A Comparison Between Three Serological Tests for
Brucella melitensis Infection in Sheep

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Abstract: A comparative study of the Rose Bengal test (RBT), Enzyme-Linked Immunosorbent Assay (ELISA) and complement fixation test (CFT) was performed to analyze sera of two Awassi flocks. Flock A was composed of 215 ewes, naturally infected with brucellosis. None of the animals were vaccinated. Flock B consisted of 336 non-infected non-vaccinated ewes. These served as negative controls. The two flocks were each divided into 4 groups, and samples were collected from one group every 6 months and subjected to bacteriologic and serologic testing. Samples from any aborted ewes were also collected immediately after abortion and 6 weeks later. During the 2 year period of study, 57 ewes spontaneously aborted, out of which *Brucella melitensis* was isolated from 16 aborted fetuses. Blood from only 2 pregnant ewes were cultured positive to *B. melitensis* from flock A, and none from flock B. RBT was demonstrated as the most suitable screening method in the field if suspected weak positive (+1) readings are considered negative. ELISA is the most specific and sensitive method since no false negative results were recorded which is in contrast to other methods. One single test is not sufficient to confirm the diagnosis of brucellosis and the combination of two tests should be performed. These are preferably the RBT and ELISA.

Key Words: Sheep, brucellosis, ELISA, RBPT, CFT

Koyunların *Brucella melitensis* İnfeksiyonunda Kullanılan
Üç Serolojik Testin Karşılaştırılması

Özet : Sığır brusollozis'i birçok ülkede başarılı bir şekilde eradike edilmiş olmasına karşın, koyun brusellozis'i halen dünyanın birçok bölgesinde endemik bir seyir izlemektedir. Teşhis ve sağaltım konularında gerçekleştirilen ilerlemelere rağmen brusellozis halen dünyada yaygın olarak görülmekte ve gelişmekte olan ülkelerde deprevalensi artmaktadır (1). Klinik ve laboratuvar çalışmaları için subakut ve kronik brusellozis infeksiyonları bir probem oluşturmaktadır.

İnfeksiyonun kesin teşhisi mikroorganizmanın izolasyonu ile gerçekleştirilir fakat serolojik testler dahapratik ve brusellozis kontrol ve eradikasyon programlarının en temel unsurlarıdır. Nevar ki, koyun serumlarında serolojik testlerin değerlendirilmesi ile ilgili çok az çalışma bulunmaktadır ve bunların sonuçları da tatmin edici değidir (2-4). Koyun brusellozis'inin rutin serolojik teşhisindt kullanılan testler arasında tüp aglutinasyon testi, Rose Bengal plate testi (RBPT) ve komplement fikzasyon testi (CFT) bulunmaktadır (5). Counter immunoelektroforezis, yukarıda bahsedilen testlerden bazılar ııle karşılaştırılmıştır (4, 6-7). Koyun bruselozis'i için kullanılan serolojik testler genellikle yanlış negatif sonuçlar verir (8) ve bu testlerden hiçbirisi tek başına koyun brusellozis'inin teşhisi için yeterli değildir (4, 9).

Bu çalışmada, Brucella melitensis ile doğal infekte olan koyunların, infekte olmayan koyunların ve spontan abort olguları görülen koyunların kan serumlarında ELISA yöntemi RBPT ve CFT ile karşılaştırılmıştır.

Anahtar Sözcükler: Koyun, brusellozis, ELISA, RBPT, CFT

Introduction

Ovine brucellosis is still endemic in many regions of the world although bovine brucellosis has been succesfully eradicated in many countries. Despite advances made in diagnosis and therapy, brucellosis is still widespread and the prevalence in many developing countries is increasing (1). Subacute and chronic infections of brucellosis are also problems to clinical and laboratory workers.

The only conclusive diagnosis is isolation of the organism but serological tests are more practical and are the backbone of brucellosis control and eradication programs. However, few studies on the evaluation of serologic methods have been conducted on sheep sera and the results obtained are not consistant (2-4). Tests used routinely in diagnosis of ovine brucellosis are the tube agglutination test, Rose Bengal plate agglutination test (RBT), and the complement fixation

test (CFT) (5). Counter immunoelectrophoresis has been compared with some of the above serologic tests (4, 6-7). Serological tests for ovine brucellosis often give false negative results (8) and no single test will detect all cases of ovine brucellosis (4, 9).

