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INDIRECT AND COMPETITIVE ELISA AS A TOOL FOR DIAGNOSIS OF BRUCELLOSIS IN VACCINATED AND INFECTED CATTLE

(With 3 Tables)

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استخدام اختبارات الاليزا الغير مباشرة والتنافسية المختلفة
لتشخيص البروسيلا فى الأبقار المحصنة والمصابة

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فى هذه الدراسة تمت مقارنة نتائج اختبار الاليزا الغير مباشر والاليزا التنافسى بنتائج الاختبارات السيرولوجية التقليدية للكشف عن وجود اجسام مضادة لميكروب البروسيلا مليتنسس. وبفحص 360 عينة سيرم لحيوانات مصابة وغير محصنة أشارت النتائج الي أن حساسية اختبار الاليزا الغير مباشرهي 98.3% ، وكانت نسبة التخصصية في 270عينة سيرم لحيوانات خالية من البروسيلا هى 98.1%. وحينما استخدم اختبار الاليزا التنافسي لنفس العدد من عينات السيرم للمجموعتين كانت النتائج بنسبة 97.2% تخصصية و100% حساسية علي التوالي. عند مقارنة خمس اختبارات سيرولوجية علي 180 عينة سيرم لحيوانات مصابة ومحصنة كانت نسبة التخصص 97.7% باستخدام اختبار الاليزا التنافسى و 93.8% لاختبار التلزن الأنبوبى المتحور باضافة الايديتا ، و88.8% باستخدام اختبار الروز بنجال و 82.7% باستخدام اختبار التلزن الأنبوبى و 72.2% لاختبار الاليزا الغير مباشر. من هذه الدراسة يمكن أن نستخلص أن: استخدام اختبار الروزبنجال يعطي نتائج ايجابية كاذبة أقل من التنائج المعطاه باستخدام اختبار الاليزا الغير مباشر في الحيوانات المحصنة، وأن اختبار الاليزا التنافسي هو اختبار تأكيدى جيد بالاضافة لأهميته للتفرقة بين الأجسام المناعية الناتجة عن التحصين من تلك التي تنتج عن العدوى بميكروب البروسيلا المعزول في مصر (بروسيلا مليتنسس).

SUMMARY

The results of an indirect ELISA (iELISA) and a competitive ELISA (cELISA) for detection of antibody to *Brucella melitensis* in cows were compared with those of conventional serological tests. The sensitivity of iELISA using 360 sera from infected not vaccinated animals was 98.3% and the specificity in 270 sera from brucellosis-free animals was 98.1%. The cELISA when tested in the same numbers of sera of the two groups

were 97.2% Specific and 100 % sensitive respectively. When comparing five serological tests on 180 sera from vaccinated-infected animals, the specificity was 97.7% for cELISA, 93.8% for EDTA-modified SAT, 88.8 % for RBPT, 82.7% for SAT and 72.2 % for iELISA. It can be concluded from this study that the RBPT gave less false positive results than the iELISA in vaccinated animals and the cELISA is a good confirmatory test with the advantage of distinguishing the antibody response due to vaccination from that resulting from infection with the local Brucella isolates(*Brucella melitensis*) in Egypt.

Key words:

INTRODUCTION

Serological detection of antibodies is usually the method of choice for control and eradication of bovine brucellosis. Several conventional serological tests have been used as a single or in combination for the diagnosis of this disease (FAO/WHO, 1986). A rapid screening test of high sensitivity is usually applied initially for the testing of sera in control programs. A positive reaction in the screening test would result in the serum being tested in a confirmatory test of higher specificity.

In most countries, agglutination tests such as the Rose Bengal plate test (RBPT) and the buffered plate antigen test (BPA) are currently used as screening tests, while other agglutination tests such as the 2-mercaptoethanol test (2ME), the Rivanol test (RIV), the EDTA modified SAT and the slow tube agglutination test (SAT) are regularly used as confirmatory tests. However, the agglutination techniques may have limitations in sensitivity resulting from prozoning (Alton *et al.,* 1975) or non-specificity because of cross-reactivity. In the first case, the result can be missed detection of positive animals and, in the second, false-positive reactors are detected due to the presence of common antigenic determinants between *B. abortus* and several other bacteria (Hurvell and Lindberg, 1973; Corbel, 1975 and Marino *et al.,* 1991).

