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INVESTIGATING THE SEROLOGICAL RESPONSE AND SAFETY OF *BRUCELLA MELITENSIS* REV.1 CONJUNCTIVAL VACCINE IN SMALL RUMINANTS

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ABSTRACT

Mass vaccination, which is one of the main control policies, provides herd immunity against infectious diseases. This could contribute to the control of the disease and eventually its eradication. The purpose of this study was to investigate the safety and humoral immune response of *Brucella melitensis* Rev.1 vaccine before the start of mass vaccination. A total of 741 sheep and goats were vaccinated conjunctivally. No adverse effect was observed after the vaccination of the animals. No abortion was seen in pregnant animals. Vaccine strain was isolated from some milk samples taken from only lactating vaccinated goats. Excretion of the vaccine strain was not intense and long-termed. Post-vaccination immune response was evaluated by serological tests, namely, Rose Bengal Plate Test, Serum Agglutination Test and Complement Fixation Test. One month after vaccination, the immune response was high, and the decrease of antibody titers was the highest four to six months after vaccination by inversely correlated with the age of the vaccinated animals. In conclusion, we observed that *Brucella melitensis* Rev.1 vaccine, used conjunctivally, was safe enough for the animals, and vaccinated animals had high vaccine-induced immune response.

Key words: *Brucella melitensis*; conjunctival; mass vaccination; safety; serology

INTRODUCTION

Brucellosis is one of the most common zoonotic diseases all around the world (Boschiroli *et al.*, 2001; Yumuk and O'Callaghan, 2012; Ducrotoy *et al.*, 2014; Hou *et al.*, 2019; Wareth *et al.*, 2019). The disease is seen over 170 countries and regions (Hou *et al.*, 2019) and more than 500,000 new cases are expected to occur annually in humans (Pappas *et al.*, 2006; Nicoletti, 2010). While these expected case numbers were revealed more than one decade ago; recent studies stated that numbers might be higher because of underestimating the real situation (O'Callaghan, 2020).

The causative agent of the disease is *Brucella* (B.) genus (Boschiroli *et al.*, 2001; Ducrotoy *et al.*, 2014; Wareth *et al.*, 2019) in which *B. melitensis* is considered to be most hazardous one (Godfroid *et al.*, 2005). Brucellosis leads to health and economic issues, particularly in endemic areas (Boschiroli *et al.*, 2001; Yumuk and O'Callaghan, 2012; Zhang *et al.*, 2018) including the Mediterranean countries (Banai, 2002; Garin-Bastuji *et al.*, 1998; Wareth *et al.*, 2019).

The control of brucellosis in animals, especially by lowering the proportion of reactor animals, provides significant support for the control of the disease in humans (Boschiroli *et al.*, 2001; Godfroid

et al., 2005; Lalsiamthara and Lee, 2017). Immunization of susceptible hosts within the endemic regions or areas with high prevalence is considered to be the only way to control and eradicate the disease respectively (Fensterbank, *et al.* 1982; Briones, *et al.* 2001; Minas, 2006; Stournara *et al.*, 2007; Nicoletti, 2010). Rev.1 vaccine is considered to be the most successful vaccine for the prevention of brucellosis in small ruminants (Fernsterbank, 1987; Blasco, 1997; Minas, 2006; Stournara *et al.,* 2007; Nicoletti, 2010).

The most effective strategy for controlling the disease is conjunctival vaccination of young and adult animals with Rev.1 vaccine (Minas, 2006). Despite being an attenuated vaccine strain, it might cause abortion because of an existing virulence (Jiménez de Bagüés *et al.*, 1989; Banai, 2002; Minas, 2006; OIE, 2018a). The recommended vaccination route and dose is the administration of the standard dose $(0.5-2.0 \times 10^9$ cfu) to $3-5$-month-old animals by either subcutaneous or conjunctival route (OIE, 2018a).

Administering Rev.1 vaccine subcutaneously with a standard dose may result in long-lasting serological responses (Jiménez de Bagüés *et al.*, 1992; Zundel *et al.*, 1992; Garin-Bastuji *et al.*, 1998; Minas, 2006; Stournara *et al.*, 2007; OIE 2018a). When this vaccine is given via the conjunctival route, however, the immunity provided is identical to the one generated by the usual technique, but the serological reaction elicited dramatically diminishes (Jiménez de Bagüés *et al.*, 1992; Garin-Bastuji *et al.*, 1998; Minas, 2006; OIE 2018a).

