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Detection *of Brucella* antigen in the aborted ovine fetal stomach contents using a modified ELISA test

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Abstract

This study was conducted on two flocks of sheep suffering from abortion in Mosul city, Iraq. The clinical findings in ewes were abortion during the 3-4 months of gestation period in the both flocks. The total percentage of abortion was 11.7 %, whereas stillbirth percentage was 4 %. *Brucella spp*. was isolated from four (33.3 %) of the 12 samples (stomach contents of the aborted fetuses). All culture – positive samples had also positive with direct smears. By a modified enzyme-linked immunosorbent assay (ELISA), *Brucella* antigens were detected in the fetal stomach contents of 5 samples. The sensitivity and specificity of the modified ELISA were 100 % and 87.5 % respectively. The test had a good negative predictive value but only a moderate positive predictive value. Therefore, the test would be useful for confirming the existence of suspect disease. Comparison of modified ELISA with bacterial isolation demonstrated a close agreement (Kappa value = 0.92). Of the 12 serum samples from aborted ewes, eight samples were positive with Rose Bengle test (66.7 %), more than 10 samples (83.3 %) were detected by indirect ELISA test.

Keywords: Ovine brucellosis, ELISA, Fetus, Stomach contents.
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الكشف عن مستضد البروسيلا في محتويات معدة اجنة الضأن المجهضة باستخدام اختبار الاليزا المحور

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الخلاصة

اجريت الدراسة على قطيعين من الضأن يعانيان من الاجهاض في مدينة الموصل / العراق، اذ لوحظ سريريا حدوث الاجهاض في النعاج خلال الشهرين ٣- ٤ من الحمل في كلا القطعين، وبلغت نسبة الاجهاض الكلية ١١,٧ %، اما نسبة المواليد الضعيفة والتي نفقت بعد الولادة فهي ٤ %. عزلت جراثيم البروسيلا من اربعة عينا ت (٣٣,٣ %) من مجموع اثنا عشر عينة (محتويا ت معدة الاجنة المجهضة). كانت كل العينات الموجبة للزرع الجرثومي موجبة للمسحة المباشرة، ولكن عند استخدام اختبار الاليزا المحور ظهر مستضد البروسيلا في ٥ عينا ت من محتويات معده الاجنة المجهضة. بلغت نسبة حساسية ونوعية اختبار الاليزا المحور ١٠٠ و ٨٧,٥ % على التوالي. اظهر الاختبار قيمة جيدة للحالات السالبة، وقيمة متوسطة في الكشف عن الحالات الموجبة، مما اشار الى امكانية استخدام الاختبار كاختبار تأكدي لتشخيص المرض في الحالات المشكوك بها. أما عند المقارنة بين اختيار الاليزا والعزل الجرثومي كانت قيمة الكابا عالية (قيمة الكابا = ٠,٩٢)، مما يعني وجود علاقة قوية بينهما. ومن مجموع اثنا عشر عينة مصل من النعاج المجهضة كانت ثمانية منها موجبة لاختبار وردية البنكال (٦٦,٧ %)، بينما كانت عشرة (٨٣,٣%) عينات من المصل موجبة لاختبار الاليزا غير المباشر.

Introduction

Ovine brucellosis is usually caused by *Brucella melitensis*, less frequently by *B. abortus*, and rarely by *B. ovis* (1,2). It is usually manifested by abortion, with excretion of the organisms in uterine discharges and in milk (2,3). Diagnosis depends on the isolation of *Brucella spp.* from aborted fetuses, milk or from tissues removed at postmortem examination (1). Alternatively, specific cell-mediated or serological responses to *Brucella* antigens can be demonstrated (3,4).

The demonstration by modified acid-fast or immunospecific staining of organisms of *Brucella* morphology in abortion material or vaginal discharges provides presumptive evidence of brucellosis, especially if supported by serological tests (5). Whenever possible, the species and biovar should be isolated, and identified by phage lysis or oxidative metabolic tests, or both, and by cultural, biochemical and serological criteria (5-7). The recently developed polymerase chain reaction (PCR) and DNA-probe methods provide additional means of detection (8,9).

