Evaluation of oral immunization of sheep with Brucella Melitensis vaccine REV.1 in combination with Flagellin against a virulent Brucella melitensis 16 M strain

[1]Lubna F. Farahat, [2]Mohamed G. Abdelwahab, [2]Mohamed H. Ebied,[2]Abdel-Moneim, Moustafa, and [1] Saleh, M.S. El-Ayouby

[1]Antigens and Serums Dep., VSVRI. Abbasia, Cairo Egypt. [2]Animal Medicine Department Faculty of veterinary Medicine Benha University, Egypt

ABSTRACT

In this work five vaccination protocols were prepared and applied these protocols in sheep. Serum samples were collected from each group at time points 25, 50, 77, 89, and 98 days post vaccination. The results revealed that humoral antibodies were detected in groups 3, 4 and 5 by using Rose Bengal test (RBT), Buffer Acidified Plate Antigen Test (BAPAT), and Tube Agglutination Test at 98 days, 77 days, and 77 days respectively, while for ELISA test revealed that were positive at 50, 89, 98, 98, and 98 days post vaccination in group 1, 2,3,4 and 5 respectively. Cell mediated immunity was evaluated by Lymphocyte Blastogenesis Assay Test and Brucellin test (Delayed Type Hyper Sensitivity Test). The results indicated that there were no significant differences in between mean of different groups at $P \leq 0.05$, So for Skin Delayed Hyper Sensitivity test, Group 1 and group 6 were negative while Group 2, 3, 4 and 5 were positive. Conclusion, animals in Groups 3, 4 and 5 had humoral immune response and can be protected from abortion in pregnant ewes and prevent infection. In this work, we evaluated to potential of three doses reduced Rev.1 mixed with E. coli flagellin which induced protection without need of adjuvant against I/P Brucella melitensis challenge. Also these data suggest that flagellin proteins might induce protective immune responses and these proteins will be a good candidate for subunit vaccine against ovine brucellosis in sheep.

Key Words: Brucella melitensis, Rose Bengal Test, Buffer Acidified Plate Antigen Test

(http://www.bvmj.bu.edu.eg) **(BVMJ-29(2): 200-207, 2015)**

1. INTRODUCTION

Brucellosis remains endemic in many developing countries (Cassataro et al., 2007). Brucellosis results in economic losses to the animal production industry by causing abortion and infertility (Blasco 2010, Nicoletti 2010). Also this pathogen causes undulant fever, arthritis, endocarditis and meningitis in human (Sergio et al., 2010). Brucella spp. can persist in unpasteurized dairy products such as raw milk, soft cheese, butter and ice-cream. *B. melitensis* strain Rev.1 is recommended as the most effective vaccine for small ruminants (Mohammad Ebrahimi et al., 2012). At this moment, three Brucella vaccines have been used in Brucellosis prevention: Strain 19, Rev.1, and RB51. However, these strains are still far from ideal. Although the smooth strains Strain 19 from *B. abortus* and Rev.1 from *B. melitensis* are able to induce effective levels of protection in cattle and in goat and sheep: respectively. These vaccines some problems when these vaccines used to vaccinate adult animals, they caused abortion in pregnant animals vaccinated with full standard doses of Rev.1 ($1\text{-}2\times10^9$ CFU) administered subcutaneously, as well as they can be secreted in milk of vaccinated animals. Besides, both of them are pathogenic to humans and interfere with the serological diagnosis because long lasting

of humoral responses (Nicoletti et al., 1990, Mohammad Ebrahimi et al., 2012). Current vaccines are effective in preventing abortion and transmission of brucellosis, but poor at preventing infection (Blasco, 1997). Reducing the dose of vaccine has been suggested as a method of avoiding this problem and accordingly, a reduced dose vaccination strategy has been widely used and has been reported as a safe and effective method for controlling small ruminant Brucellosis (Blasco 1997). Flagelin, the major structural protein of the flagelar filament of Gram-negative bacteria, is an extra-ordinarily potent inducer of innate immunity. (Honko and Mizel, 2004). Vaccination with oral flagellar protein H7 to induce protection against B.melitensis Rev.1 infection in sheep and detect the protection level of combined reduced dose of Rev.1 vaccine and flagellar protein orally then Compare between the different types of vaccination to Subcutaneous full dose of Rev.1 vaccine by using humoral and cell mediated immune response.