Veterinary vaccines must be tested in the field for safety and effectiveness before being given to animals (OIE 2018b). Field experiments on these two subjects are carried out in target animals (EMEA, 2001; OIE, 2018b). According to the findings of a serological study at a national extend, the herd prevalence rate in small ruminants was found to be 22.5 %. According to these quite high figures of herd prevalence, a new vaccination strategy was launched in Türkiye, in which mass vaccination for sheep and goats were vaccinated by Rev.1 vaccine through conjunctival route (MFAL, 2012). In this context, the aim of the study was to investigate the serological response and safety of the conjunctival Rev.1 vaccine prior the beginning of the mass vaccination program of small ruminants in Türkiye.

MATERIAL AND METHODS

A total of 334 sheep and 407 goats that include pregnant (n = 32) and lactating (n = 239) animals were vaccinated in this study, as shown in Table 1. This field study was carried out in 5 farms and 3 different provinces, because it was recommended to use host animals and to organize the field studies in different geographical locations (VICH, 2008; OIE, 2018b).

The study was conducted after the necessary permission (dated 28.06.2010, numbered B.12.0.K KG.0.19/108-02/15-2467-48891-025366) obtained from the General Directorate of Food and Control of Ministry of Agriculture and Forestry related to field trials of conjunctival anti-brucella vaccines in sheep and goats. As there is a need to have a control group to compare the results (VICH, 2008; OIE, 2018b), all the animals that were not vaccinated, were included in the control group. Farms II and III were regarded as infected farms, because positive results were obtained serologically and bacteriologically from blood, vaginal swab and milk samples prior the vaccination.

All the animals, included in the vaccinated group, were selected randomly. In this study, 3 different batches (BM-K/09/01 BM-K/09/02 and BM-K/09/03 of BRUPEN-M) of vaccine with the doses of 1.56, 1.76, 1.63×10^9 cfu/dose were used. These batches were produced at The National Laboratory for Brucellosis, which already produces anti-Brucella vaccines.

Safety of vaccines was evaluated following a single dose or a repeated application according to the recommendations for use (EMEA, 2001; OIE, 2018b). We monitored the general health status of the animals

Table 1. Total number of vaccinated goats and sheep

Yearling lambs	Lactating		Pregnant		Buck	Ram	Kid	Lamb	Total	
	Goats	Sheep	Goats	Sheep					Goats	Sheep
142	206	33	12	20	15	1	174	138	407	334

for 21 days in order to detect an unexpected systemic or local side effects after the first administration of the vaccines. The focus of the field safety trials is the potential local and systemic reactions like allergic reactions, mortality or fever (EMEA, 2001).

It is necessary to utilize an overdose test, particularly for live vaccines, such as Rev.1, to be able to investigate the specific disease manifestations (OIE, 2018b). For the overdose test, some of the animals (n = 23) were vaccinated with an overdose (4.7–5.7 x 10^9 cfu/dose) of the vaccine. Randomly selected animals from both the vaccinated and the control group were selected and their body temperatures were monitored on the first 3 days after vaccination.

Two weeks after vaccination, nasal and conjunctival samples from the vaccinated animals and control animals, representing 10 % of the population, were collected. Collected milk samples from the 20 % of the lactating animals for 3 months after vaccination were examined bacteriologically.

Vaginal swabs were collected at 2-week intervals during one month before delivery. Vaginal swabs, colostrum and milk samples from the vaccinated pregnant animals were collected at 2-week intervals during 3 months after delivery. Samples were cultured based on the recommendations in the OIE manual (OIE, 2018a).

Vaccinated animals were bled before vaccination (0 day) and then, on a monthly basis, during 6 months post-vaccination period for serological evaluation. In order to assess humoral immune response, vaccinated group is composed of animals of different ages and physiological conditions. Blood samples were also taken from the control group representing the 10 % of the animals.

Rose Bengal Plate Test (RBPT), Serum Agglutination Test (SAT) and Complement Fixation Test (CFT) were performed according to the methods described in their manuals. An animal displaying 30 or more IU/ml was regarded as positive for SAT and sera, containing 20 or more International CFT Unit/ml, were considered as positive for CFT (OIE, 2018a).

RESULTS

Neither systemic nor local clinical signs were observed during 21 days after vaccination. There were no symptoms related to conjunctivitis in the vaccinated animals. Body temperatures were within acceptable limits in both vaccinated and control animals. *Brucella* isolations from conjunctival and nasal swabs are shown in Table 2.