No serological test is appropriate for all epidemiological situations (6). The buffered *Brucella* antigen tests (Rose Bengal plate agglutination test and buffered plate agglutination test) are suitable for screening herds and individual animals (10). The reactivity of positive samples should be confirmed by the complement fixation test or by enzyme-linked immunosorbent assay (ELISA), both of which can also be used for both screening and confirmation (11,12). The serum agglutination test is inferior to other tests in specificity and sensitivity, and is not recommended if other procedures are available (12). Another immunological test was developed for detection of *brucella* antigen in the bovine fetal stomach contents as agar gel precipitation and counter – immunoelectrophoresis (13). Counter – immunoelectrophoresis also was used for detection of *brucella* antigen in the stomach contents of aborted buffalo fetuses (14), while coagglutination test was used for detection of *Brucella* antigens in aborted ovine fetal stomach contents (15). The aim of the study to describe a simple and rapid method (Modified ELISA) for detection of *Brucella* antigen in aborted ovine fetal stomach contents.

Materials and methods

The study was conducted in April 2008 on two flocks of sheep in the Mosul city (Badoosh and Zumar) covering 120 and 52 sheep for each respectively. Complete examination was performed to all aborted animals and 12 were subjected to thorough postmortem examination. The two flocks were not vaccinated against brucellosis.

The following specimens were collected for diagnosis of the disease:

1. Bacterial isolation trials from stomach contents of the aborted fetuses were made on the modified *Brucella* agar medium supplemented with antibiotic (Himedia, India) and incubated at 37 ^{o}C in an atmosphere of 10 % CO_2 and in air for 6 days and then *Brucella* colonies were identified and typed according to conventional methods (4). Suspected colonies were further identified and subcultured on *Brucella* spp. agar slants. We identified *Brucella* spp. isolates according to morphologic characteristics, microscopic appearance. *Brucella spp.* isolates were typed according to their CO_2 requirement, H_2S production, and growth in the presence of basic fuchsin and thionin at final concentrations of 20 µg/ml, as described (4, 5).
2. Direct smear were prepared from aborted fetal stomach contents, then stained with Koster stain and examined under light microscope (16).
3. Detection of the brucella antigen from aborted fetal stomach contents by modified ELISA: Apart of the samples was heated for 1 hr at 70 ^{o}C and centrifuged. The precipitates were washed twice with phosphate buffered saline (PBS) and centrifuged. The precipitates were then used for ELISA. A hyperimmune antibrucella serum for using in the modified ELISA was prepared in sheep by four biweekly injections subcutaneously with live *Brucella* vaccine (Rev.1 strain of *Brucella melitensis*) produced in (CZ Veterinaria, Spain) with the standard dose of 1×10^9 colony forming units (CFU) (17).

   Modified ELISA was done according to the general principals of immunoblotting (18). Brifly, 20µl of each of the samples and controls (BPS) were applied to nitrocellulose membrane (Amersham). The membrane was blocked in 5 % skim milk in PBS for 45 minutes. Membrane was washed three times, 5 min each with PBS- T (PBS containing 0.05 % Tween 20), was placed in 1: 100 dilution of anti brucella serum in PBS- T and incubated at room temperature for 1 hr. The membranes were then incubated in conjugate goat antimouse IgG horse – radish peroxidase (Svanovir ®, Sweden) diluted 1: 1000 in PBS – T for 1 hr. The same washing procedure was repeated after incubation with the anti – sheep peroxidase conjugate (Svanovir ®, Sweden), followed by reaction with a chromogen – substrate solution consisting of 30 µl of 30 % H_2O_2 in 50 ml of PBS mixed with 30 mg of 4 – Chloro – 1 – naphtol in 10 ml of cold methanol. The results were determined by observation of violet – stained spots on the membrane.
4. Serum samples: 12 blood samples from all aborted ewes (12) were collected by venipuncture at 15 days after abortion. Rose Bengal (RBT), and indirect ELISA were used to detect antibodies against brucellosis. The Rose Bengal test was performed as described by (19). A

commercially available indirect ELISA (Brucella – Ab I-ELISA kit Svanovir ®, Sweden) was used to detect specific antibodies for *Brucella spp.* according to manufacturer´s instruction.