2. MATERIALS AND METHODS

2.1. Experimental animals:

Thirty-one sheep, aged between three to six months and some of sheep are pregnant, non-pregnant males. It selected from a known Brucellosis free flock with no history of abortion after apply of serological tests (RBT, BAPT, TAT) for about 3 successive months to give negative results to considered free from brucellosis. Sheep were divided into six groups, group 1 were 5 animals each vaccinated orally with 60 µg flagellin only for three successive doses one week interval, group 2 were 6 animals each vaccinated orally with reduced dose of local prepared Rev.1 vaccine $2x10^8$ CFU and flagellin 60 µg mixed together and give 3 successive doses one week interval, group 3 were 5 animals each vaccinated subcutaneously with 60 µg flagellin three successive doses one week interval, group 4 were 5 animals each vaccinated subcutaneously full dose of local prepared Rev.1 vaccine ($1\text{-}2x10^9$ CFU) only, group 5 were 5 animals each vaccinated subcutaneously full dose of local prepared Rev.1 vaccine ($1\text{-}2x10^9$ CFU) and 180 µg of flagellin, group 6 were 5 animals served as control group they were given orally PBS as shown in Table.1.

2.2. Types of different immune potentiation (adjuvants):

a. Flagellin, local prepared from virulent strain of E. coli O157:H7. Dosage:0.2 ml of flagellin contains 60µg of flagellin. (McNeily et al., 2008). A total (31) sheep were divided in to 6 groups: Show table (2).

2.3. Brucella strains:

2.3.1. Brucella melitensis Rev.1:

A vaccine strain was kindly obtained from seed strain (obtained from National Veterinary Services Laboratories "NVSL", 1800 Dayton Avenue, Ames, Iowa, 50010, USA) obtained from VSVRI in Abbasia, Cairo

2.3.2. Brucella melitensis strain 16M:

It was supplied by USDA, USA, National Veterinary Services Laboratories "NVSL", Ames, Iowa, 50010. Strains (3.1.2.1, 3.1.2.2., 3.1.2.3) were reconstituted in 10 ml diluent (0.75 M NaCl, pH 6.4).

2.3.3. Brucella abortus strain RB51:

Brucella abortus strain RB51 a vaccinal strain, is kindly provided by private cattle farm, lyophilized vaccine vials of 5 doses, each and the dose of (3.4×10^{10}CFU), lyophilized vaccine, serial No. 1472, Professional Biological Company, 4950 York St., Denver, Colorado 8021. USA. The vaccine vial was reconstituted in 10 ml diluent (0.15M NaCl, pH 6.4).

2.4. E. coli O157:H7 (EHEC):

Strains was tested and confirmed by standard technique. The strain was kindly provided by serological Unit of Animal Health Research Institute, Dokki, and Giza, Egypt.

2.5. Preparation of H7 flagellin:

H7 flagellin was prepared according to El-Ayouby et al. (2008). H7 flagellin was examined by SDS-PAGE as described in (He and Keel, 1994). Purified flagellin H7 protein was determined.

2.6. Brucella antigens:

2.6.1. Rose Bengal Antigen:

Prepared in VSVRI, Abbasia, Cairo accpording to Alton et al. (1988).

2.6.2. Buffer Acidified Plate Antigen:

Prepared in VSVRI, Abbasia, Cairo accpording to Alton et al. (1988). *2.1.4.3. Tube*

2.6.3. Agglutination Antigen (B. abortus):

Prepared in VSVRI, Abbasia, Cairo, according to Alton et al. (1988).

