Veterinary Research 5 (2): 31-36, 2012
ISSN: 1993-5412

Comparative Studies of Different Serological Tests for Diagnosis of Brucellosis in Vaccinated Sheep with Special Reference to Competitive ELISA

[1]Al-Hassan M. Mustafa, [2]Mahmoud R. Abd Ellah, [3]Essam El-Din M. Elbauomy and [2]Ali H. Sadiek
[1]Animal Health Research Institute, Assiut, Egypt
[2]Department of Animal Medicine, Faculty of Veterinary Medicine, Assiut University, Egypt
[3]Department of Brucellosis, Animal Health Research Institute, Dokki, Giza, Egypt

Abstract: The purpose of the current study was to compare different serological test for diagnosis of brucellosis in vaccinated sheep. A total of 40 sheep that belonged to a governmental farm at El-Badary city, Assiut Governorate, Egypt were subjected to study during the period from May 2009 to January, 2010. Sheep (8-12 months old) that proved free from brucella infection were vaccinated and included in the study. A total of 190 blood serum samples were aseptically collected from animals under investigation before vaccination and at days 30, 90, 150 and 240 post-vaccination. Generally the number of positive reactors after vaccination where higher by using Buffered Acidified Plate Antigen Test (BAPA), Rose Bengal Plate Test (RBPT) and Rivanol Test (Riv. T) than Competitive Enzyme Linked Immunosorbent Assay (cELISA). The number of positive reactors was 12, 10, 16 and 16 at 30, 90, 150 and 240 days, respectively by using BAPA, RBPT and Riv. T. On the other hand, the numbers of positive reactors were 7, 3, 13 and 13 at 30, 90, 150 and 240 days, respectively by using cELISA. It could be concluded that differentiation between naturally infected sheep with brucellosis and vaccinated ones still acts as a major problem in sheep herds at 1st month post vaccination. Conventional tests cannot differentiate between vaccinated and infected sheep. The cELISA test gives accurate results at 90 days or more post vaccination.

Key words: Brucella, sheep, cELISA, vaccine, post vaccination, Rivanol test, Egypt

INTRODUCTION

Sheep brucellosis is an important, worldwide spreading infectious zoonotic disease affecting both animal and human as well and causes great economic loses. *Brucella melitensis* is a cause of important diseases of small ruminants: abortions, stillbirths, retention of fetal membranes and post-parturient genital infections (Kimberling, 1988; Hugh-Jones *et al*., 1995; Corbel, 1997; Garin-Bastuji *et al*., 1998).

The definitive diagnosis for brucellosis requires the recovery of the organism however, it is difficult to recover from life infected animals therefore, diagnosis has been based mostly on the results of serological tests (Hamdy, 1997).

A panel of tests has been validated and approved for diagnosing brucellosis in cattle (Office International Epizooties, 2004). For ovine and caprine brucellosis caused by *Brucella melitensis* only the Rose Bengal Plate Test (RBPT) and the Complement Fixation Test (CFT) are currently accepted by the European Union and the Office International des Epizooties (European Council Directive, 1991; Office International Epizooties, 2004). The lack of Diagnostic Sensitivity (DSn) of both the RBPT and CFT makes implementation of a test and slaughter policy for brucellosis eradication in small ruminants less effective than in cattle (Nicoletti, 1969). Efforts to improve serological diagnosis of brucellosis in small ruminants have led to the development of new tests such as the indirect Enzyme-Linked Immunosorbent Assay (iELISA), Fluorescence Polarisation Assay (FPA) and Competitive ELISA (cELISA) (Blasco *et al*., 1994; Jacques *et al*., 1998; Nielsen and Gall, 2001). All of these tests appear to be more efficacious than the RBPT and CFT in identifying infected animals.

