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Immunological Response of Awassi Sheep to Conjunctival Vaccination against Brucellosis Disease in Mount Lebanon

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ABSTRACT

This study was conducted in order to study the immune responses of Awassi lambs to *Brucevac* vaccine (Rev. 1 strain) applied in conjunctival way by the use of the 2 serological tests c- ELISA and Rose Bengal Plate test. Awassi sheep in three farms were used where animals in each farm were conjunctival vaccinated with Brucevac (Rev.1 vaccine) (2 x 10^9 viable organisms) and were later divided into 3 groups (each of 7 animals). Group 1 consisted of female kids aged between 3 and 6 months; group 2 consisted of female kids aged between 9 and 11 months; while group 3 consisted of females aged between 12 and 18 months. All animals were bled before vaccination to confirm that they are *Brucella* free. Blood samples from each group were collected periodically at 3, 6 , 9 and 12 months after vaccination. Blood samples were subjected to Rose Bengal Plate test and to C-Elisa test. The results showed that the 3 flocks of ewes showed a high serological response following vaccination and peak at 90 days post vaccination, the PI values start to drop at 180 days post vaccination to reach a minimum at 360 days. The 3 flocks of sheep were considered seronegative based on c-ELISA test; flock 1 showed the highest seroconversion rate throughout the study while flock 2 showed the lowest seroconversion, the average PI of this flock became negative less than 30%, 360 days post vaccination. c-ELISA test showed higher sensitivity than Rose Bengal test and lower specificity.

***Key words*:** Awassi, *Brucella melitensis,* c-ELISA, Rose Bengal test

Introduction

Brucellosis is a major zoonotic disease, widely distributed in both humans and animals, especially in the developing world. The occurrence of the disease in humans is largely dependent on the animal reservoir and high rates of brucellosis infection in sheep and goats usually cause the greatest incidence of infection in humans (WHO, 1998). Brucellosis, caused by *Brucella melitensis*, in small ruminants is essentially an acute disease of the pregnant ewes, causing abortion in the later part of pregnancy as the main symptom (Alton, 1987). This zoonosis has great socio-economic impact worldwide, and, despite the control and eradication campaigns implemented in the second half of the 20th century, the disease persists as a major threat to food safety and public health in vast areas of the planet (Corbel, 1989; Acha and Szyfres, 2001; Nicoletti, 2002). Brucellosis, particularly *Brucella melitensis*, has been identified as the major zoonotic disease of public health importance in Mediterranean and Middle East regions. However, its prevention and control poses several problems to national authorities, particularly to the veterinary services (WHO, MZCP workshop, 1998). In those Mediterranean and Middle East countries where brucellosis control programs have been implemented, several technical problems still pose a great challenge to their veterinary services, such as animal movement control and identification, vaccination coverage and the emergence of *B. melitensis* as a cattle pathogen (WHO, MZCP workshop, 1998). In Lebanon, brucellosis in sheep and goats is very frequent. A mini-serological survey done by the Ministry of Agriculture in some private farms showed that almost all of the farms had infected animals. *B. melitensis* is the main agent of infection in all ruminants. The last screening revealed an incidence of 18% in cattle and 9.2% in sheep and goats. In a last report in 1998, it was mentioned that about 800 human cases are reported annually to the Ministry of Health (Refai, 2002).

Vaccination is considered a powerful tool for small ruminant brucellosis control, and is recommended by the WHO as a measure to prevent the disease dissemination (by reducing the excretion of microorganisms from the infected animals, the contamination of the environment and the rates of infection of exposed animals) and to reduce human brucellosis. The main aspects to consider for the implementation of a vaccination campaign, in order to properly immunize the animals while reducing interference with the diagnosis of infection, are related to the vaccine to be used, the animal classes to be vaccinated, the appropriate dose and the application route. Several studies and the extensive field use of Rev 1 vaccine confirmed its good immunizing behavior against *B.melitensis* (Alton and Elberg, 1967; Elberg, 1981; Elberg, 1996; Blasco, 1997). However, because of some

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associated problems with Rev1 application, there is a preferential selection of young animal's vaccination, between 3 to 6 months of age, as the target of vaccination, especially in campaigns combining test and slaughter with vaccination. On the other hand, there is no sufficient prove that the immunity is lifelong, as referred by some authors, and population protection obviously depends on the average age of culling of vaccinated animals (WHO, 1998). Vaccination of all animals is therefore recommendable in many circumstances, to obtain a good protection of the population and to rapidly reduce prevalence (Alton, 1987; Garin-Bastuji, 1995; Blasco, 1997; WHO, 1998).

