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USING OF BRUCELLA CELL ENVELOP AS AN ANTIGEN IN DIAGNOSIS OF OVINE BRUCELLOSIS

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SUMMARY

In this study serum samples from 35 Brucella culture positive and 80 Brucella culture negative sheep were used to evaluate the use Whole Cell Sonicate (WCS) and Cell Envelop (CE) antigens prepared from Brueclla melitensis type 3 (field strain) for ELISA. These samples were also tested using Rose Bengal test (RBT), Buffered acidified plate test (BAPT), Rivanol test (Riv. T.) and Tube agglutination test (TAT). The results showed that 35 (100%) of the examined sera from the culture positive group were positive in BAPA, RBT and TAT tests while 34 (97.14%) were positive for Riv. T. However, from 80 animal from the culture negative group, 7 (8.75%), 8 (10%), 5 (6.25%), and 8 (10%) were positive for RBT, BAPA, Riv. T and TAT respectively. The serum samples of these animals were then tested by indirect ELISA using WCS and CE antigens. From the 35 culture positive animals 35 (100%) showed positive results, while, from the 80 culture negative animals 5 (6.25%) and 2 (2.5%) were positive in WCS and CE ELISA respectively. The sensitivity of ELISA with both WCS and CE antigens was 100%. It is clear that WCS and CE detect all culture positive sheep. The specificity of ELISA for WSC was 93.75% and for CE was 97.5%. In conclusion CE seems to be effective for diagnosis of *Brucella melitensis* infection in sheep by ELISA.

INTRODUCTION

The bacteria of the genus Brucella are gram negative intracellular parasites of both human and animals. Among Brucella species, *Brucella melitensis* may cause abortion in sheep and goats, which results in huge economic losses particularly in Mediterranean countries (Zygmunt et al., 1994). *Brucella melitensis* is the most virulent species of all the Brucellae (OIE, 1996). The virulence is partly measured by their capacity to cause brucel-

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losis also in cattle and human beings that are not considered natural or preferred hosts (Elberg, 1981), even though in these species the disease may sporadically lead to abortion. Moreover, *Brucella melitensis* is excreted in the milk of infected cows thus transmitting the disease to the suckling neonatals. Conventional serological tests e.g. Tube agglutination, Rose Bengal and Complement fixation tests are the standard tests used to detect ovine brucellosis (OIE, 2000). These tests principally measure antibody to smooth Brucella lipopolysaccharidies (LPS) which may lead to some undesired reactivities. However, there are contradictory reports on the value of the above tests especially when applied to sheep (Blasco, et al., 1994b). Indirect enzyme linked immunosorbent assay (ELISA) was developed using LPS antigen. However, although the ELISA showed better sensitivity, the test lacked specificity due to interference by vaccinal antibodies and antibodies induced by cross reacting microorganisms. These problems were largely overcome by the introduction of the competitive ELISA as stated by Nielsen et al. (1995). Thus, it is reasonable to assume that the use of other surface antigen in indirect ELISA may also circumvent these problems.

A variety of *Brucella melitensis* surface antigens contribute in the diagnosis of infection in sheep. Although the internal antigen may be considered as an excellent antigen for its specificity, practical use seems to be limited since the antibodies can not detected in the early stages of infection (Serikawa et al., 1989).

Sonication damages the cell wall by high frequency sound waves. It uses to disrupt cell membranes and release cellular contents. The cells are disrupted in a buffer that has been chosen to keep the target protein in an active form (Gensel et al., 1990). So, bacterial extracts prepared by sonication contained a complete mixture of all the protein bands and LPSs (Baldi et al., 1999). Gram-negative bacteria including Brucellae have a cell envelope consisting of an inner membrane and an outer membrane that are separated by a periplasmic space containing the peptidoglycan layer (Martinez De Tejada and Moriyon, 1993).