In the present study the enzyme-linked immunosorbent assay (ELISA) was compared with the RBT and the CFT on sera of sheep naturally infected with *Brucella melitensis*, non-infected sheep and sera from sheep that suffered spontaneous abortion.

Materials and Methods

Sampling

Two flocks (A&B), situated in the northern Jordan 15 kilometers apart were studied. Both flocks adopted a semi-intensive husbandary system, with one lambing season spread over approximately two months from the last week of October to the end of December. Both flocks used their private pasture and no mixing with other sheep or animals. Neither flock was vaccinated against Brucella. Flock (A) consisted of 220 breeding ewes, aged 2-4 years and showed clinical and serological evidence of brucellosis. Flock (B) consisted of 336 breeding ewes, aged 2-6 years and had no clinical or serological evidence of Brucella. This flock was used as a negative control. The two flocks were both divided into 4 groups, and followed during 1989-1990 seasons inclusive. Sera were collected from all aborted ewes immediately after abortion and 6 weeks later. To ensure each animal was sampled during pregnancy, sera were collected routinely at 6 month intervals (4 times in total) during the study. Sera were kept in aliquots at -20ºC untill analysed.

Bacteriological Examination

All aborted fetuses were brought to the laboratory during the study. The fetuses were dissected and 1 ml of stomach contents was inoculated in liquid media (tripticase in soy broth), incubated at 37ºC for 2-3 weeks, then cultured on Brucella-media at 37ºC for a further 4-7 days. Brucella isolates were identified using the methods described elsewhere (10). One ml of the blood sample was cultured in an identical manner.

Serological tests

a. The RBT was performed with standard Brucella antigen (Diagnostics Pasteur, France). Positive results were scored from slight agglutination (+1) to complete agglutination (+4).

b. The ELISA method utilised anti-Brucella IgG anti-

bodies at a dilution of 1/44 and determined using kits (supplied by The Central Veterinary Laboratory, New Haw, United Kingdom). S-19 antigen, positive and negative control sera were used to standardize the test. Test samples were measured photometrically in a cuvette of $1cm^3$ light path at 405 nm. The cut-off value was determined at two times the mean absorbance value of 8 standard negative sera. Positive results were divided into 4 categories according to he degree of absorption as follows: 0.32 or less as negative, +1=0.33 - 0.48 absorption, +2 = 0.49 - 0.64, +3 = 0.65 - 0.8 and +4 > 0.81.

c. The CFT method was carried out according to the method described previously (11), using Behrinwerke (Marburg, Germany) reagents. A titration of hemolysin and antigen was performed before the test. The minimum hemolytic dose (MHD) was also estimated for each run using 3% sensitized sheep RBC withlsever's solutioompt andne buffer saline in u-shaped microtiter plates. Two MHD units were used throughout the test. The end point titer was taken as the first well showing approximanetly 50% lysis of indicator cells. Each sample was tested in duplicate in a serial dilution of 1/10, 1/20, 1/40, 1/80 and 1/160. Negative samples were checked by testing in serial dilution up to 1024 times. The test was performed in the cold with the appropriate controls at 4°C for 10h. Absence of hemolysis at 1/10 serum dilution was taken as negative.

Results

During the survey period flock (A) had abortions in 57 of the ewes and 5 ewes died. Of the 57 aborted fetuses, *B. melitensis* were isolated from 16 fetuses. Only two blood samples cultured positive for *B. melitensis*. Flock (B) had only 2 cases of abortion, and no bacteria were isolated from either.

Table 1.   Comparison of RBT and ELISA in testing of 215 sheep sera for brucellosis.

RBT	-ve	+1	+2	+3	+4	Total
ELISA						
-ve[a]	50[b]	71	5	1	0	127
+1	5	7	3	7	0	22
+2	1	3	2	21	2	29
+3	1	4	2	13	5	25
+4	1	1	0	5	5	12
Total	58	86	12	47	12	215

a : -ve indicates negative,   b : indicates the number of sera

Table 2. Comparison of CFT and ELISA in testing of 215 sheep sera for brucellosis.