2.7. Evaluation of humoral immune response of vaccinated sheep by using

Serological tests: Blood samples were collected from all groups of sheep (G1-G6) every 2 week until the end of the experiment 12weeks (for 3months). Sera were stored at -70ºC. After inactivation and examined by Brucella antigen. All sheep were tested for anti-Brucella before vaccination, on the day of Brucellin inoculation and at days 15, 30, 45, 60 and 75 post Brucellin inoculation and days examined for lymphocyte transformation test (LTT).

2.8. *Rose Bengal Test (RBT):* was applied according to Alton et al., 1988.

Buffer Acidified Plate Antigen Test according to Alton (1988). *Tube Agglutination Test (TAT)* according to Alton et al, 1988).

2.9. ELISA Test according to (Alton et al., 1988). For evaluation of humoral immune response

2.10. Brucella vaccine:

Brucella melitensis Rev.1 vaccine.from (VSVRI).

2.11. RB51 Brucellin: Professional Biological Company, 4950 York Street, Denver, Colorado 80216.

2.12. Evaluation of cell mediated immune response by

Delayed Type Hypersensitivity test according to Araya et al., 1989. Lymphocyte Blastogenesis Assay test: for evaluation of cell mediated immune response according to Slater et al., 1963.
Serum samples were collected from all sheep groups vaccinated and control every 2 weeks post vaccination. The sera were inactivated at 56°C for 30 minutes, and then stored at -20C until used in HI test.

2.13. Heparinized blood samples:

Jugular blood samples from vaccinated and non-vaccinated sheep were collected with anticoagulant (Heparin 20-40 IU/ml) every 2 weeks post vaccination for evaluation of cell mediated immune response by Lymphocyte Blastgenesis assay.

2.14. Culture media:

Tryptone soya agar: Tryptone soya agar medium with bovine serum 5-10 % prepared according to method of. For growth of Brucella strains. Alton et al. (1988)

3. RESULTS:

3.1. Result of humoral immune response:

A-Serological test:
Group (1) oral vaccination with flagellin and group (6) injected with PBS. Humoral immune response gave (-ve) results for 98 days from vaccination. Group (2, 3, 4, and 5) gave humoral results as in table (3) after 98 days from vaccination. *B-ELISA test:* showing table no. (7) and fig. (3).

3.2. Result of cell mediated immune response of sheep groups:

Results of lymphocyte blastogenesis assay: To further investigate the cellular immune response induced by the different type vaccines in sheep groups. As shown in table (4) and in Fig. (1). Table (5) showed that in group 2 oral vaccination reduce Rev.1 with flagellin exhibited a similar degree of blast genesis to group (5) vaccinated was S/C full

dose Rev.1 and flagellin. Also group (2) induced a higher degree of blast genesis than group (4) vaccinated with full dose of Rev.1 vaccine S/C after 3 weeks and 6 weeks and 8 weeks from vaccination. There are no significant differences between mean of different groups at $P \leq 0.05$. table (5). Lymphocyte assays sheep were immunized with PBS or Rev.1 S/C or orally with and without flagellin. Lymphocyte proliferation responses were measured at 3 weeks and 3 weeks after last immunization. Fig (2).

3.3.Comparison of different groups vaccinated of sheep

Table (6) group (2) oral vaccinated with reduced dose Rev.1 and flagellin gave 3 successive doses with high blastogenesis $1.052 \neq 0.81$ and gave Brucellin test after 1 month from vaccination (7.03 mm) and humoral immune response gave [1 (+ve) and 5 (-ve)] for RBT, BAPT, TAT to 50 days after vaccination in sheep 2 group.