In this study whole cell sonicate were prepared from *Brucella melitensis* biovar 3 (Field strain), Also, the cell envelop (CE) antigen was extracted from the same field strain of Brucella and characterized using SDS-PAGE, Then, enzyme linked immunosorbent assay (ELISA) was applied using both preparations as antigens to detect ovine antibodies for Brucella infection.

MATERIALS AND METHODS

Antigen preparation: Whole cell sonicate (WCS) was prepared from heat killed *Brucella meitensis* biovar 3. Cells were sonicated at 30 HZ for 15 minutes (Funk et al., 2005).

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Cell envelop preparation (CE): A modification of the procedure of Rosenbusch (1974) by Gamazo and Moriyon, (1987) was used for cell envelop preparation. Acetone killed bacteria were sedimented by centrifugation (7500 Xg, 15 minutes at 4°C), washed twice with saline and suspended in a small amount of 10 mM HEPEs- 5mM Mg Cl2. The cells were then disintegrated with cell homogenizer and after removal of the unbroken cells by low-speed centrifugation, the cell envelop fraction was collected by ultracentrifugation (80000 Xg, 2 hours), suspended in 10 mM Trihydrochloride (pH 7.5), and stored at -20°C till used.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was employed to check the purity of cell envelop proteins preparation and to detect its molecular weight. The cell envelop proteins preparation was stained by Coomassie blue stain and analysed in SDS-PAGE according to Sambrook, et al. (1989).

Serum samples: These were collected from 35 naturally infected sheep from a known flock with *Brucella melitensis* infection. These animals were culture positive for *Brucella melitensis* biovar 3. Negative control sera were collected from 80 culture negative sheep maintained in Brucella free unvaccinated flock.

Serological tests

- **Conventional serological tests:** These included Rose Bengal test (RBT), Buffered acidified plate test (BAPT), Rivanol test (Riv. T.) and Tube agglutination test (TAT) and applied according to Alton et al. (1988). The antigens of these tests were obtained from Veterinary Vaccines and Sera production and Researches institute, Abbassia.

- **Indirect ELISA:** Ovine serum samples were tested by ELISA for antibody reactivity using antigens prepared from whole cell sonicated (WCS) and cell envelop (CE) of *Brucella melitensis* biovar 3. Ninty six wells polystylene plates were coated with 100 µL of 0.1 mg/ml WCS or 0.1 mg/ml CE in phosphate buffer saline (PBS), pH 7.2, for 18 hours at room temperature (100 µL per well) as recommended by Tabatabia and Deyoe (1984). Tested sera were diluted 1:100 in PBS containing 0.05% Tween 20 (PBS-T). After incubation for 2 hours at 37°C, binding of antibodies was detected by using peroxidase-Labeled rabbit anti-sheep immunoglobulin G (IgG) diluted 1/10000 in PBS-T. After incubation for 1 hour at room temperature plates were filled with a substrate solution containing 4 mM H_2O_2 and 1 mM ABTS (2, 2-azeno-di-C3-ethylbenzthiazoline- sulfonic acid) in 50 mM sodium citrate, pH 4.2. Washing between incubation periods were performed with 0.9% NaCl solution con-

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taining 0.005% Tween 20 (NaCIT). Plates were shaken for 1 hour at room temperature and optical density values at 414 were recorded within automatic ELISA reader.

The samples considered positive in a cut off value of OD ≥ 0.2.

Calculation of Sensitivity and Specificity: A (2x2) Table was designed to calculate specificity and sensitivity by using the criteria of truenegative and true-positive responders from the predetermined brucellosis status of the animals depending upon the bacteriological examination results following the steps of Baum et al. (1995) as following:-

$$\text{Sensitivity} = \frac{\text{True Positive}}{\text{True Positive + False Negative}}$$

$$\text{Specificity} = \frac{\text{True Negative}}{\text{True Negative + False Positive}}$$

The Gold Standard used in this study for true positive animals of Brucella infection was positive bacteriological examination even for those animals showed negative serological reactions.