Competitive ELISA was initially developed to improve the Diagnostic Specificity (DSp) of immunoassays for brucellosis especially when sera were tested from vaccinated cattle (Nielsen *et al*., 1989; MacMillan, 1990). Since, the cELISA does not use a species specific immunoglobulin conjugated with enzyme, it can easily be adapted for detecting *Brucella* sp. infections in deferent animal species (Gall and Nielsen, 1994; Nielsen *et al*., 1995, 1996). The purpose of the current study was to compare different serological test for diagnosis of brucellosis in vaccinated sheep.

Corresponding Author: Al-Hassan M. Mustafa, Animal Health Research Institute, Assiut, Egypt

MATERIALS AND METHODS

Animals: A total of 40 sheep that belonged to a governmental farm at El-Badary city, Assiut Governorate, Egypt were subjected to study during the period from May 2009 to January, 2010. Sheep (8-12 months old) that proved free from brucella infection by using RBPT, TAT, BAPA and Riv. T were vaccinated and included in the study.

Samples: A total of 190 blood serum samples were aseptically collected from animals under investigation during the period of study. Blood samples were collected before vaccination and at days 30, 90, 150 and 240 post-vaccination. Blood samples (10 mL) were obtained from each examined animals by using a double jack needle inserted in the jugular vein after cleaning and disinfection of the site of the puncture with tincture iodine. Samples were were centrifuged at 3000 rpm for 15 min. The collected sera were transferred into sterile tubes which were coded and kept at -20°C up to the time of the test (Alton *et al*., 1988).

Vaccine: The used Rev. 1 vaccine was Ocurev vaccine (CZ Veterinaria S.A. Reg. No.: 1481 ESP) which is an attenuated, smooth strain of *Brucella melitensis*, streptomycin non-dependent, isolated from streptomycin dependent cells which was obtained from the virulent strain 6056. Each dose (1 drop) of the reconstituted vaccine contains: 1-2×10^9 cfu of *Brucella melitensis*, Rev. 1 in smooth phase, Patent Blue V (E-131)…… 0.01%.

Serological tests

Rose Bengal Plate Test (RBPT): The technique was carried out according to Morgan (1967). The test was done at one dilution, any degree of agglutination within 4 min (≥25 IU mL^{-1}) was considered as positive.

Buffered Acidified Plate Antigen Test (BAPAT): The technique was carried out according to Alton *et al*. (1988). The test was only done at one dilution, any agglutination within 8 min (≥20 IU mL^{-1}) was considered positive.

Rivanol Test (Riv. T): The technique was carried out according to Alton *et al*. (1988). The sample considered positive when agglutination occur at any dilution.

Competitive Enzyme Linked Immunosorbent Assay (cELISA) method: The test was done using COMPELISA kit obtained from Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15 3NB United Kingdom.

RESULTS AND DISCUSSION

Generally, the number of positive reactors after vaccination where higher by using BAPA, RBPT and Riv. T than cELISA. The number of positive reactors was 12, 10, 16 and 16 at 30, 90, 150 and 240 days, respectively by using BAPA, RBPT and Riv. T. On the other hand, the numbers of positive reactors were 7, 3, 13 and 13 at 30, 90, 150 and 240 days, respectively by using cELISA (Table 1).

At the 30 days post vaccination, only 30% of vaccinated sheep showed positive reaction with BAPA, RBPT and Riv. T while cELISA gave 17.5% positive reactors of vaccinated sheep.

At 90 days post vaccination, only 25% of vaccinated sheep showed positive reaction with BAPA, RBPT and Riv. T while cELISA gave 7.5%.

At 150 days post vaccination, abrupt rise in the number of positive cases were obtained by BAPA, RBPT and Riv. T which showed 40% positive reactors; 32.5% true positive (positive isolation) and 7.5% false positive (negative isolation) while cELISA test showed 32.5% positive reactors.

At 240 days post vaccination, the same occurred as at 150 days but the number of vaccinated animals became only 30 animals as 10 of them were slaughtered due to infection.