Alternative routes of application were also tested and it is now generally accepted that the conjunctival route is better than the subcutaneous route, for both young and adult vaccination, inducing a good immunity, minimizing the long-term persistence of vaccine antibodies and reducing the allergic response (Fensterbank *et al.*, 1985; Blasco, 1997; Garin-Bastuji *et al.*, 1998).

In this context, this research was carried out to study the immune responses of Awassi lambs to Brucevac vaccine (Rev. 1 strain) applied in conjunctival way by the use of the 2 serological tests c- ELISA and Rose Bengal Plate test.

Materials and Methods

The experiment was conducted in Mount Lebanon region in order to evaluate the immunological response of Awassi sheep to conjunctival vaccination of Brucevac vaccine (Rev.1 strain of *Brucella melitensis*). The study took place from May 2011 to May 2012 in 3 different Awassi farms in Mount Lebanon about 1000- 1200 m above sea level. Experimental flocks were sound and apparently healthy and were randomly selected from brucellosis free stocks. Farm 1 counted 90 lactating ewes, farm 2: 130 lactating ewes while farm 3 counted 157 lactating ewes. The 3 farms were raised in extensive system and used to migrate in search of water and pasture.

The 3 farms were vaccinated in the month of May; this allows the flock specially the lactating ewes to build immune response before the mating period between end of August and mid of October.

Experimental design:

Following conjunctival vaccination of the 3 farms with Brucevac (Rev.1 vaccine) (2 x 10^9 viable organisms). Animals in each farm were later divided into 3 groups (of 7 animals); the selected animals were ear tagged. Group 1 consists of female kids aged between 3 and 6 months; group 2 consists of female kids aged between 9 and 11 months; while group 3 consists of females aged between 12 and 18 months. All animals were bled before vaccination to confirm that they are Brucella free. Blood samples from each group were collected periodically at 3, 6, 9 and 12 months after vaccination. Blood samples were subjected to Rose Bengal Plate test and to C-Elisa test.

Statistical analysis:

The results were expressed as a percentage inhibition (% Inh) against antigen (Inh *(%)* = 100 − [*(*OD test sample*/*mean OD of conjugate control*)* ×100]). The cut-off was set at 30% inhibition. Average inhibition values for each group were submitted to one way ANOVA under Sigma Stat program where days after vaccine administration is taken as the factor.

Results

No clinical signs were detected after vaccination. No febrile response was detected in any of the ewes in either of the vaccinated groups during the fourteen days following vaccination. Of the ewes vaccinated 20% presented a slight reaction at the site of administration, which resolved within one to two weeks later.

In farm (1), the mean PI (Percent inhibition value) for the different groups of lambs is represented in table 1.

After vaccination, the 3 groups of lambs vaccinated with Brucevac (Rev.1) became positive to c-ELISA test peaking at 90 days post vaccination; the PI value starts to drop after that in the 3 groups of lambs; however, all animals remain positive at 180 and 270 days post vaccination. It is only group 3 females (vaccinated at the age of 12 to 18 months) that becomes negative at 360 days post vaccination.

Table 1: Mean P1 value for the different groups of lambs in Farm (1).

	Days post vaccination				
Flock	0	90	180	270	360
Group 1	13.3±2.3a	90.9±10.7b	84.7±13.5b	60.7±12.5c	40.6±9.8d
Group 2	17.4±5.4a	86.9±11.2b	76.8±9.6c	55.4±7.9d	37.4±7.6e
Group 3	18.5±8.7a	84.7±14.3b	70.7±10.8c	36.5±8.7d	25.6±4.5e

a,b,c,d,e: Within group, between days, p<0.01.

The serological response of the different animals in this flock identified by ELISA test is represented in table 2.

Table 2: Serological response (percentage) of the different animal groups by ELISA test.

	Days post vaccination					
Lambs %		0	90	180	270	360
Negative	0<PI<30	100.00				47.62
Weak positive	30<PI<50				57.14	42.86
Positive	50<PI<70		14.29	61.90	42.86	9.52
Strong positive	PI>70		85.70	38.10		

Before vaccination, all animals in the different groups of flock 1 were considered negative (PI<30%); 90 days post vaccination, 85.7% of the lambs revealed highly positive by c-Elisa test while 14.29% were positive (50<PI<70%). The PI value of all the tested animals start to drop starting from day 180; and consequently 47.62% of the tested animals were negative while 42.86% of them were weak positive 360 days post vaccination. When submitted to Rose Bengal Test, the sera were given note from 0 (non agglutination) to 3, 4 (Strong agglutination); the percentage of animals declared positive by Rose Bengal Test is represented in table 3.