Table 3 illustrates the number of bacteria, as colony-forming units, identified during 15 days after vaccination in conjunctival or nasal areas of sheep and goats, respectively. According to the results, the isolation rate of vaccine strain from these areas was found to be 60 – 70 %. However, the bacterial growth was very low, and it included less than 10 CFU even at the peak stage, as illustrated in Table 3. No vaccine-induced abortion or premature delivery was detected in any of the 32 pregnant animals (20 sheep, 12 goats).

Clinical and bacteriological findings of pregnant and lactating sheep and goat are shown in Table 4. *B. melitensis* Rev.1 vaccine strain isolation rate in sheep from samples taken after parturition was found to be 10 % (n = 2). No vaccine strain isolation was obtained from milk samples (n = 7) of the lactating sheep after vaccination. No isolation occurred in all the samples belonging to control group, pregnant (n = 3) or lactating sheep (n = 5). The persistence of isolation lasted for a month, but after the first inoculation the growth level decreased gradually and was not observed afterwards.

Table 2. Rev1 isolation results in the ocular and nasal area after vaccination

Animal	Vaccinated sheep	Control group (sheep)	Vaccinated goats	Control group (goats)
Number of swab samples	33	8	41	10
Number and percentage of isolation (Ocular area)	21 (63.6 %)	0	30 (73.1 %)	0
Number and percentage of isolation (Nasal area)	19 (57.5 %)	0	28 (68.3 %)	0

Table 3. Post-vaccination results of conjunctival and nasal swabs in sheep and goats

Swab samples	1st day CFU		2nd day CFU		3rd day CFU		7th day CFU		10th day CFU		15th day CFU	
	S	G	S	G	S	G	S	G	S	G	S	G
Conjunctival	8.9	9.6	5.7	6.7	3.3	4.2	1.7	2.4	0.9	1.0	0	0
Nasal	6.1	7.1	5.0	5.8	1.9	3.8	1.1	1.7	0	0.4	0	0

Sheep: S, Goat: G

Table 4. Clinical and bacteriological findings of sheep and goat after vaccination

	Vaccinated sheep		Vaccinated goat	
	Pregnant	Lactating	Pregnant	Lactating
Number of sheep	20	7	12	42
Abortion	-	-	-	-
Rev.1 isolation from vaginal swabs	2 (10 %)	-	3 (25 %)	-
Rev.1 isolation from colostrum	2 (10 %)	-	3 (25 %)	-
Rev.1 isolation from milk	2 (10 %)	-	3 (25 %)	-
Rev.1 excretion during lactation	-	-	-	1 (2.4 %)

Table 5. Serological responses in the post vaccination period (Farm I, IV, V)

Period	1 month after			2 months after			3 months after			4 months after		
Test	RBPT	SAT	CFT	RBPT	SAT	CFT	RBPT	SAT	CFT	RBPT	SAT	CFT
Positivity Rate %	82.9	67.2	55.9	66.9	49.6	27.5	45.2	21	4.9	8.4	4.9	0

B. melitensis Rev.1 vaccine strain isolation rate from goat samples taken after the parturition was 25 % (n = 3). Two of three positive isolations were obtained from pregnant animals in the infected goat farms (Farm II, III). The persistence of isolation lasted for 6 weeks, but after the first inoculation the growth level has decreased in the following passages. There was no any persistent excretion of vaccine strain in any of the animals. No vaccine strain isolation was done from milk samples (n = 42) of the lactating goats during 3-month post-vaccination period except in one sample. No isolation occurred in any of the samples belonging to the control group, pregnant (n = 2) or lactating goats (n = 10). In this study, the isolation from milk and vaginal secretion was done from sheep and goat for 4 and 6 weeks after vaccination, respectively.

Serological test results are presented in Table 5. The animals in the control group were seronegative during the study. The results of Farm II and III were excluded from Table 5, since they were regarded as the infected farms.

Another finding is that positive RBPT and SAT results lasted longer than the results of CFT. Higher antibody titers were detected in animals vaccinated with an overdose. On the other hand, no significant difference was observed related to the disappearance of antibodies between normal dose and overdose. Over-dose administration did not cause any persistent serological response. Table 6 shows three serological results of tested sheep/goats (n = 20) and lamb/kids, respectively (n = 10) of infected Farm-II and III in the post-vaccination period.