The principal objective of using the serological tests in brucellosis control and eradication programs is to detect infected animals that may cause spread of the disease. One of the most important drawbacks of serological testing is the existence of false positive reactors that appear as a result of vaccination.

Although, conventional serological techniques suffer from several drawbacks, poor performance and lack of standardization, RBPT has been used as a screening test of Brucella infection (MacMillan, 1990). The RBPT had been implemented in diagnosis of brucellosis in small ruminants however, the specificity and sensitivity of the RBPT in sheep and goats are still unclear (Blasco *et al*., 1994; Erganis *et al*., 2005).

The serological responses following infection with smooth *Brucella* species are directed predominantly against the Smooth Lipopolysaccharide (S-LPS). Thus, in humans as well as in animals, the diagnosis of brucellosis is usually based on the detection of specific antibodies against S-LPS (Alton *et al*., 1988; Wright and Nielsen, 1990).

Enzyme Linked Immunosorbent Assay (ELISA) for diagnosis of brucellosis has several advantages when compared with other tests. Firstly, it is a direct method of identification of specific antibody and therefore, it is not prone to false positive reactions. Secondly, it is more

Table 1: Number of positive and negative reactors at different times post vaccination

	BAPA		RBPT		Riv. T		*cELISA	
Days	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
0	-	40	-	40	-	40	-	30
30	12	28	12	28	12 1/400	28	7	33
90	10	30	10	30	10 1/200	30	3	17
150	(13 true) (3 false)	24	(13 true) (3 false)	24	16 1/200	24	13	7
240**	(13 true) (3 false)	14	(13 true) (3 false)	14	16 1/400	14	13	7

*Only 130 of representative blood serum samples (30, 40, 20, 20 and 20 blood samples at days 0, 30, 90, 150 and 240, respectively were subjected to cELISA test; **The no. of animal became 30 at 240 days post vaccination because 10 of them were slaughtered with the history of abortion

sensitive than other agglutination tests and thus has the potential to detect infected animals. Thirdly, the antibody enzyme conjugate employed has light chain reactivity and thus is able to detect all classes of antibody. A combine determination of all classes of antibody allows accurate serological diagnosis at any stages of disease. Fourthly, ELISA results provide an epidemiological tool for investigating the infective status of flocks (Rahman, 2003). In addition, the enzyme immunoassays are objective and easy to perform and may be automated to permit the processing of a large number of sera within a short time (Delgado *et al*., 1995).

During the period of the study some vaccinated sheep became seropositive. This is suggestive that they came in contact with field strain of *Brucella* sp. or the vaccinal strain Rev. 1.

All serum samples from this study were subjected to BAPA and RBPT tests as screening tests and Rivanol test as confirmatory test. All samples reacting positively to the BAPA, RBPT and Riv. T were submitted for cELISA test.

At the 30 days post vaccination, only 30% of vaccinated sheep showed positive reaction with BAPA, RBPT and Riv. T while cELISA gave 17.5% positive reactors of vaccinated sheep. These results are in agreement with Fensterbank *et al*. (1985) who reported that 44% positive reactors with RBPT test, Marin *et al*. (1999) who reported that 14% positive reactors with cELISA and with Jalali *et al*. (2003) who reported that 42% positive reactors with RBPT test and 22% positive reactors with cELISA test. On the other hand, the 30 days post vaccination results are disagreed with Marin *et al*. (1999) who reported that most of the vaccinated animals gave positive reactions with BAPA and RBPT tests as RBPT test gave 100% positive reactors, Stournara *et al*. (2007) who reported that 95% positive reactors with RBPT test and 85% positive reactors with cELISA test and with Al-Hankawe (2009) who reported that 100% positive reactors with RBPT test and 100% positive reactors with cELISA test.