RESULTS

- In this study Cell envelop extracted from *Brucella melitensis* biovar 3 and subjected to SDS-PAGE, revealed 10 protein bands of molecular weight 89.22, 53.81, 43.27, 40.73, 38.26, 36.09, 34.19, 27.02, 19.25 and 10.59 kDa (Photo, 1).
- A total of 115 ewes including 35 animals selected from a flock with a history of Brucella infection which was confirmed by positive *Brucella melitensis* cultures in addition to other 80 serologically negative animals from a non infected flock which also proved to be bacteriologically negative. The results illustrated in Table (1) and figures (1&2) revealed that the examined sera of the infected group were 100% positive in BAPA, RBT and TAT tests, while 34 (97.14%) were positive for Riv. T. However, for the sera collected from 80 from the bacteriologically negative animals, 5 (6.25%), 4 (5%), 3 (3.75%), and 5 (6.25%) were positive for BAPA, RBT, Riv. T and TAT respectively.

The serum samples of these animals were also tested by indirect ELISA using WCS and CE (Tables 2 & 3 and figures 1 & 2). All examined sera of Brucella culture positive animals (100%) showed positive results using both WCS and CE. While, from the 80 culture negative animals only 5 (6.25%) and 2 (2.5%) were positive using WCS and CE respectively.

In Brucella culture positive animals the mean optical density for positive ELISA was 0.321 in WCS and 0.551 in CE. While for culture negative animals the mean optical density for positive ELISA was 0.368 and 0.401 in WCS and CE respectively and 0.121 and 0.108 for the negative ELISA in both used antigens respectively.

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Table (1): Results of serological tests among the examined sera collected from bacteriologically positive and negative sheep

Bacteriological status	BAPAT		RBT		Riv.T.		TAT	
	No.	%	No.	%	No.	%	No.	%
Culture Positive (35)	35	100	35	100	34	97.14	35	100
Culture negative (80)	5	6.25	4	5	3	3.75	5	6.25
Sensitivity	100%		100%		97.14		100%	
Specificity	93.75%		95%		96.25%		93.75%	

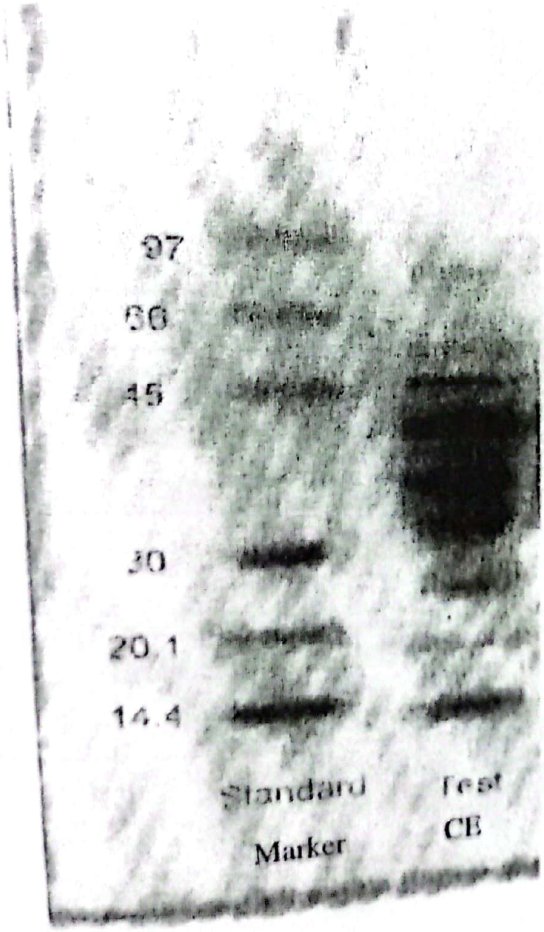

Photo (1): SDS-PAGE analysis of Cell envelop extracted from ***Brucella melitensis*** biovar 3.