Table. 1 Groups of experimental animals (sheep)

Groups	Group (1)	Group (2)	Group (3)	Group (4)	Group (5)	Group (6)
Type of injected material	Flagellin only	Reduced dose of local prepared Rev.1 vaccine +flagellin. Mixed together.	Flagellin only	Full dose of local prepared of Rev.1 vaccine without flagellin.	Full dose of local prepared of Rev.1 vaccine+flagellin mixed together.	(PBS) Phosphate Buffer Saline
No.of dose	3 successive dose about 1 week interval.		3 successive dose	Only one dose.		
Time			1week intervals between 1st, 2nd and 3rd dose.		.	
Dosage of each material	Dose of flagellin 60µg	Reduced dose of Rev.1 (1-2×10^8(CFU) Dose of flagellin: 60µg	Dose of flagellin 60µg	Dose of local prepared of Rev.1 vaccine(1-2×10^9CFU)	Full dose of local prepared of Rev.1 vaccine (1-2×10^9(CFU) Dose of flagellin:180µg	
Route of injection	orally		S/C	S/C	S/C	Oral

Table.2 Result Rose Bengal Test (RBT), (BAPA), (TAT) for detection of humoral immune responses of sheep groups:

Animal groups	Sheep groups Mean optical densities					
	Group (1)	Group (2)	Group (3)	Group (4)	Group (5)	Group (6)
B- Humoral immune response RBT, BAPA TAT	All(-ve) for 3 month	1/6 1(+ve) 5(-ve) for 50 day.	3/2 3(+ve) 2(-ve) After 98 day.	1/5 1(+ve) 5(-ve) After 89 day.	2/3 2(+ve) 3(-ve) After 98 day.	(-ve) all control

Table (3): Humeral immune response (ELIZA test) between different treated groups at different times after sheep vaccination.

Group	25 days	50 days	77 days	87 days	98 days
Group 1	0.205 ± 0.001	0.192 ± 0.001	0.153 ± 0.001	0.137 ± 0.001	0.124 ± 0.002
Group 2	0.263 ± 0.001	0.253 ± 0.001	0.233 ± 0.001	0.199 ± 0.003	0.159 ± 0.007
Group 3	0.262 ± 0.001	0.207 ± 0.001	0.202 ± 0.001	0.193 ± 0.001	0.184 ± 0.001
Group 4	0.243 ± 0.001	0.219 ± 0.006	0.197 ± 0.002	0.190 ± 0.002	0.183 ± 0.001
Group 5	0.337 ±0.002	0.233 ± 0.001	0.253 ± 0.001	0.251 ± 0.008	0.198 ± 0.011
Group 6	0.162 ± 0.001	0.152 ± 0.001	0.142 ± 0,001	0.132 ± 0.001	0.122 ± 0.001
Mean	0.245 ± 0.010	0.209 ± 0.006	0.197 ± 0.007	0.184 ± 0.008	0.162 ± 0.006

ANOVA showing difference in ELIZA test between different treated groups at different times after sheep vaccination.

Table (4): Lymphocyte blastogenesis assay after vaccination for the different treated groups of sheep

Group	3 Weeks	6 Weeks	8 Weeks	Mean
Group 1	0.240 ± 0.078	0.457± 0.058	0.777± 0.246	0.491± 0.100
Group 2	0.514± 0.027	0.680± 0.195	1.206± 0.364	0.800± 0.149
Group 3	0.333± 0.035	1.121± 0.223	0.492± 0.095	0.648± 0.118
Group 4	0.33± 0.021	0.561± 0.200	0.859± 0.351	0.597± 0.135
Group 5	0.451± 0.051	0.756± 0.163	1.176± 0.268	0.794± 0.126
Group 6	0.157± 0.011	0.739± 0.033	0.182± 0.016	0.359± 0.072
Mean	0.345± 0.028	0.719± 0.072	0.782± 0.117	0.615± 0.056

ANOVA for lymphocyte blastogenesis assay

Source of variance	S.E.	df	M.S.	F-value	Sig.
Between groups	2.226	5	0.445	2.826	0.022
Between times	3.352	2	1.676	10.639	0.000
group * time interaction	3.427	10	0.343	2.175	0.029
Error	11.344	72	0.158		

Table (5): Cell mediated immune response for the different treated groups of sheep Judged by skin delayed hyper sensitivity test (SDHT).

