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Safety and efficacy of reduced doses of *Brucella melitensis* strain Rev. 1 vaccine in pregnant Iranian fat-tailed ewes

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Summary

Brucellosis is one of the most important zoonotic diseases and is a significant cause of abortion in animals. *Brucella melitensis* strain Rev. 1 is recommended as the most effective vaccine for small ruminants but the application of full doses in adult animals is restricted. This study was conducted to determine a proper reduced dose of vaccine which confers protection but which is not abortifacient in Iranian fat-tailed sheep. A total of 51 non-vaccinated pregnant ewes were divided into three main groups and several subgroups. Ewes in different groups were vaccinated at different stages of pregnancy and various subgroups were subcutaneously immunised with different quantities of the micro-organism (7.5×10^6, 10^6, 5×10^5). Ewes again became pregnant a year later and were challenged with the wild-type strain to evaluate the protection conferred. Results revealed that the proportion of vaccination-induced abortions was significantly higher in ewes immunised with 7.5×10^6 Rev. 1 organisms than in those which received 10^6 or 5×10^5 bacteria. While 80% of non-vaccinated ewes aborted after challenge, none of the vaccinated ewes aborted post-challenge. This study indicated that a reduced dose of Rev. 1 vaccine containing 10^6 or 5×10^5 live cells could be safely used to induce protection in Iranian fat-tailed sheep at various stages of pregnancy.

Keywords

Brucella melitensis, Brucellosis, Ewe, Fat-tailed ewes, Iran, Sheep, Strain Rev. 1, Vaccine.

Sicurezza ed efficacia di dosi ridotte di vaccino *Brucella melitensis* ceppo Rev. 1 in pecore dalla coda grassa iraniane (fat-tailed ewes) in stato di gravidanza

Riassunto

La brucellosi, zoonosi di primaria importanza, è causa rilevante di aborto negli animali. Il vaccino Brucella melitensis *ceppo Rev. 1 è da considerare il più efficace per i piccoli ruminanti, ma l'applicazione nella dose prevista su animali adulti è sconsigliata. Questo studio è stato condotto per determinare la giusta dose ridotta di vaccino che possa garantire la protezione, ma che non risulti abortiva nelle pecore iraniane dalla coda grassa (fat-tailed ewes). Cinquantuno pecore gravide, non vaccinate, sono state suddivise in tre gruppi principali e ulteriormente ripartite in sottogruppi.*

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*Quelle sottoposte a vaccinazione (in diverse fasi della gravidanza) sono state immunizzate per via sottocutanea con quantità diverse di microrganismo (7,5 × 10^6, 10^6, 5 × 10^5). Le pecore gravide, in seguito, sono state trattate con l'inoculazione del ceppo selvatico per valutare il livello di protezione conferita. I risultati hanno rivelato una percentuale di aborti significativamente più alta in pecore immunizzate con 7,5 × 10^6 Rev. 1, rispetto a quelle trattate con 10^6 o 5 × 10^5 batteri. Delle pecore non vaccinate l'80% ha abortito dopo il challenge, al contrario di quelle vaccinate. Questo studio ha dimostrato che una dose ridotta di vaccino Rev. 1 (contenente 10^6 o 5 × 10^5 microrganismi) è innocua ed in grado di indurre protezione in pecore iraniane dalla coda grassa (*fat-tailed ewes*) nelle varie fasi della gravidanza.*

Parole chiave

Brucella melitensis, Brucellosi, Ceppo Rev. 1, Iran, Pecora, Pecora dalla coda grassa, Vaccino.

Introduction

Small ruminant brucellosis caused by *Brucella melitensis* is an endemic zoonosis in Iran which is of paramount importance due to its public health hazards (18, 23). It also results in economic losses to the animal production industry by causing abortion and infertility (6, 17). Vaccination of sheep and goats is considered as the main control strategy for which attenuated live *B. melitensis* strain Rev. 1 is used (6, 10). It is recommended to immunise lambs and kids at 3 to 7 months of age with full doses of the vaccine containing 10^9 bacteria (22). However, vaccination of replacement animals is not sufficient for success in the control of the disease, especially in countries with high prevalence and uncontrolled animal movements (6, 16). Additionally, production of small ruminants in nomadic and low socio-economic conditions and strong possibilities of transboundary spread of the disease from surrounding countries due to illegal livestock imports are factors which create many of the difficulties encountered while combating the disease in many areas of Iran (13). In such a situation, mass vaccination (vaccination of the entire flock at the same time) may be a more feasible strategy (6, 17). Nevertheless, applying full doses of the vaccine may induce abortions in pregnant animals and result in long-lasting humoral responses that interfere with serological diagnosis (5).