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Table (2): Results of ELISA using *Brucella melitensis* WCS coated plates among the examined samples.

Bacteriological status	Positive for ELISA			Negative for ELISA			Sensitivity	Specificity
	No. of positives	%	Mean optical density	No. of positives	%	Mean optical density		
Culture Positive 35	35	100	0.321	0	0	0	100%	93.75%
Culture negative 80	5	6.25	0.368	75	93.75	0.121		

*Cut off value for OD ≥ 0.2

Table (3): Results of ELISA using *Brucella melitensis* CE coated plates among the examined samples.

Bacteriological status	Positive for ELISA			Negative for ELISA			Sensitivity	Specificity
	No. of positives	%	Mean optical density	No. of positives	%	Mean optical density		
Culture Positive 35	35	100	0.551	0	0	0	100%	97.5%
Culture negative 80	2	2.5	0.401	78	97.5	0.108		

*Cut off value for OD ≥ 0.2

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Figure (1): Prevalence of Brucela positive reactors to the used serological tests among the Culture Positive animals (35)

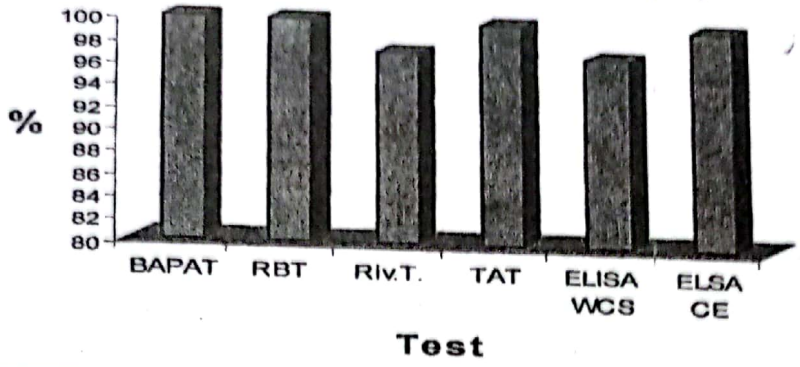
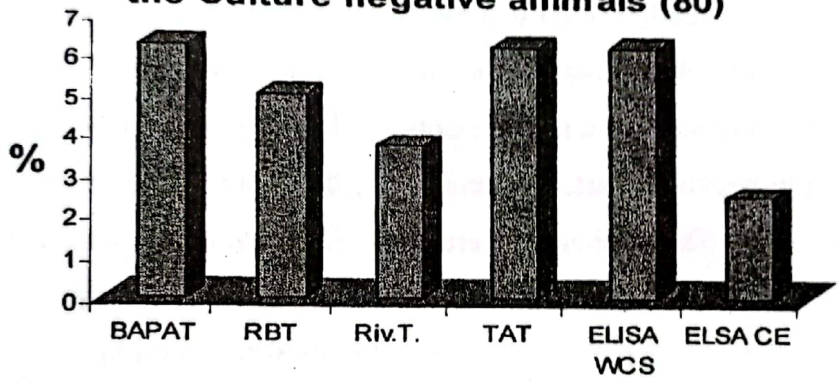


Figure (2): prevalence of Brucella positive reactors to the used serological tests among the Culture negative animals (80)



DISCUSSION

Definitive diagnosis of ovine brucellosis requires lanoratory confirmation as clinical finding such as abortion in females and infertility in males are not specific to this disease (Young, 1994). Diagnosis of *Brucella melitensis* infection in sheep is largely depends upon both bacteriological examination and serological methods (Nielsen et al., 2004).

