

## *Use of BCG vaccine for enhancement the immune response of sheep to Rev.1 Vaccination*

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**This study was conducted to evaluate the protective effect of *Brucella melitensis* Rev.1 and the use of BCG vaccine as immunostimulant by subcutaneous injection in Guinea pigs. Lab. animals were divided into 8 groups: Combined Rev.1 and BCG vaccines injected in the 1<sup>st</sup> group & both vaccines were injected simultaneously in the 2<sup>nd</sup> group. Three groups sensitized with BCG vaccine then injected with Rev.1 vaccine one week, two week & three weeks intervals respectively. Other two groups were injected with BCG and Rev.1 vaccine individually. The last one was unvaccinated control. All injected animals showed resistance to infection with 16 M strain (90 %, 80 %, 80, 60 %, 70 %, 0 % and 70 % respectively). Thus animals vaccinated with bivalent Rev.1 and BCG vaccines (in one shot) showed the best protective level to infection.**

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Brucellosis is a major zoonotic disease that causes abortion in wild and domestic animals and undulant fever in humans. The disease is transmitted to humans by consumption of dairy products or by direct contact with infected animals or carcasses (Young 1983 and 1995). On contact, brucellae penetrate the skin or mucosal membranes and enter the lymph nodes, which become hemorrhagic, resulting in bacteremia, which facilitates dissemination throughout the body. During the early phase of infection, brucellae invade macrophages, adapt to the acidic environment, and multiply in the vacuolar compartments (Porte *et al.*, 1999). *Brucella* prevents phagosome lysosome fusion (Baldwin and Winter, 1994; Pizarro-Cerda *et al.*, 1998). The infection involves many tissue types and organs. The spread of the disease is controlled in developed countries by livestock testing, vaccination, and slaughter programs, (Corbel, 1997).

The Elberg *B. melitensis* Rev.1 strain (Porte *et al.*, 1999) is the small ruminant's equivalent vaccine as is *B. abortus* S19 in cattle. Since 1957, when first used in international pilot scale studies, this vaccine had been used worldwide showing excellent results in the control of *B. melitensis* (Alton and Elberg, 1967; Elberg, 1981 and 1996). Because of its wide use different producers became interested in mass production of the vaccine for commercial purposes. The seed stock had been distributed among the producers, each propagating it similarly, however, losing to a certain extent the quality of

the vaccine. As a result some countries have encountered adverse effects with the vaccine. These results led to application of the Rev-1 vaccine in a regional control program for the Middle East countries, proposed by the FAO/WHO/OIE expert committee.

*Bacillus of Calmette and Guerin* (BCG) is a well known immunostimulant agent against a variety of human and animal diseases. BCG or its active fragments have proved to be effective therapy and can stimulate a cross resistance to unrelated organisms including protozoa, fungi, bacteria and viruses as well as to certain transplanted tumors (Barakat *et al.*, 1984).

Therefore the present study was planned to clarify the following aspects: detecting the ability of Rev-1 vaccine to combat challenge with *Brucella* virulent strain (16 M) alone and in combination with BCG vaccine.

### **Materials and methods**

**Experimental animals.** One hundred and twenty Guinea-pigs from 300-400 grams weight were used. All animals were *Brucella* free through serological screening using *Brucella* agglutination test. They were divided into eight groups each of 15 animals.

***Brucella* strain.** Virulent *Brucella melitensis* 16M.

(USDA , vs. Nat. Vet, services lab, Ames Iowa 50010 USA )

***Brucella* antigens.** Rose Bengal (RBA) and Tube agglutination antigens. They were prepared in VSVRI .Abbassia , Cairo,Egypt