To overcome these drawbacks and limitations, vaccination with reduced doses of strain Rev. 1 was introduced (2, 9), but there is much controversy over its usefulness. While use of reduced doses of Rev. 1 vaccine is recommended by some authors in mass vaccination campaigns (1, 17, 20), there are studies which show that the vaccination of pregnant animals even at reduced doses is not without adverse effects (see a review in 5). Several reduced doses have been studied on adult animals in various experiments (5, 17), but there is still no consensus on a defined range of viable cells which is safe and effective in pregnant sheep (17). Moreover, breed susceptibility has been implicated in the occurrence of vaccine abortifacient effects (17). Therefore, the present study was designed to evaluate safety and efficacy of various reduced doses of the vaccine in Iranian fat-tailed ewes at different stages of pregnancy.

Material and methods

Animals and vaccination protocols

In this randomised experimental field trial, 51 non-vaccinated clinically healthy adult Iranian fat-tailed ewes, which were non-reactive in Rose Bengal test (RBT) and the serum agglutination test (SAT), were selected from a known brucellosis-free flock with no history of abortion. All animals were kept in the same conditions and fed a similar diet. After a period of nutritional flushing, ewes were mated with healthy rams and the time of mating was recorded for each ewe. Pregnant animals were randomly divided into three main groups (n = 17 per group), and then each group was divided into four subgroups (three subgroups of 5 ewes and one subgroup with 2 ewes as controls). Vaccination was performed using different doses of vaccine at different months of pregnancy as shown in Table I.

Table I
Vaccination protocols for different groups and subgroups

Group	Subgroup	Number of ewes	Month of pregnancy at the end of which vaccination was performed	Number of Rev. 1 bacteria per dose
1	1	5	First	7.5×10^6
	2	5		10^6
	3	5		5×10^5
	Control*	2		–
2	1	5	Second	7.5×10^6
	2	5		10^6
	3	5		5×10^5
	Control	2		–
3	1	5	Third	7.5×10^6
	2	5		10^6
	3	5		5×10^5
	Control	2		–

* in control animals, the same volume of normal saline was injected subcutaneously instead of the vaccine

Ewes were observed daily following vaccine or placebo administration to detect any induced abortion until parturition.

Vaccine

The Rev. 1 vaccine utilised in the study was produced at the Razi Vaccine and Serum Research Institute of Iran according to standard procedures (3) for which the original seed was supplied by Veterinary Laboratories Agency in Weybridge.

Serological examination

Blood samples were collected monthly after vaccination of all groups for three months, and samples were examined serologically using both the RBT and SAT. Antigens for these tests were produced and standardised in the Razi Vaccine and Serum Research Institute as described previously (3, 22). Briefly, *B. abortus* strain 99 was cultured on potato agar in Roux flasks, and incubated at 37°C for 72 h. The bacteria were harvested using 0.5% (v/v) phenol in 0.85% (w/v) NaCl solution (phenol saline). The suspension was heated at 80°C for 90 min to kill the organisms and centrifuged at 23 000 g for 10 min at 4°C. To produce RBT antigen, the cell sediment was re-suspended in phenol saline (1 g in 22.5 ml) and stained by adding 1 ml of 1% (w/v) Rose Bengal (Fluka, Buch) in sterile distilled water to each 35 ml of the suspension. After stirring the mixture for 2 h at room temperature, it was filtered through sterile cotton and cells were deposited in a refrigerated centrifuge at 10 000 g. Stained cells were re-suspended in buffered diluent (21.1 g NaOH and 95 ml lactic acid in 961 ml phenol saline) with a pH adjusted to 3.65 ± 0.05 to give a suspension containing 8% packed cell volume (PCV). Finally, RBT antigen was standardised in such a way as to produce agglutination with 1/45 dilution of the World Organisation for Animal Health (*Office International des Épizooties*: OIE) international standard serum (OIEISS) (obtained from the Veterinary Laboratories Agency in Weybridge), but not with its 1/55 dilution. To perform the RBT, equal volumes of the antigen and serum sample were mixed for agglutination formation. For preparation of SAT antigen, after re-suspension of killed cells sediment in phenol saline, the antigen was standardised to produce 50% agglutination with 1/650 titre of OIEISS. To perform the test, a phenol saline prepared from 5% (w/v) NaCl solution was used to dilute the antigen and serum samples. Antibody titres of the samples were determined in terms of international units (IU) per ml.