Table 6. Serological responses of sheep and goats in the post vaccination period (Farm II, III)

The period following the vaccination	RBPT – AR		SAT (IU/ml) Average titer		CFT (ICFTU/ml)	
	Sheep/Goat	Lamb/Kids	Sheep/Goat	Lamb/Kids	Sheep/Goat	Lamb/Kids
Instantly	++	+	257.8	89.7	169.5	68.2
1 month later	++	+	376.4	117.6	197.2	56.2
2 months later	++	+	253.4	97.6	117.5	44.2
3 months later	+	+	198.0	53.2	95.3	34.1
4 months later	+	+	143.5	33.1	67.2	18.7
5 months later	+	-	97.5	11.1	39.5	-
6 months later	+	-	38.3	-	16.8	-

AR: Agglutination Reaction

DISCUSSION

This study aimed at evaluating the safety and serological responses of vaccination of goats and sheep by conjunctival *B. melitensis* Rev.1 vaccine. During 21 days after vaccination, neither systemic nor local clinical signs were observed, which is in agreement with previous studies (Jiménez de Bagüés *et al.*, 1989; Zundel *et al.*, 1992). The animals in the control group were negative during the study, as reported in the previous studies (Zundel *et al.*, 1992, Stournara *et al.*, 2007). This shows that even after lambing, the vaccinal strain was unlikely to be discharged extensively into the environment by any of the vaccinated animals (Stournara *et al.*, 2007). Therefore, vaccination of non-pregnant animals might be safer not only for animals but also for the environment (Stournara *et al.*, 2007).

According to the ocular and nasal swab results in Table 3, excluding the negative results of the control group, the isolation rate of vaccine strain from these areas was found to be 60 – 70 %. However, the bacterial growth was very low, and it included less than 10 CFU even at the peak stage. Regarding the results of bacterial growth, conjunctival vaccination can be considered safe for the vaccine practitioners and the environment.

The amount of bacterial growth in Table 3 was slightly higher in goats than in sheep. Clinical response after Rev.1 vaccination might be more serious in goats than in sheep depending on the well-known high-level susceptibility of goats to brucellosis (Zundel *et al.*, 1992). Another finding related to bacterial growth level is that the isolation from ocular samples included more CFUs than the nasal samples. This correlation

was also similarly investigated in a previous study with nasal, ocular and buccal swab samples (Zundel *et al.*, 1992). In this study, at the end of 15 day period, no isolation was observed, which was in line with the results of Zundel *et al.* (1992).

Vaccination of pregnant animals either with subcutaneous or conjunctival route always includes an abortion risk (Zundel *et al.*, 1992; Blasco, 1997; Garin-Bastuji *et al.*, 1998). However, in this study, vaccinations of pregnant sheep and goats did not cause any abortion. The reason behind this might be the administration of vaccine in the last months of pregnancy, which is considered to be a safer period for vaccination of pregnant animals (Jiménez de Bagüés *et al.*, 1989; Blasco, 1997). Abortion risk due to the Rev. 1 vaccine strain depends on the pregnancy phase and the vaccine dose (Minas, 2006). Vaccination of animals in the second or third month of pregnancy leads to more abortion than the last month of pregnancy (Jiménez de Bagüés *et al.*, 1989; Olsen and Stoffregen, 2005). Late lambing period, lactation, or before mating are recommended periods for vaccination (Blasco, 1997; Minas, 2006; OIE, 2018a).

Infected animal can excrete *brucella* micro-organisms by their milk and vaginal secretions (Ducrotoy *et al.*, 2014). The attenuated vaccine strain could be excreted via milk or vaginal secretions with a high or low amount of bacterial burden, as it was indicated in the previous studies (Fensterbank *et al.*, 1985; Fensterbank *et al.*, 1987; Jiménez de Bagüés *et al.*, 1989; Zundel *et al.*, 1992; Banai, 2002; Godfroid *et al.*, 2005). In this study, the isolation from milk and vaginal secretion was done from sheep and goats of 4 and 6 weeks after vaccination, respectively. These results

are compatible with those of the previous research (Jiménez de Bagüés *et al.*, 1989; Zundel *et al.*, 1992).

Rev. 1 excretion was detected only in one goat among the lactating sheep (n = 7) and goats (n = 42) and this result supports the recommendation of vaccination in the last months of pregnancy or during the lactation (Jiménez de Bagüés *et al.*, 1989; Blasco 1997; Minas, 2006). Behind this suggestion is that the excretion of vaccine strain in milk is thought to be tolerable (Minas 2006). However, it is recommended that *B. melitensis* Rev.1 vaccine should be given to only young, not lactating animals (Banai, 2002).

