods used to detect antibodies to *B. ovis*, I-ELISA has been shown to be the most sensitive and specific test. As a diagnostic serological method, the ELISA has important advantages over other serological tests commonly used for the diagnosis of ovine brucellosis, such as providing readily measurable results and being easy to perform and standardize [7,15,20,21].

This study compared the results of five serological tests. The percentage of positive results was 11% for ELISA with HSE-RB51, 3.3% for ELISAs with HSE-M as well as commercial indirect *B. ovis* ELISA kit, 7.6% for RSAT with *B. abortus* S99 and 2.7% for RSAT with *B. canis* M- strains *(Table 1)*. These results might showindicated that brucellosis caused by smooth speciesis more prevelant than those caused rough species These results are not surprising since small ruminant brucellosis caused by *B. melitensis* is endemic in Turkey [6,22]. Only two serum samples were positive for all the tests except S-RSAT. These two serum samples (189 and 419) might be from real *B. ovis* infected animals. Three serum samples were positive by only R-RSAT. RSAT is a screening test with which some false positives results might be seen [15,23].

Two of the serum samples were found positive by only 3 ELISAs. This was in agreement with the findings that ELISA has been proven to be more sensitive thanagglutination-based techniques [6,21].

Various ELISA-based methods for serodiagnosis of brucellosis have been proposed and used with various success rates depending on the antigens used in the assay. In this study, hot saline extract (HSE) was used as antigen for the ELISA, which has been shown to be a complex antigen, mainly composed of outer membrane proteins (OMPs) and rough lipopolysaccharide (R-LPS) [8,14,16]. In this study, two serum samples were positive to all tests except R-RSAT, this might suggest that OMPs can be shared between rough and smooth brucellae [1,21,24].

Among three ELISAs, HSE-RB51 ELISA showed the highest amount of seropositivity. This finding was not consistent with the findings of Nielsen et al.[14] in which *B. abortus* RB51 RLPS based ELISA gave the best spesificity and sensitivity results. This discrepancy suggests that the presence of OMPs in our HSE extract of *B. abortus* RB51 could explain why the HSE-ELISA was more sensitive than RLPS-ELISA.

In our study I-ELISA- *B. canis* and commercial I-ELISA-*B. ovis* kit detected the same serum samples. Since *B. ovis* shares antigenic components with *B. canis* [15,25,26], it would seem that both strains might be used as an antigen with the same results.

As conclusion, the similar results were obtained from the same serum samples by both commercial I-ELISA and HSE-M-ELISA. The results indicated that HSE-M-antigen could be used in ELISA for serologic diagnosis of *B. ovis* infection in sheep. Since 14 serum samples were found as positive by only HSE-RB51-ELISA, it was assumed that this test could be less specific than other tests used in the study. Although R-RBPT is a screening test, it showed the lowest seropositivity in the study. This could be explained by its less mucoid nature than other natural rough species. In

this context, we also concluded that R-RBPT using *B. canis* M(-) strain could be specific and practical screening test for serologic diagnosis of infection caused by rough strains.

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