Challenge with wild-type strain

To assess protection conferred by vaccination over pregnancy, ewes were subcutaneously challenged with 4×10^9 colony-forming units

(cfu) of *B. melitensis* strain 16M (supplied by the Veterinary Laboratories Agency in Weybridge) at the end of the first month of a second pregnancy that occurred one year later. An ampoule of dried *B. melitensis* strain 16M was re-suspended in phosphate-buffered saline (PBS) and inoculated on *Brucella* agar (BD, Sparks, Maryland) slopes. After incubation for 48 h at 37°C, the organisms were harvested into PBS. The number of live bacteria in 1 ml of the suspension was determined by preparing serial decimal dilutions and colony counting. Based on the result, the suspension was diluted in PBS to contain 4×10^9 cfu per ml. One ml of this final dilution was injected subcutaneously behind the shoulder of each animal. All animals were observed daily for detection of abortion until delivery.

Isolation of *Brucella* from aborted foetuses

For microbiological examination of abortions induced after vaccination during the first pregnancy or following challenge over the second pregnancy, specimens obtained from the lung, spleen, liver and foetal stomach contents of aborted foetuses were cultured on *Brucella* agar media (BD, Sparks, Maryland) containing 5% (v/v) inactivated equine serum and *Brucella* selective supplement (Oxoid, Basingstoke, Hampshire) in accordance with the instructions of the manufacturer. Identification of strains was performed using methods described previously (3).

Statistical analysis

A comparison of the proportion of vaccine-induced abortions between different groups/subgroups and controls and also the rate of post-challenge abortions between vaccinated animals and non-vaccinated controls was done using Fisher's exact test for which a p-value of ≤0.05 was considered significant.

Results

Vaccination-induced abortions

Three ewes in group 1 aborted after vaccination. Two of these ewes had received 7.5×10^6 cfu, and abortion occurred 28 days and 42 days post-vaccination. Vaccine strain was isolated from both foetuses. The third one aborted one day post vaccination which was administered with 10^6 cfu and the foetus gave negative results for the vaccine strain.

In each of groups 2 and 3, one pregnant animal aborted approximately one month post vaccination; both had received 7.5×10^6 cfu. Rev. 1 organisms were isolated from the aborted foetuses of these animals.

The rates of vaccination-induced abortions in different groups and subgroups are illustrated in Tables II and III. Although the proportion of post-vaccination abortions increased while vaccination was performed during early pregnancy and with higher doses of the vaccine, the differences observed in various groups and subgroups were not statistically significant in comparison to controls (p >0.05).

However, a comparison of the proportion of post-vaccination abortions in ewes receiving 7.5×10^6 Rev. 1 organisms with that of other vaccinated animals revealed a significantly lower risk of abortion when vaccination was performed at doses of 10^6 or 5×10^5 ($p = 0.036$).

Table II
Number of abortions following vaccination of different groups

Group	No. of animals	No. of abortions (%)	p-value versus controls
1	15	3 (20%)	0.3
2	15	1 (6.7%)	0.7
3	15	1 (6.7%)	0.7
Controls	6	0 (0%)	–

Antibody response following vaccination

Serological evaluation of animals revealed that all vaccinated ewes were positive to the RBT and SAT within two months post vaccination. Three months after the injection of the vaccine, vaccination-induced antibodies dropped in all animals immunised with different doses at different months of pregnancy to levels which were not detectable in the tests (Fig. 1).