**Freeze dried vaccines.** Smooth *Brucella*

**Table (1): animal vaccination and challenge scheme.**

Animal group	Guinea pigs No.	Vaccination						Challenge
		1 <sup>st</sup> vaccination			2 <sup>nd</sup> vaccination			
		Type of vaccine	Vaccination condition	dose	route	Type of vaccine	interval	
1	15	BCG + Br. m. Rev-1	Mixed combined	0.1 ml + 1 ml	s/c	Non	-	Each guinea pig was inoculated intraperitoneally with virulent <i>B. melitensis</i> 16 M (5x10 <sup>5</sup> CFU) 4 weeks post last vaccination
2	15	BCG and Br. m. Rev -1	Simultaneous At the same time	0.1 ml + 1 ml	s/c	Non	-	
3	15	BCG	1st preliminary	0.1 ml	s/c	Br. m. Rev -1	1 week	
4	15	BCG	1st preliminary	0.1 ml	s/c	Br. m. Rev -1	2 weeks	
5	15	BCG	1st preliminary	0.1 ml	s/c	Br. m. Rev -1	3 weeks	
6	15	BCG	Single	0.1 ml	s/c	Non	-	
7	15	Br. m. Rev -1	Single	1 ml	s/c	Non	-	
8	15		Control unvaccinated guinea pigs					

- Br. m. Rev-1 = *Brucella melitensis* Rev-1 vaccine. s/c = subcutaneous.
- \* = Rev -1 1x10<sup>8</sup> CFU and BCG 2X10<sup>4</sup>

*melitensis* Rev-1 vaccine. Freeze dried BCG vaccine. (VSVRI .Abbassia , Cairo,Egypt).

**Potency test.** (Alton, 1988 ; OIE 2000) All groups were challenged with virulent *Brucella melitensis* 16M (5x10<sup>5</sup> CFU I/P) four week after vaccination with Rev.1 . The guinea pigs injected with *B. melitensis*, were weighed and killed after 8 weeks post –inoculation. The spleen of each animal was weighed and counted for *Brucella* 16M .

**Blood sampling.** Heparinized blood samples: Samples were collected from each group at 4, 11, 18 and 25 days for estimation of cell mediate immune response. Serum samples were collected every 2 weeks for evaluation of humoral immune response.

**Evaluation of the immune response.** Humoral immune response: Rose Bengal (RBT) and Standard tube agglutination tests (STAT) were performed according to Alton,(1988).

Cell mediated immune response:It was evaluated by lymphocyte transformation test according to Lucy(1974) and (1977) modified by Charles *et al.*,(1978) ; Lee (1984) as follow: 2 ml of blood was obtained from vaccinated Guinea pigs using heparinized syringe.

The blood was diluted 1: 1 with PBS (pH 7.2) then carefully layered on the surface of

equal volume of lymphocyte separation medium (Ficol Hypaque) (Flow Lab.UK) in a sterile plastic tube. The tube was centrifuged at 2400 rpm for 30 minutes and the separated buffy coat cells were washed three times in RPM 1. Viable lymphocytes were adjusted to a final concentration of 5 X 10<sup>6</sup> (cells / ml RPM I containing foetal Calf Serum (FCS) and each well of the tissue culture microplates received 0.5 X 10<sup>6</sup> cells in 0.1 ml. Flat bottomed sterile 96 well tissue- culture microplates were used for this purpose and the wells PHA (10 ul) stimulated cultures, control unstimulated cells and control medium without cells. The plates were covered and incubated at 37°C in humid CO<sub>2</sub> atmosphere (5 - 10 %) for 72 hours. Evaluation of lymphocyte blastogenesis by MTT (Mosmann, 1983): In a dark place, MTT was added as 10.0 ul MTT/ well. Following incubation, 50 ul of 10% SDS solution were added for each well and reincubated overnight at 37°C in 5% CO<sub>2</sub>.The plates were read at 570 nm by micro ELISA reader.

**Experimental design.** G.Pigs was divided into eight groups each of 15 animals as shown in Table (1)

## Results and Discussion

**Table (2): Comparison of humeral immune response between groups of G.pigs vaccinated with *B.melitensis* Rev.1 and BCG vaccine S/C and challenge with 5x10<sup>5</sup>CFU *B. melitensis* 16M.**

Weeks post Rev-1 vaccination	Results of Rose Bengal test ( +ve %)								Results of Tube agglutination test ( mean IU/ml)							
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	60 %	80 %	80 %	-	-	80 %	-	-	93	106	106	-	-	106	-
6	60 %	80%	100 %	100%	-	-	100 %	-	93	186	106	106	-	-	106	-
<b>Animal challenged with virulent <i>B. melitensis</i> 16M 5x10<sup>5</sup>CFU 8 weeks after vaccination with <i>B.melitensis</i> Rev-1.</b>																
10	60 %	80 %	80 %	80 %	80 %	100 %	80 %	100 %	134	40	134	40	134	640	160	848
12	-	100%	100%	100 %	100 %	100 %	100%	100 %	-	268	268	160	160	744	186	848
14	-	100%	100%	100 %	100 %	100 %	100 %	100 %	-	134	160	106	106	744	268	848
16	-	100%	100 %	100 %	100%	100 %	100 %	100 %	-	80	93	93	93	848	160	848

Group 1: Mixed BCG+B .melitensis Rev-1.