Table 3: Percentage of positive animals by Rose Bengal test by Rose Bengal test.

	Days post vaccination				
Lamb %	0	90	180	270	360
0	90.50				33.33
1	9.50			19.05	47.62
2				38.10	9.52
3			47.62	28.57	4.76
4		100.00	52.38	14.29	4.76

According to table 3, before vaccination 2 lambs out of 21 showed weak agglutination (note of 1) and were considered as positive; while with c-ELISA test all the animals were proved negative before vaccination. 90 and 180 days after vaccination, all the lambs were confirmed strong positive (note of 3 and 4), at 270 days post vaccination, 42.86% of the lambs were strong positive (note 3 and 4); while the percentage of strong positive with c-ELISA at this period was zero. At 360 days post vaccination 33.33% of the animals were confirmed negative with Rose Bengal Test; compared to 47.62% with c-ELISA test. Table 4 represents a comparison between the result of Rose Bengal test and those of c-ELISA test.

Table 4: Comparison between Rose Bengal test and c- Elisa test.

Number of lambs by test			Days post vaccination				
			Day 0	Day 90	Day 180	Day 270	Day 360
Negative	c-Elisa	PI<30	21	0	0	0	10
	Rose Bengal	0	19	0	0	0	7
weak positive	c-Elisa	30<PI<50	0	0	0	12	11
	Rose Bengal	1	2	0	0	4	9
Positive	c-Elisa	50<PI<70	0	1	13	9	2
	Rose Bengal	2	0	0	10	8	2
strong positive	c-Elisa	PI>70	0	20	9	0	0
	Rose Bengal	3, 4	0	21	11	9	2

At day 0, 21 animals were negative with c-ELISA test compared to 19 with Rose Bengal Test; the number of strong positive is higher with Rose Bengal test than with c-ELISA test at the different period of the study. The number of strong positive lambs 90, 180, 270 and 360 days post vaccination were (21, 11, 9 and 2 with Rose Bengal test) compared to 20,9,0,0 with c-ELISA test. At 360 days post vaccination the number of animals confirmed negative was higher with c-ELISA test (10/21) than with Rose Bengal Test (7/21).

The seroconversion of sheep lambs in flock 2 to conjunctival vaccination with Brucevac vaccine (Rev.1) identified by ELISA test is represented in table 5.

Table 5: Seroconversion of sheep lamb in flock 2 to conjunctival vaccination with Brucevac vaccine by ELISA test.

Flock 2	Days post vaccination				
	0	90	180	270	360
Group 1	0±0a	80.5±9.4b	72.5±9.8c	50.3±10.4d	34.1±8.5e
Group 2	0±0a	74.3±8.7b	69.5±11.3c	40.7±6.5d	30.4±8.7e
Group 3	0±0a	69.6±7.4b	63.7±10.4c	35.9±5.7d	23.7±3.4e

a,b,c,d,e: Within group, between days, p<0.01.

The lambs of group 1 (3 to 6 months) were able to achieve the highest PI value at 90 days post vaccination (80.5±9.4%) compared to group 2 (9 to 11 months) and group 3 (12 to 18 months). They were also able to maintain higher PI value throughout the different period of the study. Before vaccination, all animals were confirmed to be negative (PI=0); Following vaccination, all animals became positive and peak at 90 days post vaccination. The immune response starts to drop starting 180 days post vaccination to 360 days post vaccination. The distribution of the animals (%) of flocks 2 according to their PI value is represented in table 6.

Table 6: P1 value of the animals of flock 2.

	Days post vaccination					
Lambs %		0	90	180	270	360
Negative	0<PI<30	100.00			33.33	66.67
Weak positive	30<PI<50			14.29	28.57	28.57
Positive	50<PI<70		42.86	42.86	33.33	4.76
Strong positive	PI>70		57.14	42.86	4.76	

At day 0, all animals in the 3 groups were confirmed negative by c-ELISA test; At day 90 post vaccination, 57.4% of the animals were strong positive while 42.86% were positive; the percentage of seropositivity starts to drop at 180 days post vaccination and 66.67% of the animals were confirmed negative by c-ELISA test at 360 days post vaccination. When submitted to Rose Bengal test, the sera give the following results as shown in table 7.

Table 7: Results of sera (lambs %) by Rose Bengal test.