Table III
Number of abortions following vaccination of different subgroups

Subgroup	No. of colony-forming units	No. of animals	No. of abortions (%)	*p*-value versus controls
1	7.5×10^6	15	4 (26.7%)	0.2
2	10^6	15	1 (6.7%)	0.7
3	5×10^5	15	0 (0%)	–
Controls	–	6	0 (0%)	–

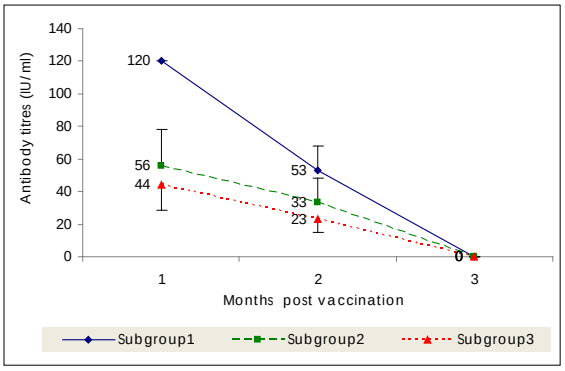

Figure 1
Mean ± standard deviation of serum agglutination test antibody titres following vaccination of different subgroups

Protection against challenge

Four ewes, a control and three vaccinated animals, did not become pregnant the following year and these data were excluded from statistical analysis. After challenge of pregnant animals with the virulent strain, none of vaccinated ewes aborted, but four control ewes aborted one to two months post challenge. The virulent strain was isolated from their aborted foetuses (Table IV).

Table IV
Number of post-challenge abortions in vaccinated and non-vaccinated animals

Vaccination	No. of aborting ewes (%)	No. of non-aborting ewes (%)	Total (%)
Vaccinated	0 (0)*	42 (100)	42 (100)
Controls (not vaccinated)	4 (80)*	1 (20)	5 (100)

* significantly different ($p = 0.000$)

Discussion

Small ruminant brucellosis caused mainly by *B. melitensis* is still a worldwide zoonosis which is of paramount importance especially in the Middle East and the Mediterranean Region due to the hazards it poses to public health and also the losses it causes to livestock production (6, 13, 17). It is well documented that prevention of human infection is highly dependent on the control of the disease in animal reservoirs (12, 14, 16, 17). Vaccination with *B. melitensis* strain Rev. 1, which is known to be the most effective vaccine for the prophylaxis of the disease in sheep and goats, is widely used all around the world as an important component of control programmes (4, 5, 6, 10, 17, 21). Protection conferred by Rev. 1 vaccine prevents abortions and reduces pathogen shedding when immunised animals are infected (4). However, the vaccine strain has drawbacks, such as inducing antibodies that interfere with serological diagnosis and retaining some degrees of virulence which can lead to abortion in pregnant animals and excretion in milk (4, 5, 21).

While the eradication of *B. abortus* infection in cattle has been achieved with success in many countries, control and eradication of *B. melitensis* in sheep and goats has been more problematic and complicated (11, 12, 17), particularly in developing countries where low socio-economic conditions, uncontrolled animal movements, transboundary spread of the disease and traditional livestock production in rural areas provide the agent with the opportunity to persist and re-emerge

(12, 13). Moreover, it is evident that vaccinating young animals only has not been effective in controlling small ruminant brucellosis in countries with high disease prevalence (4, 6, 12, 14, 16). Although mass vaccination is considered as a more effective and practical strategy in these situations (4, 6, 16, 17, 18), the adverse effects of the vaccine restrict its utilisation in adult animals. Hence, the use of reduced doses of Rev. 1 vaccine have been recommended (1, 17, 20) but there are doubts about whether vaccination of adult animals with reduced doses confers a good level of protection and can obviate problems with abortifacient effects (5, 6). Additionally, in Iran, due to the lack of adequate financial resources to compensate abortions induced by vaccination and unavailability of the vaccine for conjunctival use, which is considered as a method with little side effects (5, 6), subcutaneous immunisation of adult sheep and goats with reduced doses of the vaccine remains the only feasible strategy for mass vaccination campaigns.