Group 3: BCG+B. melitensis Rev-1 ( 1 week interval).

Group 5: BCG+B. melitensis Rev-1 (3 weeks interval).

Group 7: B. melitensis Rev-1 vaccine.

Group 2: Simultaneous BCG+B. melitensis Rev-1.

Group 4: BCG+B. melitensis Rev-1 (2weeks interval).

Group 6: BCG vaccine.

Group 8: control un vaccinated guinea pigs.

**Table (3): Spleen count assay among Guinea pigs vaccinated with Rev-1 with or without BCG vaccine and challenged with 5x10<sup>5</sup> CFU *B. melitensis* M16**

Groups of Guinea pigs	Group1	Group2	Group3	Group4	Group5	Group6	Group7	Group 8
Mean body weight in grams	360.3	424.8	375	536.1	508.9	542	361.6	365
Mean spleen weight in grams	0.6	0.9	1.10	1.4	0.9	1.62	0.62	1.95
Mean spleen weight/ body weight ratio	0.17	0.21	0.29	0.26	0.18	0.30	0.17	0.65
Mean colonies / whole spleen	50	200	200	430	266	16.600	250	20.500
Mean colonies / gram spleen	83.3	222.2	181.1	307.1	295.5	10.246	403.2	10.51
Log Y	1.5	1.9	1.9	2.2	2.0	3.6	2.0	3.7
Protection	P	P	P	P	P	Non-P	P	Non-P
percent of protection and non-protection	90%	80%	80%	60%	70%	0%	70%	0%

Animals: Challenge 8 weeks after vaccination with 5x10<sup>5</sup> CFU *B. melitensis* M 16. Animals: slaughtered 8 weeks after challenge.

According to OIE (2000) X= number of virulence Brucella organisms per spleen. Y= log (X / log X) = response and protected animals. <2.5

Group 1: Mixed BCG+B. melitensis Rev-1.

Group 3: BCG+ B.melitensis Rev-1 ( 1 week interval).

Group 5: BCG+ B.melitensis Rev-1 (3 weeks interval).

Group 7: B.melitensis Rev-1 vaccine.

Group 2: Simultaneous BCG+B.melitensis Rev-1.

Group 4: BCG+ B.melitensis Rev-1 (2weeks interval).

Group 6: BCG vaccine.

Group 8: control un vaccinated guinea pigs.



**Table (4): Results of lymphocyte transformation test in Guinea pigs vaccinated with *B. melitensis* Rev-1 and or BCG**

Animal groups	Results of lymphocyte transformation test			
	4 days	11 days	18 days	25 days
1	0.495	0.399	0.312	0.300
2	0.426	0.323	0.305	0.300
3	0.400	0.372	0.331	0.303
4	0.403	0.386	0.356	0.319
5	0.411	0.391	0.364	0.327
6	0.282	0.388	0.385	0.375
7	0.420	0.390	0.308	0.300
8	0.197	0.223	0.219	0.187

Group 1: Mixed BCG+B. melitensis Rev-1.

Group 2: Simultaneous BCG+ B. melitensis Rev-1.

Group 3: BCG+ B. melitensis Rev-1 ( 1 week interval).

Group 4: BCG+ B. melitensis Rev-1 (2weeks interval).

Group 5: BCG+ B. melitensis Rev-1 (3 weeks interval).

Group 6: BCG vaccine. Group 7: B. melitensis Rev-1 vaccine.