Statistical analysis: The sensitivity, specificity, positive and negative predictive values were calculated. The Kappa value was used to evaluate the correlation between modified ELISA and bacterial isolation (20,21)

Results

Clinical examination showed abortion occurred during the 3-4 months of gestation period in the both flocks. Table 1 illustrate the number of aborted ewes and average of their gestation period in months. The total percentage of abortion was 11.7 %, while stillbirth percentage was 4 %.*Brucella spp*. was isolated from 4 (33.3 %) of the 12 samples (stomach contents of the aborted fetuses). All culture – positive samples had also positive results with direct smears (stained with Koster stain). By modified ELISA, *Brucella* antigens were detected in the fetal stomach contents of 5 samples (42.0 %). Other culture – negative samples were also negative with modified ELISA test (Table 2).

Table 1: Numbers of aborted animals and average of their gestation period in months.

Regions	Total lambing	Abortion No (%)	Stillbirth No. (%)	Gestation period (Months)
Badoosh	120	8 (9.6 %)	3 (3.6 %)	3 - 4
Zumar	52	12 (6.3 %)	4 (2.0 %)	3 - 3.5
Total	172	20 (11.7%)	7 (4.0 %)	3 - 4

Table 2: Results of the tests used for detection of brucella antigens in stomach contents of the aborted fetuses.

Samples	Bacterial culture	Direct smears	Modified ELISA
1	-	-	-
2	+	+	+
3	-	-	-
4	+	+	+
5	-	-	-
6	+	+	+
7	-	-	-
8	-	-	-
9	-	-	-
10	+	+	+
11	-	-	-
12	-	-	+
Total	4 (33.0 %)	4 (33.0 %)	5 (42.0 %)

(-) negative results, (+) positive results.

From the results, it appeared that the modified ELISA has a high sensitivity (100 %), but only moderate specificity (87.5 %). The test also has a good negative predictive value(100 %) and moderate positive predictive value (80 %). Therefore, the test is would be useful for confirming the existence of suspected disease. Comparison of modified ELISA with bacterial isolation demonstrated a close agreement (Kappa value = 0.92). Of the 12 serum samples from aborted ewes, eight samples were positive with Rose Bengle test (66.7 %), more than 10 samples (83.3%) were detected by indirect ELISA test.

Discussion

Brucellosis is widely regarded as an extremely insidious disease, demanding the most exhaustive care in diagnosis and many cases are more difficult to diagnosis than others, and a few are extremely difficult to pick up, and could easily be missed altogether (1,2). In this study, *Brucella spp*. was isolated from 4 (33.3 %) of the 12 samples (stomach contents of the aborted fetuses). All culture – positive samples were also positive with direct smears (stained with Koster stain). By modified ELISA, *Brucella* antigens were detected in the fetal stomach contents of 5 samples (42.0 %). Other culture – negative samples were also negative with modified ELISA test. According to the results of this study modified ELISA is able to detect *Brucella* antigens in fetal stomach content because the modified ELISA has a high sensitivity (100 % = false negative rate of 0%), but only moderate specificity (87.5 % = false positive rate of 12.5%). The test also has a good negative predictive value (proportion of modified ELISA negative animals which do not have disease = 100 %) and moderate positive predictive value (proportion of modified ELISA positive animals which have the disease = 80 %) (20). Therefore, the test would be useful for confirming the existence of suspected disease because the test has a few false positive rate and moderate positive predictive value (21).

Comparsion of modified ELISA with bacterial isolation demonstrated a close agreement (Kappa value = 0.92).The Kappa test can be used to measure the level of agreement beyond that which may be obtained by chance. The Kappa lies within a range between -1 and +1 (20). (20), (21) were noted that the Kappa value gives no which of the tests is better and that a good agreement may indicate that both test are equally good or equally bad.

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