The diagnosis of brucellosis in cattle is frequently complicated particularly when live vaccines such as strain 19 of *B. abortus* are used on a large scale. The occurrence and persistence of serum antibodies following *Brucella* strain 19 vaccination is the major disadvantage since antibodies may interfere with detection of brucellosis infected cattle. The antibody response induced by these vaccines is difficult to distinguish from that of natural infection by conventional serological tests (FAO/WHO, 1971). Several supplementary serological tests such the

agglutination with 2 mercaptoethanol, complement fixation and agar-gel immunodiffusion containing a soluble polysaccharide antigen have been shown to differentiate to some extent the antibody response of vaccinated from infected animals (Diaz *et al.,* 1979; Alton *et al.,* 1988).

In the recent past, indirect enzyme linked immunosorbent assay (iELISA) and competitive enzyme linked immunosorbent assay (cELISA) have been much popular and extensively used. Despite excellent performance and superiority of iELISA over conventional serological tests for diagnosing brucellosis in various species of animals the assay is not able to distinguish animals infected with virulent *Brucella* or vaccinated with *B. abortus* strain 19 vaccine. The cELISA is a multi-species assay which has the ability to determine the antibody to *B. abortus* in various species of animals and generally do not react with sera containing residual antibody from vaccination with *B. abortus* strain 19. However, in Egypt *B.melitensis* organism is the main isolate from different species of animals infected with Brucella (Refai, 2002). Therefore, we must distinguish between antibodies due to infection with *B. melitensis* and that of *B. abortus* strain 19 vaccine.

The objective of the present study was to validate the diagnostic performance characteristics of the competitive ELISA to the indirect ELISA and to the standard serological techniques on sera from negative, infected not vaccinated and *B. abortus* strain 19 vaccinated-infected cattle.

MATERIALS and METHODS

Test Sera:

Negative sera: A total of 270 sera were obtained from herds in an area without recent history of infection or vaccination against brucellosis.

Positive sera: A total of 540 serum samples were obtained from herds from which *B. melitensis* was isolated. These sera were segregated into two groups:

1- 360 sera from herds infected not vaccinated with *Brucella* strain 19 vaccine.

2- 180 serum samples from vaccinated cows with *Brucella* strain 19 vaccine which showing some abortions and reproductive disorders.

Serological Tests:

1- Conventional tests:

The tests used were the RBPT and the SAT as described by (Alton *et al.,* 1988) and the EDTA modified SAT according to (MacMillan, 1990). In the RBPT any degree of agglutination was considered to be

positive. For the SAT, visible agglutination at the dilution of 1/40++ or more was considered to be positive and for the EDTA modified SAT, visible agglutination at the dilution of 1/10 was considered to be positive.

2- Indirect ELISA:

An ELISA kit (Brucelisa) provided by the VLA, (an executive agency of the Department for Environment, Food and Rural Affairs), which contained all the necessary reagents was used. The test was performed according to the manual which is accompanied with the kit. Briefly, a primary dilution of 1/40 of all test and control sera were made by adding 25 µl of serum to 1 ml. of diluting buffer (5 Tablets of Phosphate Buffer Saline (PBS),0.5 ml of phenol red indicator and 250 µl of Tween 20 to 500 ml of distilled water). The plate was prepared by adding 80 µl of diluting buffer to all wells. Then, 20 µl of each of primary diluted samples were added to all prepared wells except columns 11 and 12 (this gives a final dilution of 1/200). Twenty microns of the primary diluted positive control were added to each of the wells in column 11 and 20 µl of the primary diluted negative control were added to each of the wells in column 12 except H12 which should have no sample added as it is to be used to blank the plate. Then, the plate was covered with the lid and incubated at room temperature for 30 minutes on a rotary shaker. The contents of the plate were shaked out and rinsed 5 times with washing solution (one ampoule of Na_2HPO_4 and 1 ml of Tween 20 to 10 liters of distilled water) and then thoroughly dried by tapping the plate on absorbent paper towel. The diluted conjugate solution, 100 µl were added to all wells. The plate was covered with a lid and incubated for 30 minutes on a rotary shaker at 160 revs/min. Then the plate was shaked out, rinsed 5 times with washing solution and then thoroughly dried (as previously described). Microplate reader was switched on and allowed to be stabilized for 10 minutes. A hundred microns of substrate solution (300 µl of ABTS chromogen to 12ml. of substrate buffer plus 60 µl of substrate (hydrogen peroxide) were added to each well and the plate was left at room temperature for a minimum of 10 minutes and a maximum of 15 minutes. To each well, 100 µl of stopping solution (one ampoule of sodium azide with 500 ml of distilled water) were added, condensation of the bottom of the plates was removed with an absorbent paper towel. Finally the pate was read at 450 nm blanked on well H12.