In the present study BAPAT, RBT, Riv. T and TAT were used as serological tests for diagnosis of *brucillosis* .The false positive reactions comparing with the results of bacteriological examination as a gold standard were 5 (6.25%), 4 (5%), 3 (3.75%), and 5 (6.25%) animals in these tests respectively (Table, 1 and Figures 1 & 2). The variation between the results of these tests was also reported by many authors including Moyer et al. (1987), Baum et al. (1995) and Shalaby et al. (2003), they found that the lower specificity and

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the false positive reactions of agglutination tests were not uncommon. The sensitivity of BAPAT, RBT, Riv. T and TAT Tests in this study were 100%, 100%, 97.14 and 100% respectively, while their specificity were 93.75%, 95%, 96.25% and 93.75% respectively. Blasco et al. (1994a) showed that antigens for brucellosis diagnosis of sheep and goats in many used official tests showed wide variation in composition and differences in sensitivity. They found that RBT and CFT sensitivities found in their work are lower than those generally reported. There are some problems of the specificity of serological tests for sheep brucellosis since antibodies against *Brucella melitensis* epitopes may be present in the animal population due to vaccination and/or of contacts with other Gram-negative bacteria (mainly, Yersinia enterocolitica O:9) sharing cross-reactive epitopes with Brucella (Garin-Bastuji et al., 2006).

Results of SDS-PAGE of cell envelop extracted from *Brucella melitensis* biovar 3 revealed 10 protein bands of molecular weight 89.22, 53.81, 43.27, 40.73, 38.26, 36.09, 34.19, 27.02, 19.25 and 10.59 kDa. This step was performed to ensure the purity of the prepared cell envelop antigen comparing with other workers. The results were comparable with those of Goldbaum et al. (1992). They found that among the immunodominant antigens, some belong to the cell envelope and correspond to both major outer membrane proteins (OMPs, 25 to 27 kDa and 36 to 38 kDa) and minor OMPs (10 kDa, 16.5 kDa, 19 kDa, and 89 kDa).

Among the 80 Brucella culture negative animals 5 (12.5%) and 2 (5%) were positive for ELISA using WCS and CE respectively (Tables, 2 &3 and Figures 1 & 2). Enzyme linked immunsobent assay using purified antigens and/or monoclonal antibodies have developed in order to eliminate the problem of lower specificity (Oncel, 2005). This technique has been evaluated for many years for their diagnostic performance to detect serum antibodies to Brucella species. Indirect ELISA have several advantages being that the antibodies to be detected reacts with the antigen without performing a secondary function such as agglutination, precipitation or activation of complement (Nielsen and Kwok, 1995). A variety of *Brucella melitensis* surface antigens contribute in the diagnosis of infection in sheep. Although the internal antigen may be considered as an excellent antigen for its specificity, its practical use seems to be limited since the antibodies can not detected in the early stages of infection (Serikawa et al., 1989). LPS is commonly used as an antigen in most indirect ELISA formate (Nielsen and Gall, 1994). Nonspecific cross-reacting antibodies in uninfected animals against Brucella Lipopolysaccharidies (LPS) have been shown due to several different pathogens including *Escherichia Coli, Yersinia enterocolitica* and *Pseudomonas salanacearum* (Lamb et al., 1979 and Nielsen and Duncan 1982). The cell envelop of Brucella spe-

cies is composed of Lipopolysaccharides (LPS), phospholipids, peptidoglycan (PG) and several proteins (Cloeckaert et al., 1992).

There was a marked difference in the antibody response (OD values) of positive sera against CE and those against WCS. The mean antibody response for ELISA positive samples with CE antigen was 0.401-0.551OD and with WCS as an antigen was 0.321-0.368 OD. While mean antibody response for negative ELISA samples with CE antigen was 0.108 OD and with WCS as an antigen was 0.121 OD (Tables 2 &3). The observes higher values of mean OD readings of ELISA for both positive and negative samples when using CE as an antigen may be explained by the nature of the CE as it is a LPS-protein complex (Cloeckoert et al., 1992).