Lambs %	Days post vaccination				
	0	90	180	270	360
0	100.00			9.52	47.62
1				23.81	23.81
2			14.29	28.57	28.57
3		19.05	33.33	23.81	
4		80.95	52.38	14.29	

At day 0, all the animals were confirmed negative by Rose Bengal test; 100% were strong positive at 90 days post vaccination compared to 57.14% with c-ELISA test; 47.62% were confirmed negative at day 360 post vaccination compared to 66.67% with c-ELISA test. The number of animals confirmed positive or negative by the 2 tests (c-ELISA and Rose Bengal) at the different period of the study are represented in table 8.

Table 8: Numbers of animals confirmed positive or negative by the 2 tests.

Number of lambs			Days post vaccination				
			Day 0	Day 90	Day 180	Day 270	Day 360
Negative	c-Elisa	PI<30	21	0	0	7	14
	Rose Bengal	0	21	0	0	2	10
weak positive	c-Elisa	30<PI<50	0	0	3	6	6
	Rose Bengal	1	0	0	0	5	5
Positive	c-Elisa	50<PI<70	0	9	9	7	1
	Rose Bengal	2	0	0	3	6	6
strong positive	c-Elisa	PI>70	0	12	9	1	0
	Rose Bengal	3, 4	0	21	18	8	0

In the 2 tests, 21/21 were confirmed negative before vaccination; at day 90, 180, 270 the number of animals confirmed strong positive is higher with Rose Bengal compared to those with c-ELISA test (21, 18, 8 vs 12, 9,1 respectively). The number of seronegative lambs at day 360 post vaccination is higher with c-ELISA compared to Rose Bengal test (14/21 vs 10/21).

In Farm 3, the mean PI values for the different group of lambs at the different period of the study are represented in table 9.

Table 9: P1 values of the different groups of lambs by ELISA test at the different period of study.

	Days post vaccination				
Flock 2	0	90	180	270	360
Group 1	0±0a	92.3±11.2b	85.4±10.7c	54.5±7.4d	39.4±6.4e
Group 2	0±0a	88.4±12.5b	76.5±9.8c	47.4±9.5d	34.3±7.6e
Group 3	0±0a	85.3±11.3b	63.9±10.3c	39.5±8.7d	26.3±5.4e

Lambs of group 1 (3 to 6 months) showed higher seroconversion in response to Brucevac vaccine than those of group 2 (9 to 11 months) and those of group 3 (12 to 18 months) and maintained higher PI values throughout the different period of the study. Before vaccination all animals were confirmed to be seronegative;

After vaccination, all the animals became seropositive and peak at 90 days; The antibody titer starts to drop after that to reach PI values of 39.4±6.4 (weak positive) in group 1, 34.3±7.6 (weak positive) in group 2, and 26.3±5.4 (negative) in group 3. The percentage of seropositivity in each period of the study is represented in table 10.

Table 10: Percentage of seropositivity in each period of the study.

Lambs %	Days post vaccination					
		0	90	180	270	360
Negative	0<PI<30	100.00			38.10	42.86
Weak positive	30<PI<50			9.52	28.57	42.86
Positive	50<PI<70		23.81	33.33	33.33	14.29
Strong positive	PI>70		76.19	57.14		

Based on c-ELISA test, all animals were confirmed seronegative before vaccination. The number of seropositive lambs raised sharply after vaccination and consequently 76.19% were confirmed strong positive at day 90 post vaccination which is represented the peak seroconversion; the PI values drop after that and 42.86% of the animals were confirmed seronegative 360 days post vaccination. Based on Rose Bengal Test, the seropositivity percent is represented in table 11.

Table 11: percent of seropositivity of Rose Bengal Test.

Lambs %	Days post vaccination				
	0	90	180	270	360
0	100.00			28.57	23.81
1			4.76	38.10	47.62
2		4.76	14.29	23.81	28.57
3		28.57	23.81	4.76	
4		66.67	57.14	4.76	

Similar to c-ELISA test, all animals were confirmed to be seronegative before vaccination; however the number of strong positive lambs post vaccination is higher than those with c-ELISA; while the number of seronegative is lower. The number of animals confirmed seropositive or seronegative in flock 3 both with c-ELISA test and Rose Bengal test is illustrated in table 12.

Table 12: Number of animals confirmed seropositive or seronegative in flock 3 by the 2 tests.