With regard to serological responses, the titer of the antibody in vaccinated young animals lasted shorter than in adult animals, as indicated in previous research (Fensterbank *et al.*, 1987; Stournara *et al.*, 2007). The response of the animals younger than 1 year-old to serological tests showed low titers of antibodies, which lasted for 2 months, whereas the response of older animals was a much higher and more long-lasting. Therefore, the vaccination of 3 to 6-month--old animals is recommended to avoid serological interference (Jiménez de Bagüés *et al.*, 1989; OIE 2018a). It should be taken into consideration that in vaccinated adults post-vaccinal titers can last for one year or more (Fensterbank *et al.*, 1987; Stournara *et al.*, 2007). On the other hand, serological titers can be higher or even persistent if adult animals are vaccinated subcutaneously (Fensterbank *et al.*, 1982; Jiménez de Bagüés *et al.*, 1989; Zundel *et al.*, 1992). In this study, results of serological test of vaccinated animals were screened 4 months after vaccination. Four-month-post-vaccination period could be used to monitor the serological status of lambs vaccinated conjunctivally (Stournara *et al.*, 2007).

Discrimination of infected and vaccinated animals becomes more difficult because of long-lasting serological response (Zundel *et al.*, 1992). When we evaluate all the results, it is possible to mention that conjunctival vaccination led to a short-term humoral response with low level antibody titers. Even though the response percentage in the first month following the vaccination was very high, the test positivity rate was, in fact, between 55.9 – 82.9 %, as indicated in Table 5. However, the response of most of the animals (92 %) to 3 serological tests was negative at the end of 4 months after vaccination. Antibody titers were partially lower, and the titers of the sera decreased earlier in CFT than other tests. This was not surprising

since this test is considered to be highly specific (OIE, 2018a).

Previous studies indicated that 4 months after vaccination, the serological status of young animals turned into negative (Fensterbank *et al.*, 1982; Fensterbank *et al.*, 1985; Jiménez de Bagüés *et al.*, 1992; Stournara *et al.*, 2007). Short-lasting serological response might be helpful for test-and-slaughter programs (Fensterbank *et al.*, 1985; Hou *et al.* 2019) after mass vaccination, because conjunctival vaccination firstly stimulates the head lymph nodes, which reduces the interference in serological tests (Jiménez de Bagüés *et al.*, 1992; Hou *et al.*, 2019).

The reason for including brucella-infected farms in this study is to evaluate the bacteriological and serological responses after the vaccination of animals in infected farms prior to mass vaccination programs. Since brucellosis is endemic in Türkiye, it was expected that many animals were serologically positive before mass vaccination. Serological response was intense and lasted longer in infected farms. This is an expected result because vaccination induced a second antigenic stimulation in infected animals, which caused to longer-lasting antibody response. Particularly, the serological response of adult animals was barely negative in CFT at the end of 6 months post-vaccination. At the end of 5 months, all the young animals from infected farm were negative to all serological tests. In these infected farms, after vaccination the number of abortions decreased, as stated by farmers. This outcome might confirm that conjunctival vaccination can be safely used in infected farms. However, excretion of field strains via milk of *B. melitensis* was detected one month after vaccination. Therefore, vaccination of infected farms was not considered to be completely sufficient to prevent *Brucella* excretion to the environment (Banai, 2002).

Several elements influence the success of control programs including prevalence, animal husbandry, serosurvey screening, vaccine availability and quality, available resources, legislative authority and intersectoral cooperation (Nicoletti, 2010). It should also be stated that the vaccination can only provide benefits for the control of the disease, but it can never be adequate to eradicate the disease (Olsen and Stoffregen, 2005; Zhang *et al.*, 2018). Increasing the vaccination coverage directly affects the seroprevalence by reducing the prevalence in well-vaccinated areas (Blasco, 1997; Zhang *et al.*, 2018). In order to

achieve successful outcome via mass vaccination, the recommended vaccination coverage is 80 % with high quality vaccines (Minas, 2006; Zhang *et al.*, 2018).

CONCLUSION

In conclusion, regarding the result of this field study, conjunctival Rev.1 vaccination provides innocuousness to host animals and it does not pose risks to practitioners and the environment. Vaccination did not lead to a transmission to the control group and no seroconversion occurred in these unvaccinated animals. The short-lasting serological response was evaluated as the beneficial effect of using conjunctival vaccination. Even though no abortion occurred in this study, abortion risk should be taken into account for the vaccination of pregnant animals.

AUTHOR CONTRIBUTIONS

Conceptualization: Gurbilek Erdenlig, S.
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Writing-review and editing: Gurbilek Erdenlig, S., Karagul, M. S.
Project administration: Gurbilek Erdenlig, S.

All authors have read and agreed to the published version of the manuscript.

INFORMED CONSENT STATEMENT

Not applicable.

DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author.

CONFLICTS OF INTEREST

There is no conflict of interest with any individual or organization regarding the materials discussed in this manuscript.

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