Groups	48 hrs	72 hrs	Mean
Group 1	5.060± 0.268	3.880± 0.222	4.470± 0.256
Group 2	6.940± 0.885	4.960± 0.557	5.950± 0.593
Group 3	7.040± 0.803	5.900± 0.612	6.470± 0.512
Group 4	9.120± 0.107	5.920± 0.404	7.520± 0.568
Group 5	10.140± 0.427	5.800± 0.584	7.970± 0.794
Mean	7.660± 0.435	5.292± 0.259	6.476± 0.302

ANOVA

Source of variance	S.E.	df	M.S.	F	Sig.
Between groups	76.227	4	19.057	12.998	.000
Between times	70.093	1	70.093	47.809	.000
group * time interaction	19.127	4	4.782	3.262	.021
Error	58.644	40	1.466		

4. DISCUSSION

Brucella is intracellular pathogen that causes abortion in domestic animals (sheep, cattle and goats) Brucella melitensis is able to invade erythrocytes in vivo but not multiply in erythrocytes (Vitry, 2014) (Fretin et al, 2005) reported that B. melitensis 16M were grown 48hr in 2yT (peptone, 16giL, yeast extract 16 g/L, NaCl 5g/L), a complete polar flagellar structure surrounded by a LPS sheath is visible by transmission electron microscopy (TEM). In this study, evaluated the motility of B. melitensis by inoculated the strain in motility medium and inoculated at 37ºC for 2weeks and examined as appeared in photo. Oral delivery of vaccines is an attractive mode of immunization because it would induce both systemic and mucosal immunity. Moreover, oral delivery has no requirement for needle administration. Orally Rev.1 administered without the need of adjuvants which induce protection against a mucosal challenge with B. melitensis 16M (Jueassat, 2011). Oral vaccines are relatively easy to administer, and their use avoids working with contaminated needles and syringes (Levine 2010). This study was conducted to administer Rev.1 vaccine by oral route by using three reduced dose (1×10^7 CFU). In this route the Rev.1 infection is mainly restricted to digestive lymph nodes. Thus the immunity conferred to similar to that induced by standard full dose subcutaneously method but the serological response reduced and the program of vaccination not limited to replacement animals, can be used in pregnant, non-pregnant and male animals) and induce life-long immunity and not followed by abortion. The oral Rev.1 vaccine followed by annual booster vaccination gives high protective vaccine. In this study we were comparison of the brucellin skin test with the lymphocyte blastogenesis assay in sheep groups vaccinated. Results of the in vitro lymphocyte assay were consistently positive for groups 2 and 5 after 8 weeks which the skin test consistently positive for G4 and 5G. The skin test in G5 gave strong reactions with mean 10.14mm, while lymphocyte assay for this group was consistently after 8 weeks after vaccination with mean 1.1756 $\pm$ 0.60, this results agreement with (Chukwu, 1986).

In this study flagellin used Rev.1 orally or S/C induced rising in the skin allergic test and lymphocyte assay. The humoral results agreed with (Clapp et al., 2011) who said the oral route vaccination gave protection for both mucosal and systemic tissues and rapidly cleaned diagnostic antibodies from

oral vaccinated animals. Results of humoral immune responses of sheep (G3) vaccinated with S/C flagellin. All animals were positive serologically which decreased gradually with 12 weeks post vaccination. These results agreed with (Lix et al., 2012) who said that some flagellin proteins of E.coli might induce protective immune responses and these proteins with be good subunit vaccine candidates.

In this study, purified H7 flagellin which serves as adjuvant and protective vaccine with live attenuated B.melitensis Rev.1 vaccine for the development of new vaccination stratigies induce and boost immune responses against Brucellosis.