ELISA	-ve	+1	+2	+3	+4	Total
CFT						
-ve[a]	42[b]	4	4	3	1	54
1/10	3	10	2	1	6	22
1/20	3	8	17	6	4	38
1/40	6	8	15	14	12	55
1/80	0	2	8	8	6	24
1/160	0	2	6	11	3	22
Total	54	34	52	43	32	215

a : -ve indicates ne   b : indicates the number of sera

Table 3. Comparison of RBT and CFT in testing of 215 sheep sera for brucellosis.

ELISA	-ve	+1	+2	+3	+4	l
CFT						
-ve[a]	52[b]	73	1	1	0	127
1/10	3	3	1	0	0	7
1/20	5	9	0	5	0	19
1/40	2	9	1x	6	4	30
1/80	0	0	6	6	2	14
1/160	0	1	2	10	5	18
Total	62	94	20	28	11	215

a : -ve indicates negative,   b : indicates the number of sera

Table 4. Evaluation of RBT in comparison to ELISA or CFT.

	RBT & ELISA	RBT & CFT	ELISA & CFT	RBT* & ELISA	RBT* & CFT
% correlation	61	60	89	87	85
% discrepancy	39	40	11	13	15

* +1 readings of RBT are considered negative

Tables 1, 2 and 3 summarize the comparison of the results of RBT, ELISA and CFT tests. Table 4 represents the results of Chi-square, McNemar test for the RBT, ELISA and CFT tests. A significant (P<0.05) difference between the number of negative sera in RBT and ELISA (127 to 58) and in RBT and CFT (127 to 62) was found. Out of the 127 negative samples, 71 showed suspected weak positive agglutination with RBT test (+1 readings). These are responsible

for the low specificity, and poor correlation (Table 4). If the +1 readings of RBT test are consideredt negative, the specificity increases from 0.39 to 0.95. The same results were obtained when RBT test was compared with CFT (Table 2 and 4).

Table 5 represents the sero-response and bacteriological isolation in 57 aborted ewes. All three tests were positive or negative in 47 animals (79%). RBT test failed to detect antibodies against Brucella (false negative) in 2 cases (3.5%) from which Brucella has been isolated and detected antibodies in 5 cases (8.7%) from which no Brucella was isolated (false positive). Again if the +1 readings in RBT test are considered

Table 5. The results of bacteriological isolation and serological tests in 57 aborted ewes.

Number of aborted ewes	Brucella isolation	RBT	ELISA	CFT
13	+	+	+	+
1	+	+	+	+
2	+	−	+	+
34	−	−	−	−
5	−	+	−	−
1	−	−	−	−
1	−	+	−	−

negative, one case was left as a false positive instead of 5. ELISA showed no false negative and only one case of false positive (1.8%) was obtained. This was comparable to that resulted from CFT which showed one case false positive and one case false negative.

Discussion

Many improvements have been made for the diagnosis of brucellosis. However, problems exist with areas such as the diagnosis of latent infections (12). This is illustrated by the current results in which there was only 2 isolations from 215 pregnant ewes. However 57 of these subsequently aborted. Furthermore, *B. melitensis* was isolated from the only 16 of 57 aborted fetuses, this may indicate the presence of other cause of abortion in the flock. The RBT has been evaluated by several workers in sheep and goats and there are inconsistancies on its effectiveness (12). The high number of false positive results and the poor correlation between RBT and ELISA and CFT tests are the result of weak suspected RBT +1 readings. This results from the examiner recording any slight agglutination which may neither be accurate nor recommended by the manufacturers as positive.

The results obtained from aborted ewes demonstrated that ELISA test is the most specific and sensitive test for the diagnosis of ovine Brucellosis. ELISA is the only test with no false negative results. However, all three tests showed false positive results.

In order to evaluate both false positive and false negative results two approaches might be considered. Firstly, ıf the weak suspected +1 readings of RBT test are considered negative, then few cases are left as false positive and good correlation with other serological test are obtained (Table 4). This is expected since RBT test detect early infection through the strong

agglutianiting IgM antibodies produced at the initial stage of infection. Subsequently, IgG1 antibodies predtominate. Although the concentration of IgG1 decreases with time, it remains detectable for a long period. Secondly, a combination of RBT and either of the two tests could detect all the positive Brucella-reactors. This minimizes the possible false negative results. This is in agreement with previous results (4, 8-9) which concluded that it was impossible to detect all infected animals using a single test. The combination of RBT with ELISA is recomended since the ELISA method is reproducible, reliable and less time consuming than the CFT.

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