The low percentage of positive reactors given by BAPA, RBPT and Riv. T at 30 days post vaccination may be attributed to the following causes: firstly, the primary conjunctival vaccination gave a weak response detected by conventional serological tests (BAPA, RBPT and Riv. tests) (Fensterbank *et al*., 1982). Secondly, the conjunctival route of vaccination significantly reduced the intensity and duration of post vaccination serological response and made the use of this vaccine compatible with brucellosis eradication programs (Jimenez de Bagues *et al*., 1992; Diaz-Aparicio *et al*., 1994; Marin *et al*., 1999; Aldomy *et al*., 2009). Thirdly, age of the animal; the younger the Rev. 1 vaccinated animals are, the lower serological interference is produced (Marin *et al*., 1999). Fourthly, the Rev. 1 vaccine has suffered from a lack of coordinated standardization in production methods leading to considerable variability in efficiency of different preparations (Blasco, 1997). Fifthly, breed of sheep; some breeds of sheep are more resistant to brucellosis than other breeds, i.e., not respond to post vaccination reaction; considerable variation in individual responses is to be expected (FAO, 2010). Sixthly, RBPT have low specificity when testing sera from small ruminants vaccinated subcutaneously with Rev. 1. However, when the vaccine is applied conjunctivally this problem is significantly reduced (FAO, 2010).

While the lowest percentage of positive reactors given by cELISA test at 30 days post vaccination may be attributed to the following causes: Firstly, the capability of the cELISA to differentiate vaccinated sheep with *Brucella melitensis* strain Rev. 1 is much better than other serological tests (Biancifiori *et al*., 2000; Jalali *et al*., 2003). Secondly, the cELISA test is based on the displacement of serum antibodies by a fixed concentration of a Mouse Monoclonal Antibody (MAb) against the common (C/Y) epitope which is the dominant epitope in the o-polysaccharides of both *Brucella abortus* and *Brucella melitensis* and is the most relevant in the serological diagnosis (Marin *et al*., 1999). Thirdly in this test, Brucella antigen is immobilized on the plate as with the indirect ELISA. Following that the serum under test and a monoclonal antibody directed against an epitope on the antigen are co-incubated. This anti brucella monoclonal antibody is conjugated to an enzyme, the presence of which is detected if it binds to the antigen. This will only occur if there is no antibody in the serum sample which is bound preferentially (Anonymous, 2001). Fourthly, the

cELISA is not significantly affected by antibodies resulting from immunization (Office International Epizooties, 2004).

At 90 days post vaccination only 25% of vaccinated sheep showed positive reaction with BAPA, RBPT and Riv. T while cELISA gave 7.5%. These results are in agreement with Stournara *et al.* (2007) who reported that RB test gave 30% positive reactors while cELISA test gave 4% positive reactors. The results at 90 days post vaccination are disagree with Delgado *et al.* (1995) who reported that RB test gave 79% positive reactors and with Al-Hankawe (2009) who reported that RB test gave 80% positive reactors while cELISA test gave 40% positive reactors.

As previously mentioned the cELISA test showed better results than other serological tests as there was about 10% reduction in 90 days than 30 days post vaccination reaction while the other serological tests (BAPA, RBPT and Riv. tests) showed only 5% reduction in 90 days than 30 days post vaccination reaction and this indicates that cELISA test is more specific and accurate than other serological tests.

At 150 days post vaccination, abrupt rise in the number of positive cases were obtained by BAPA, RBPT and Riv. T which showed 40% positive reactors; 32.5% true positive (positive isolation) and 7.5% false positive (negative isolation) while cELISA test showed 32.5% positive reactors. These results are in agreement with Fensterbank *et al.* (1985) who reported that conjunctival vaccination with Rev. 1 gave only 40% protection to the animals against natural infection of sheep with *Brucella melitensis* strain and with Neto and Yolanda (2002) who reported that 20% of vaccinated sheep were infected post vaccination. So we should persuade the dramatical increase of these positive cases to realize the main reasons for this phenomenon. This phenomenon could be attributed to the following causes: firstly, vaccinal strains excreted from urine, vaginal discharges and milk of vaccinated sheep (Fensterbank *et al.*, 1985). Secondly, it is clearly evident that an extrinsic brucella infection was introduced to the premises which came from surrounding unvaccinated sheep or surrounding yards (Neto and Yolanda, 2002). Thirdly, vaccination alone will not eradicate brucella as the immunity produced by brucella vaccines are not absolute and can be circumvented by increasing the level of infection (Seleem *et al.*, 2010).