In this study use of flagellin of E. coli $O:_{157}:H_7$as adjuvant to evaluate for its ability to enhance immune responses to the live Rev.1 brucellosis vaccine three reduced doses of Rev.1 vaccine administrated mixed with flagellin orally. From fig (11) ELISA results indicated that adjuvant H_7 increased synthesis of antibodies against smooth Brucella LPS in ELISA test. In group (3) administration of three doses (120 µg protein) of flagellin H_7 adjuvant with one week interval which induced humoral response 62.91 ELISA unit. This results showed that immunization with H_7 only S/C could be provided specific IgG to smooth LPS antigen. From these results, the flagellar antigens proteins could be produced humoral responses in sheep vaccinated that indicated H_7 could be useful candidate for the developments of subunit vaccine against brucellosis this results agreed with (Li, xianbo, 2012). In group (4) administration of Rev.1 vaccine full dose S/C without adjuvant H_7 induced of humoral responses lower than group (2, 3, 5). In fig (11) ELISA results of different groups H_7 as adjuvant had different stimulating effect after administration with (S/C or reduced doses). In group (5) administration of Rev.1 vaccine S/C full dose (2×10^9CFU) with simultaneous administration of H_7adjuvant in this group induced high synthesis of specific IgG to smooth LPS which enhanced the immunogenic properties to 97.92 ELISA unit at 25 DPV. In group (2) ELISA test fig (11) indicate the best vaccination three reduced doses of Rev.1 vaccine (2×10^7CFU) combined with flagellin H_7 which induced specific IgG to smooth LPS antigens ELISA titers (IgG) high antibody responses at 25,50 DPV. In this group titer lower after 98 DPV.

5. REFERENCES

Alton, G.G., Jones, L.M., Angus, R.D., Verger, J.M. 1988. Techniques for Brucellosis Laboratory. Institute National De Le Recherche Agronomique (INRA) Paris.

Arenas-Gamboa, A.M., Ficht, T.A., Davis, D.S., Elzer, P.H., Kahl-McDonagh, M., Wong-Gonzalez, A., Rice-Ficht, A.C. 2009. Oral vaccination with microencapsuled strain 19 vaccines confers enhanced protection against *Brucella abortus* strain 2308 challenge in red deer (Cervus elaphus elaphus). J Wildl Dis. 45(4):1021-9.

Blasco, J.M. 1997. A review of the use of *B. melitensis* (Rev.1) vaccine in adult sheep and goats. Prev. Vet. Med., 31(3-4):275-283.

Blasco, J.M. 2010. Control and eradication strategies for *Brucella melitensis* infection in sheep and goats. Prilozi, 31:145-165.

Cassataro, J., Velikovsky, C.A., Bruno, L., Estein, S.M., Barrera, S., Bowden, R., Fassati, C.A., Giambartolomei, G.H. 2007. Improved immunogenicity of a vaccination regimen combining a DNA encoding Brucella melitensis outer membrane protein 31 (Omp31) and recombinant Omp31 boosting. Clin. Vacc. Immunol. 14(7):869-874.

Clapp, B., Skyberg, J.A., Yang., Thornburg, T., Walters, N., Pascual, D.W. 2011 Protective live oral brucellosis vaccines stimulate Th1 and th17 cell responses. Infect Immun. 79(10): 4165-74.

Chukwu. 1986. Comparison of the brucellin skin test with the lymphocyte transformation test in bovine brucellosis.J. hyg. Camb. 96:403-413.

Fretin, D.1., Fauconnier, A., Köhler, S., Halling, S., Léonard, S., Nijskens, C., Ferooz, J., Lestrate, P., Delrue, R.M.,

Danese, I., Vandenhaute, J., Tibor, A., DeBolle, X., Letesson, J.J. 2005. The sheathed flagellum of *Brucella melitensis* is involved in persistence in a murine model of infection. Cell Microbiol. 7(5):687-698.

Honko, A.N., Mizel, S.B., 2004. Mucosal administration of flagellin induces innate immunity in the mouse lung.Infection and Immunity, 72(11): 6676-6679.