Analysis of the results: Colour development within a well indicated that the sample tested had antibodies to *Brucella*. A positive/negative cut-off was calculated as 10% of the mean of the optical density (OD) of the 8

positive control wells. Any test sample giving an OD equal to or above this value should be considered as being positive.

3-Competitive ELISA:

An ELISA kit (Compelisa) provided by the VLA, (an executive agency of the Department for Environment, Food and Rural Affairs), which contained all the necessary reagents was used. The test was performed according to the manual which is accompanied with the kit. Briefly, the conjugate solution was prepared and diluted to working strength with diluting buffer (5 tablets of PBS), 0.5 ml of phenol red indicator and 250 µl of Tween 20 to 500 ml of distilled water). For each tested serum, 20 µl were added per well. The columns 11 and 12 were left as controls of which 20 µl of the negative one were added to wells A11,A12, B11,B12,C11 and C12, while 20µl of the positive one were added to wells F11,F12,G11,G12,H11 and H12. The remaining wells of the columns 11 and 12 that have no serum added, act as the conjugate controls. To all wells, 100µl of the prepared conjugate solution were dispensed which give a final serum dilution of 1/6. Then the plate was vigorously shaken on the microtitre plate shaker for 2 minutes. The plate was covered with the lid and incubated at room temperature (21˚C ± 6 ˚C) for 30 minutes on a rotary shaker at 160 revs/min. The contents of the plate were shaked out and rinsed 5 times with washing solution (one ampoule of Na_2HPO_4 and 1 ml of Tween 20 to 10 liters of distilled water) and then thoroughly dried by tapping on absorbent paper towel. The microplate reader was switched on and allowed the unite to be stabilized for 10 minutes. The substrate and chromogen solutions were prepared immediately before use (One tablet of urea H_2O_2 in 12 ml of distilled water, when dissolved the OPD tablet was added and mixed thoroughly) of which 100 µl were added to each well. Then, the plate was left at room temperature for a minimum of 10 minutes and a maximum of 15 minutes. Slowing the reaction by adding 100 µl of stopping solution to all wells (One ampoule of citric acid with 38 ml of distilled water). Condensation of the bottom of the plate was removed with absorbent towel and then the plate was read at 450 nm.

Analysis of the results: The lack of colour development indicated that the sample tested was positive. A positive/negative cut-off was calculated as 60% of the mean of the optical density (OD) of the 4 conjugate control wells. Any test sample giving an OD equal to or below this value should be regarded as being positive.

Sensitivity and Specificity: The sensitivity of tests used on sera from infected cattle and the specificity in brucellosis free animals was

calculated according to Thrusfield (1986) and followed the formulae for each test:

$$\text{Sensitivity} = \frac{\text{Total number of positive results}}{\text{Total number of positive animals sampled}}$$

$$\text{Specificity} = \frac{\text{Total number of negative results}}{\text{Total number of negative animals sampled}}$$

Bacteriological Evaluation:

Milk samples (n=130) were collected under sterile conditions (30 samples from Brucella free animals, 40 samples from infected not vaccinated ones and 80 samples from vaccinated cows showing some abortions and reproductive disorders. All samples were cultured in selective solid media and the suspected isolates were identified as *B. melitensis* according to MacMillan (1990).

RESULTS

Negative sera:

The specificities of the five serological tests performed on sera from 270 brucellosis-free cows are presented in Table (1).

Positive sera:

1- The sensitivities of the five serologic tests performed on 360 *Brucella*-infected not vaccinated animals are presented in Table (2).

2- The specificity of the five serological tests performed on 180 vaccinated *Brucella*-infected animals are presented in Table (3).

Table 1: The Specificity of Different Tests on 270 Sera From Brucellosis-Free Cows.