The sensitivity of ELISA with both WCS and CE antigens was 100%. It is clear that WCS and CE detect all culture positive sheep. The specificity of ELISA for WSC was 93.75% and for CE was 97.5% (Tables 2 &3). WHO, (1980) reported that the use of whole bacterial cells in which, there is a large amounts antigen determents as antigens for brucellosis diagnosis in ELISA is accompanied by limitation in their sensitively and specificity. In the other hand sonication of cells is reported to improve the quality of the produced antigen as it helps in removal cellular detritus and also to disaggregate possible clumps due to repeated centrifugations and to improve accessibility of OMPs. On sonicated cells, the binding of the anti- Omp25 MAbs was slightly increased (Cloeckaert et al., 1996). Sonicated antigens were also used in dermal sensitivity tests to prevent the primary toxicity caused by Brucella OMP containing LPS (Winter et al., 1983).

It is clear that using CE as an antigen for ELISA detects all culture positive sheep for Brucella (100% sensitivity) without reduction in detection of negative culture sheep (specificity) and achieves higher antibody response in positive animal sera. This is may be due to the fact that most detectable important protein found within the Brucella cell envelop. Carle et al. (2006) found that all virulent Brucella proteins were detected in the membranes, some of them localized in characteristic patterns in the cell envelope.

In conclusion CE seems to be more effective for diagnosis of *Brucella melitensis* infection in sheep.

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إستخدام الغلاف الخلوى للبروسيلا كانتيجين فى تشخيص البروسيلا فى الأغنام

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استخدم في هذه الدراسة عدد ٣٥ اغنام ايجابية وعدد ٨٠ اغنام سلبية للعزل البكتريولوجي لميكروب البروسيلا وذلك لتقييم استخدام المستخلص الكامل للخلية باستخدام الموجات الصوتية وكذلك الغلاف الخلوي كانتيجينات مستخلصة من البروسيلا مليتنسيز النوع (٣) في اختبار الاليزا. وقد تم اختبار عينات من الحيوانات السابقة بالاختبارات السيرولوجية للبروسيلا ومنها اختبار الروزبنجال والاختبار الشريحي المحمض التعادلي واختبار الريفانول واختبار التلزن الانبوني. وقد اظهرت النتائج ان ٣٥ (١٠٠%) من حيوانات المجموعة الايجابية للعزل البكتريولوجي كانت ايجابية باختبارات الروزبنجال والشريحي المحمض التعادلي والتلزن الانبوني بينما كان هناك ٣٤ عينة (٩٧,١٤%) ايجابية باختبار الريفانول، وعلى الجانب الآخر في مجموعة الحيوانات السلبية للعزل البكتريولوجي لميكروب البروسيلا كان هنا ٧(٨,٧٥%) و٨ (١٠)% و٥ (٦,٢٥%) و٨(١٠%) ايجابية لاختبارات الروزبنجال والشريحي المحمض التعادلي والريفانول والتلزن الانبوني على الترنيب. وتم بعد ذلك اختبارنفس العينات باختبار الاليزا وذلك باستخدام الانيجينات المحضرة من كل من المستخلص الكامل للخلية باستخدام الموجات الصوتية وكذلك الغلاف الخلوي وكانت النتيجة ايجابية لعدد ٣٥ (١٠٠%) باستخدام النوعين من الانتيجينات اما في المجموعة السلبية للعزل البكتريولوجي للبروسيلا فكانت ايجابية لعدد ٥ (٦,٢٥%) و ٢ (٢,٥%) للنوعين من الانيجينات على الترتيب وكانت الحساسية للنوعين ١٠٠% اما الخصوصية فكانت ٩٣,٧٥% و٩٧,٥% على الترتيب، وبذلك كان من الواضح من هذه الدراسة ان استخدام الغلاف الخلوى لميكروب البروسيلا يمكن ان يكون اكثر ملائمة في تشخيص العدوى بميكروب البروسيلا مليتنسيز في الاغنام.