Lee, L.F., 1984. Proliferative response of chicken B and T lymphocyte to mitogen. Vet. Med; 15:44-52.

Leonard, S., Ferooz, V., Halnc, I., Danese, D., Fretin, A., Tibor, S., de walque, X., De Bolle, Letesson, J.J. 2007. FtcR is a new master regulator of the flagellar system of *B.melitensis* 16M with homologs in Rhizobieceae. Bacterial. 189:131-141.

Levine, M.M., 2010. Immunogenicity and efficacy of oral vaccines in developing countries: lessons from live cholera vaccine. BMC Biol. 8:129-139.

Li, x., Xu, J., Xie, Y., Qiu, Y., Fu, S.,Yuan, X., Ke, Y., Yu, S., Du, X., Cui, M., Chen, Y., Wang, T., Wang, Z., Yu, Y., Huang, K., Huang, L., Peng, G., Chen, Z., Wang, Y. 2012. Vaccination with recombinant flagellar proteins FlgJ and FliN induce protection against *Brucella abortus* 544 infection in BALB/c mice. Vet. Microbiol. 161(1-2):137-44.

Lucy, F.L., 1977. Chicken Lymphocyte stimulation by mitogens. A micro assay with whole blood cultures. Avian Dis., 22:296-307.

Mayer, S.P., Ritts, G.D., Johnson, D.R. 1974. Phyto haemagglutinin induced leukocyte blastogenesis in normal and avian leucosis virus infection in chicken cells. Immunoi. 27:140-146.

McNeilly, T.N., Naylor, S.W., Mahajon, A., Mitchell, M.C., McAteer, S., Deare, D., Smith, D.G.E., Cristopher, Low, J., Gally, D.L., Huntley, J.F. 2008. Escherichia coli O157:H7 colonization in cattle following systemic and mucosal immunization with purified H7 flagellin.Infect. Immun.76(6): 2594-2602.

Mohammad Ebrahimi (1), Ramin Bagheri Nejad (2), Saeed Alamian (2), Ladan Mokhberal Safa (3), Fatemah Abedini (4), Rainak Ghaderi(5) and Hamid Reza Jalali (2) 2012. Safety and efficacy of reduced doses of *Brucella melitensis* strain Rev.1 vaccine in pregnant Iranian fat tailed ewes. Veterinaria Italiana, 48(4): 405-412.

Nicoletti, P. 1990. Vaccination. In: Nielsen K and Duncan J.R,eds, Animal Brucellosis. CRC Press, Inc. Boca Raton, Fla.: 283-299.

Nicoletti, P. 2010. Brucellosis: past, present and future, Prilozi, 31, 21-32.

Office International des Epizooties (OIE) 2004. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 5th Ed., Office International des Epizooties, Paris, France: 598-606.

Pappas, G., Akritidis, N., Bosilkovski, M., Tsianos, E., Brucellosis. N Engl J Med. 352(22):2325 - 2336.

Scudiero, D.A., Shoemaker, H., Paull, K.D., Monks, A., Tierney, S., Nofziger, T.H., Currens, M.J., Seniff, D., Boyd, M.R. 1988. Evaluation of soluble tetrazolium / Formazan Assay for cell growth and drug sensitivity in culture using human and other tumer cell lines. Cancer Res. 48:4827-4833.

Slater, T.F., Sawyer, B.C., Striiuli, U. 1963. Biochim. Biophys. Acta, 77: 383.

Vitry, M.A., Hanot Mambres, D., Michael Deghelt, Katrin Hack, Atnaud Machelart, Frederic Lhomme, Jean-Marie Vanderwinden, Marjoree vermeersch, carl Be Trez, David perez –Morga, Jean – Jacques Letesson, Eric Muraille, 2014. Brucella melitensis invades Murine Erythrocytes during infection. Obiettivi e Documenti Veterinari, 17(11):71-74.