In our study, a reduced dose of 7.5×10^6 cfu induced abortion in a considerable number of pregnant ewes, although it was not significant in comparison to controls, but the vaccine strain was isolated. When the proportion of abortions in ewes receiving 7.5×10^6 cfu was compared to that in ewes immunised with a dose of 10^6 or 5×10^5, the difference was significant ($p = 0.036$). As Rev. 1 strain was not isolated from the only aborting ewe vaccinated with a dose of 10^6 cfu which aborted one day post vaccination, this might be either spontaneous or caused by vaccination stress (7). Irrespective of this, none of other animals vaccinated with 10^6 and 5×10^5 cfu from one to three months of gestation aborted post immunisation. This result is in contrast with field observations reported in Spain where vaccination of over a million sheep and goats with a reduced dose of 10^6 cfu led to thousands of abortions (5). The differences between our results and those observed in Spain might be due to either the different breed susceptibility or factors governing field conditions. Crowther *et al.* (7) also reported a high rate of abortions after vaccination of pregnant Cyprus fat-tailed ewes with reduced doses of 10^6 cfu which does not concur with our results, but the abortifacient effects of this vaccine dose in their study was not conclusive. However, the results obtained in the present study concur with other experiments performed using similar reduced doses subcutaneously (8, 15, 19). Thus, although our data favour low dose vaccination at less than 5×10^6 cfu, such controversy does not eliminate the risks of abortion and vaccine strain excretion when designing a national programme.

All reduced doses of the vaccine used in this study, even doses as low as 5×10^5 cfu, conferred a level of immunity which protected ewes against abortion caused by wild-type challenge. Vaccination at different months of pregnancy did not make any difference in the protection conferred. Considering these findings, one can conclude that doses of 5×10^5 to 10^6 cfu can be applied safely for the immunisation of pregnant Iranian fat-tailed ewes to prevent infective abortions. Prevention of abortion is known as an effective method of disease control (4).

Evaluation of serological responses to different doses indicated that antibody titres decreased after two months post vaccination in all vaccinated animals and none of them were positive in SAT and RBT three months post vaccination which is compatible with previous findings (7, 9, 19). In this study, we neither evaluated vaccine strain secretion nor challenge strain in the milk of vaccinated ewes for which further studies are required. This should be taken into account regarding public health hazards it may pose while applying reduced doses of the vaccine in the field.

Conclusions

To conclude, our study revealed that while all reduced doses of Rev. 1 vaccine used could induce protective immunity in Iranian fat-tailed sheep at different stages of pregnancy which protected animals against abortion caused by wild-type infection, a dose containing 5×10^5 or 10^6 of Rev. 1 organisms was safer and did not raise serious concerns about serological interference. However, a

thorough field investigation is required in Iran to assess cost-effectiveness of mass vaccination using reduced doses of Rev. 1 vaccine for adult animals. It is also suggested that conjunctival administration of standard or reduced doses of the vaccine in local sheep breeds be evaluated to determine its efficacy and safety.