Test	Negative	Positive	Specificity
RBPT	270	0	100%
SAT	268	2	99.2%
EDTA-m-SAT	270	0	100%
iELISA	265	5	98.1
cELISA	270	0	100%

RBPT=Rose Bengal Plate Test

SAT=slow tube agglutination test
EDTA-m-SAT= EDTA modified SAT
iELISA=-Indirect ELISA
cELISA=Competitive ELISA

Table 2: The Sensitivity of Different Serologic Tests on 360 Sera from *Brucella*-Infected Not Vaccinated Cows.

Test	Positive	Negative	Sensitivity
RBPT	360	0	100%
SAT	304	56	84.4%
EDTA-m-SAT	339	21	94.1%
iELISA	354	6	98.3%
cELISA	350	10	97.2%

(See Table 1 for key)

Table 3: The Specificity of Different Serological Tests on 180 Sera from Strain 19 Vaccinated- Infected Cows.

Test	Positive	Negative	Specificity
RBPT	20	160	88.8%
SAT	31	149	82.7%
EDTA-m-SAT	11	169	93.8%
iELISA	50	130	72.2%
cELISA	4	176	97.7%

(See Table 1 for key)

Bacteriological Results:

Sixteen *Br.melitensis* biovar 3 were isolated from 130 milk samples where no isolates from Brucella free animals, 12 isolates from infected not vaccinated ones and 4 isolates from vaccinated cows showing some abortions and reproductive disorders. Sera from animals from which by Alton *et al*. (1988) were isolated showing positive results in all the above mentioned serological tests.

DISCUSSION

A simple, rapid and inexpensive serological test that will detect infected animals early in the incubation period and at all stages of the disease and that does not detect antibody in vaccinated animals is still to be found. Nevertheless, a great deal of improvement was achieved recently either by the introduction of enzyme immunoassays (Wright *et al.,* 1990) or by vaccination of animals with reduced dose of Strain 19 *B. abortus* vaccine (Plommet and Fensterbank, 1984).

In the present study two different enzyme immunoassays were compared with conventional tests on sera of negative, infected not vaccinated and vaccinated-infected animals. The specificity of the enzyme immunoassays was high, 98.1% for the iELISA and 100% for the cELISA on sera from brucellosis-free animals (Table 1). In this situation, the iELISA was the test which gave the highest rate of false positive reactions (Nielsen, 1995; Saravi *et al.,* 1995; OIE, 1996).

The discrepancy between the higher number of reactors detected by RBPT than the other tests (Table 2) is due to the fact that RBPT is a highly sensitive test which can detect low titre as in cases of chronic brucellosis, that not be considered as positive by the quantitative tests. (Nicoletti and Milward, 1983).

From the above mentioned results, the difference between SAT and EDTA modified SAT in such sera are though to contain IgM molecules which cause agglutination of *B.abortus* test antigen in a nonimmune manner, namely by binding by Fc region. This binding is inhibited by EDTA, while specific binding by the IgM, Fab region (induced by infection with *Brucella* organisms) is unaffected (MacMillan, 1990). The sensitivities of the ELISAs were 98.3% for the indirect and 97.2% for the competitive in infected not vaccinated animals (Table 2). Except for the RBPT, the two ELISAs were more sensitive than the other tests. These findings are in accordance with others (Sutherland, 1984; Dohoo *et al.,* 1986; Uzal *et al.,* 1996; Al-Farwachi *et al.,* 2009) in that RBPT was found to be a good screening test, although some authors (Saravi *et al.,* 1990) have found an unacceptable false negative rate with the RBPT.

In the vaccinated -infected group (Table 3), the iELISA was the most sensitive test, giving a high percentage of positive results. This may be explained by the use of a polyclonal antibovine IgG (H+L) conjugate which measures all isotypes present in the sera. On the other hand, the competitive ELISA with *B. melitensis* coated plate differentiates 4 animals, from which *B.melitensis* organisms were isolated, from *Brucella*

strain 19 vaccinated group. Some authors speculate that it can be a result of antigen presentation on the test and the antibody affinity (Wright *et al.,* 1990; Nielsen *et al.,* 1992; Uzal *et al.,* 1996; Aguirre *et al.,* 2002; Chand and Puran, 2006).

The results presented above raise the conclusion, it is advisable to use the RBPT as a screening test and cELISA as a confirmative test in those areas where strain 19 vaccination is routinely applied. On the other hand in areas free of vaccination perhaps the most advisable test as a confirmative one could be the iELISA because of its ability to detect small amount of IgG1.

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