Number of lambs			Days post vaccination				
			Day 0	Day 90	Day 180	Day 270	Day 360
Negative	c-Elisa	PI<30	21	0	0	8	9
	Rose Bengal	0	21	0	0	6	5
weak positive	c-Elisa	30<PI<50	0	0	2	6	9
	Rose Bengal	1	0	0	1	8	10
positive	c-Elisa	50<PI<70	0	5	7	7	3
	Rose Bengal	2	0	1	3	5	6
strong positive	c-Elisa	PI>70	0	16	12	0	0
	Rose Bengal	3, 4	0	20	17	2	0

Before vaccination 21 animals/21 were confirmed to be seronegative both with c-ELISA and Rose Bengal test; following vaccination, the number of strong positive animals is higher with Rose Bengal test (20, 17 and 2) than those of c-ELISA test (16, 12, 0) at day 90, 180, 270 respectively; The number of seronegative animals at day 360 post vaccination is higher with c-ELISA test than those of Rose Bengal test.

In Brief, the trend of seroconversion following vaccination in the 3 flocks is illustrated in figure 1.

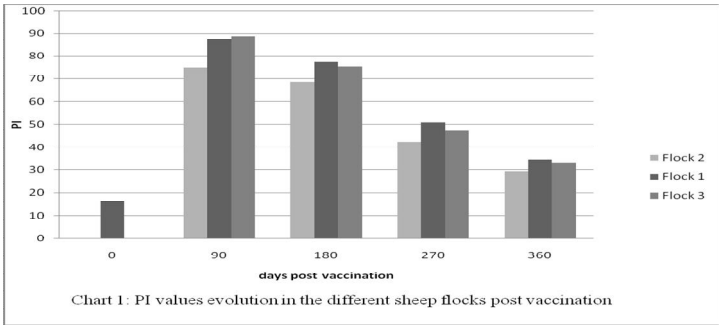

Fig. 1: PI values evolution in the different sheep flocks post vaccination.

According to Fig. (1), the 3 flocks of ewes showed a high serological response following vaccination and peak at 90 days post vaccination, the PI values start to drop at 180 days post vaccination to reach a minimum at 360 days.

As noted before, the 3 flocks of sheep were considered seronegative based on c-ELISA test; flock 1 showed the highest seroconversion rate throughout the study while flock 2 showed the lowest seroconversion, the average PI of this flock became negative less than 30%, 360 days post vaccination.

According to figure (2), c-ELISA test showed higher sensitivity than Rose Bengal test and lower specificity. Therefore at 360 days post vaccination, the number of seropositive ewes is higher with Rose Bengal than with c-ELISA test. This is due to the fact that the low reaction to Rose Bengal test (note 1) is considered positive while with c_ELISA test is taken as negative (PI<30%). In average the correlation between the 2 tests was 64 while the discrepancy was 26%.

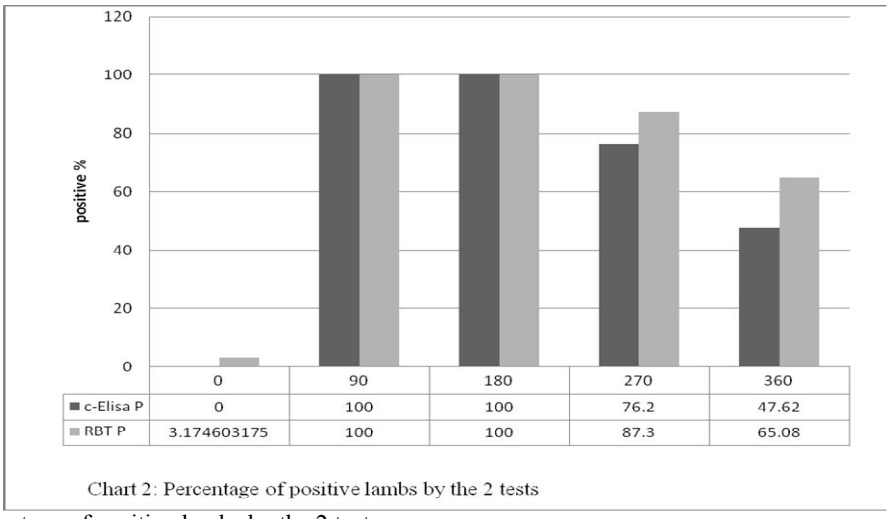

Fig. 2: Percentage of positive lambs by the 2 tests.