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References

1. Al-Khalaf S.A., Mohamad B.T. & Nicoletti P. 1992. Control of brucellosis in Kuwait by vaccination of cattle, sheep and goats with *Brucella abortus* strain 19 or *Brucella melitensis* strain Rev. 1. *Trop Anim Health Prod*, **24**, 45-49.
2. Alton G.G. 1970. Vaccination of goats with reduced doses of Rev. 1 *Brucella melitensis* vaccine. *Res Vet Sci*, **11**, 54-59.
3. Alton G.G., Jones L.M., Angus R.D. & Verger J.M. 1988. Techniques for the brucellosis laboratory. Institut National de la Recherche Agronomique, Paris, 190 pp.
4. Banai M. 2002. Control of small ruminant brucellosis by use of *Brucella melitensis* Rev. 1 vaccine: laboratory aspects and field observations. *Vet Microbiol*, **90**, 497-519.
5. Blasco J.M. 1997. A review of the use of *B. melitensis* Rev 1 vaccine in adult sheep and goats. *Prev Vet Med*, **31**, 275-283.
6. Blasco J.M. 2010. Control and eradication strategies for *Brucella melitensis* infection in sheep and goats. *Prilozi*, **31**, 145-165.
7. Crowther R.W., Orphanides A. & Polydorou K. 1977. Vaccination of adult sheep with reduced doses of *Brucella melitensis* strain Rev. 1: safety and serological responses. *Trop Anim Health Prod*, **9**, 85-91.
8. Delgado S., Cármenes P. & Fernández M. 1995. Seroprevalences and lack of abortions after vaccination of Churra sheep with reduced doses of Rev. 1 *Brucella melitensis* vaccine by subcutaneous or conjunctival routes. *Prev Vet Med*, **23**, 153-161.
9. Elberg S.S. 1981. Rev. 1 *Brucella melitensis* vaccine. Part II 1968-1980. *Vet Bull*, **51**, 67-73.
10. Garin-Bastuji B., Blasco J.M., Grayon M. & Verger J.M. 1998. *Brucella melitensis* infection in sheep: present and future. *Vet Res*, **29**, 255-274.
11. Godfroid J. & Käsbohrer A. 2002. Brucellosis in the European Union and Norway at the turn of the twenty-first century. *Vet Microbiol*, **90**, 135-145.
12. Godfroid J., Cloeckaert A., Liautard J.P., Kohler S., Fretin D., Walravens K., Garin-Bastuji B. & Letesson J.J. 2005. From the discovery of the Malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. *Vet Res*, **36**, 313-326.
13. Gwida M., Al Dahouk S., Melzer F., Rosler U., Neubauer H. & Tomaso H. 2010. Brucellosis – regionally emerging zoonotic disease? *Croat Med J*, **51**, 289-295.
14. Jelastopulu E., Bikas C., Petropoulos C. & Leotsinidis M. 2008. Incidence of human brucellosis in a rural area in western Greece after the implementation of a vaccination programme against animal brucellosis. *BMC Public Health*, **8**, 241.
15. Jimenez de Bagues M.P., Marin C.M., Barberan M. & Blasco J.M. 1989. Responses of ewes to *B. melitensis* Rev. 1 vaccine administered by subcutaneous or conjunctival routes at different stages of pregnancy. *Ann Rech Vet*, **20**, 205-213.

16. Minas A., Minas M., Stournara A. & Tselepidis S. 2004. The 'effects' of Rev-1 vaccination of sheep and goats on human brucellosis in Greece. *Prev Vet Med*, **64**, 41-47.
17. Nicoletti P. 2010. Brucellosis: past, present and future. *Prilozi*, **31**, 21-32.
18. Refai M. 2002. Incidence and control of brucellosis in the Near East region. *Vet Microbiol*, **90**, 81-110.
19. Sales Henriques H.L.R., Hueston W.D., Hoblet K.H. & Shulaw W.P. 1992. Field trials evaluating the safety and serologic reactions of reduced-dose *Brucella melitensis* Rev. 1 vaccination in adult sheep. *Prev Vet Med*, **13**, 205-215.
20. Scharp D.W., Al-Khalaf S.A., Al-Muhanna M.W., Cheema R.A. & Godana W. 1999. Use of mass vaccination with a reduced dose of Rev. 1 vaccine for *Brucella melitensis* control in a population of small ruminants. *Trop Anim Health Prod*, **31**, 135-141.
21. Schurig G.G., Sriranganathan N. & Corbel M.J. 2002. Brucellosis vaccines: past, present and future. *Vet Microbiol*, **90**, 479-496.
22. World Organisation for Animal Health (OIE: Office International des Épizooties). 2009. Caprine and ovine brucellosis (excluding *B. ovis*). *In* Manual of diagnostic tests and vaccines for terrestrial animals. OIE, Paris (www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.07.02_caprine_Ovine_bruc.pdf accessed on 18 November 2012).
23. Zowghi E., Ebadi A. & Yarahmadi M. 2008. Isolation and identification of *Brucella* organisms in Iran. *Iranian J Clin Infect Dis*, **3**, 185-188..