These infected animals were with a history of abortion and this infection was confirmed by isolation of brucella organisms from slaughtered ones. Isolation was from supramammary, retropharyngeal lymph nodes and spleen of slaughtered animals. The spleen and lymph nodes (supramammary and prefemoral) are the best sites for obtaining samples for isolation during post-mortem examination (Marin *et al.*, 1996). But it is evident that there was an increase in the number of positive reactors (3 positive reactors) obtained by BAPA, RBPT and Riv. T than cELISA test. These positive reactors were with no history of abortion and so we considered them as false positive reactors due to vaccinal antibodies and not infected strain. These results are in agreement with Marin *et al.* (1999) who reported that RBPT showed 19% positive reactors and with Stournara *et al.* (2007) who reported that RBPT showed 25% positive reactors.

At 240 days post vaccination, the same occurred as at 150 days but the number of vaccinated animals became only 30 animals as 10 of them were slaughtered due to infection. These results are in agreement with Neto and Yolanda (2002) who reported that RBPT test showed 20% positive reactors. BAPA, RBPT and Riv. T gave the same percentage of positive reactors as they can detect IgG_1, IgG_2 and IgM at different degrees.

These conventional serological tests (BAPA, RBPT and Riv. T) showed the highest percentage of false positive reactors so we cannot depend upon these tests to discriminate vaccinated sheep from infected ones but we can depend upon these tests as screening tests to detect infected from healthy ones of unvaccinated sheep.

The cELISA test showed the lowest percentage of false positive reactors at 30 and 90 days post vaccination and gave nil result at 150 and 240 days post vaccination as described before so cELISA succeeded in discriminating high percentage of vaccinated animals from infected ones, cELISA is considered a good tool and we can depend upon this test in detection of vaccinated sheep especially 90 days or more post vaccination.

The high specificity of cELISA test is attributed to the using of Monoclonal Antibodies (MAb) which eliminate the low affinity vaccinal antibodies (Marin *et al.*, 1999; Stournara *et al.*, 2007). The intensity and duration of serological response to vaccination with the Rev. 1 vaccine is dependent upon the age of the animals at the time of the vaccination this type of immunological response to vaccination is documented for sheep by Jimenez de Bagues *et al.* (1992) and Stournara *et al.* (2007).

The cELISA test not only simplifies laboratory procedures and facilitates the interpretation of the results, but presents other advantages as following: Firstly, the cELISA does not use a species specific immunoglobulin conjugated with enzyme, it can easily be adapted for detecting *Brucella* sp. infections in different animal species (Gall and Nielsen, 1994; Nielsen *et al.*, 1995, 1996). Secondly, the assay is simple to perform and can be standardized with ease (Portanti *et al.*, 2006). Thirdly, the

cELISA is relatively simple and can be performed in a relatively short time (approximately 1 h and 30 min) and therefore can be readily adapted to non-reference laboratories (Minas *et al*., 2008). Fourthly, it provides high sensitivity and specificity and the ability to detect the non-agglutinating antibodies which is a great importance in the chronic and localized forms of the disease (Weiner *et al*., 2010).

CONCLUSION

It could be concluded that differentiation between naturally infected sheep with brucellosis and vaccinated ones still acts as a major problem in sheep herds at 1st month post vaccination. Conventional tests cannot differentiate between vaccinated and infected sheep. The cELISA test gives accurate results at 90 days or more post vaccination.

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