Discussion

Despite advances in molecular techniques, serology remains at the forefront of eradication and surveillance programs for veterinary diseases and is an important tool in the fight against human brucellosis (McGiven, 2008). The immunological tests conducted on the three groups of lambs in the 3 different flocks of sheep vaccinated with Brucevac Rev.1 vaccine indicated that no previous exposure to Brucella had taken place in these animals. Those ones which were bled before vaccination were negative for brucellosis in the 2 tests RBT amd c-ELISA, with the exception of 2 animals in flock 2 that show weak seropositivity in RBT test (note of +1). No significant local or systemic reaction was observed during the first weeks of the study period.

In our studies all serological tests RBT and c-ELISA detected a high serological response in the different group of lambs at 3, 6 months post conjuntival vaccination with Brucevac vaccine where the percentage of seropositive animals was 100% in the 2 periods, the PI values drop 270 days post vaccination to reach a minimum values at 360 days post vaccination. This raises a question about the intensity and the duration of protection conferred by conjunctival vaccination and the dose of the vaccine. These results are in agreements with the result obtained by Fensterbank *et al.*,1985; Jiménez de Bagués *et al.*, 1992; Díaz-Aparicio *et al.*, 1994; Marín *et al.*, 1999) who reported that the level of protection conferred by the conjunctival vaccination is similar to that obtained in the traditional subcutaneous vaccination, but the intensity and the duration of protection is lower. According to those authors, this characteristic enables the discrimination between vaccinated and infected ewes as reported by Jalili *et al.* (2000). In another study (jalili *et al.*, 2000), approx. 90% of the vaccinated animals scored negative with the C-Elisa. This confirms that the low sensitivity obtained is most likely due to the discriminating feature of the C-ELISA, a characteristic which is rather poor with Rose Bengal. According to this author, the low reactivity in the small number of c-ELISA positives (10/43) suggests antibodies from a cross reacting organisms or possibly interference from antibodies due to vaccination and not to infection as the results from other tests are mostly to be negative. The seroconversion obtained in the different group of sheep in response to conjunctival vaccination with Brucevac vaccine is higher than that obtained by Aldomi *et al.* (2009) in Shami goat and the intensity of protection is longer. According to this author only 20% of the vaccinated kids remain positive at 24 weeks post vaccination. While in our studies, average of 40% of tested lambs remain positive with c-ELISA test up till 360 days post vaccination; the percentage of seropositive with RBT at 360

days post vaccination is 65% with RBT test. However, the seroconversion of the sheep to conjunctival vaccination is age dependent with high PI values recorded in young lambs (3 to 6 months old). The high PI value and the high number of strong positive individuals may put a question mark on the use of the vaccine in mature reproductive ewes and its virulence that may lead to abortion.

The discrepancy between RBT and c-ELISA was also reported by Jalili *et al.* (2000) and Abuharfeil and Abu Shehada (1998). For these authors, RBT was demonstrated as the most suitable screening method in the field if suspected weak positive (+1) readings are considered negative. In our study, 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.

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 result from the examiner recording any slight agglutination which may neither be accurate nor recommended by the manufacturers as positive. ELISA is the only test with no false negative results. However, all other serological tests showed false positive results (Abu Harfeil and Shehada, 1998). 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. This is expected since RBT test detect early infection through the strong agglutianiting lgM antibodies produced at the initial stage of infection. Subsequently, lgG1 antibodies predtominate. Although the concentration of lgG1 decreases with time, it remains detectable for a long period. Secondly, a combination of RBT and c-ELISA tests could detect all the positive Brucella reactors. This minimizes the possible false negative results. This is in agreement with previous results which concluded that it was impossible to detect all infected animals using a single test. The combination of RBT with ELISA is recommended since the ELISA method is reproducible (Abuharfeil and Abu Shehada, 1998).

Conclusion

When *Brucella melitensis* Rev. 1 vaccine is administered subcutaneously, an intense and long lasting antibody response can be induced, which interferes with serological testing. This disadvantage was partially overcome by the use of the conjunctival route when administering the vaccine as done in this study. This route of vaccination significantly reduces the intensity and duration of the post vaccination serological response and makes the use of this vaccine compatible with brucellosis programs, even when these are based on a test-and-slaughter policy. Although conjunctival vaccination has been proved to be effective, the safety and the duration of the immunity conferred by this method of vaccination are still the subject of controversy and needs more studies. According to this study, conjunctival vaccination with Brucevac vaccine was able to produce strong immune response in 3 flocks of sheep up to 6 months of vaccination where all animals (100%) were confirmed positive after that the immune response starts to drop at 270 days post vaccination to reach minimum PI values at 360 days post vaccination.

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