Group 8: un vaccinated control Guinea pigs

Results are shown in Tables 2, 3 and 4. The humoral immune response of different groups of vaccinated guinea pigs was evaluated using Rose Bengal and tube agglutination tests (Table 2). At the fourth week after vaccination the 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup> and 7<sup>th</sup> groups gave 60%, 80%, 80% and 80% positive respectively in Rose Bengal test and 93, 106, 106 and 106 IU/ml respectively in tube agglutination test. While the 1<sup>st</sup>, 5<sup>th</sup>, 6<sup>th</sup> and 8<sup>th</sup> groups gave negative in Rose Bengal and tube agglutination tests.

At the 6<sup>th</sup> week after vaccination the 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup> and 7<sup>th</sup> groups gave 60%, 80%, 100%, 100% and 100% positive respectively in Rose Bengal test and 93, 186, 106, 106 and 106 IU/ml. respectively in tube agglutination test. While 5<sup>th</sup>, 6<sup>th</sup> and 8<sup>th</sup> groups gave negative results in both tests.

Two weeks after challenge with the virulent *Brucella* strain all the groups gave positive results in Rose Bengal and tube agglutination tests. While at the 4th, 6th and 8th weeks after challenge only the 1st group gave negative with the both tests.

Control unvaccinated guinea pigs showed high antibody titers after challenge with virulent *Brucella* strain (100% +ve with Rose Bengal and 848 IU/ml with tube agglutination test).

The potency of different preparations was evaluated as shown in Table (3). The 1st group gave the best results (log Y 1.5 and gave 90% protection) while the 6th group gave 0% protection. Lymphocyte transformation test was performed and the results were shown in Table

(4). The 6th group (vaccinated with BCG only) was the least titer among the different groups, while the 1st group (vaccinated with mixed bivalent vaccine) gave the highest titer at the 4th day.

The members of the genus *Brucella* are gram-negative, facultative intracellular coccobacilli that cause brucellosis in many animal species and humans. The protective immune response against *Brucella* bacteria involves both humoral and cell-mediated immunity (Al-Mariri *et al.*, 2002). Acquired immunity against intracellular bacteria is T-cell dependent which means T cells play the major role in protection against these organisms. Immunity against *Brucella* species depends on antigen-specific T-cell mediated killing of this organism (Oliveira *et al.*, 1998). In addition to the central role of the macrophage in *Brucella* infection, others cells of the immune system are influenced by the interactions between bacteria and host. These cells can counteract the intramacrophagic development of the bacteria and finally influence the further development of the host defense (Oliveira *et al.*, 1998; Dornand *et al.*, 2002)

The present study describes the cross immunity between Rev-1 and BCG vaccines.

On injection of BCG and Rev-1 vaccines in guinea pigs with intervals 1, 2 and 3 weeks gave protection percent 80 %, 60 % and 70% respectively , as will as Rev-1 vaccine. This means that BCG vaccine did not enhance the protective effect of Rev-1 vaccine in these

circumstances.

On the other hand, vaccination of guinea pigs with the mixed vaccine (Rev-1+ BCG vaccine) gave 90% protection against *Brucella melitensis* virulent strain (16 M).

Among non-specific immunity, BCG vaccine gave no protection against challenge with *Brucella melitensis* virulent strain (16 M).

These results disagreed with Elberg *et al.*, (1957) who studied the non-specific immunity, where vaccination by BCG or by an effective anti brucellosis reagent induces protection in either cases against both *Mycobacterium tuberculosis* and *Brucella melitensis*. Also Barakat, (1979) studied the use of BCG as a heterologous vaccine against *Corynebacterium bovis*. BCG vaccine proved to be very efficient against caseous lymphadenitis of sheep.

Buddl and Thomson (1995) showed that S/C vaccination of calves with 104 –106 CFU of BCG caused a significant reduction in the prevalence and severity of T.B. lesions. Subcutaneous vaccination of BCG results in the induction of cell mediated immunity as manifested by (CD4+ T helper type) cells as well as (CD8+cytotoxic T-lymphocyte). These responses are accompanied by the synthesis non-specific and cell mediated immunity.

In tables 2, 3, 4 BCG vaccine alone failed to provide protection against virulent 16M *B. melitensis* challenge. Combined vaccines BCG and Rev.1 increased the level of protection against pathogenic 16M in comparison to use Rev.1 vaccine alone (table 3). Table 3 may explain that using both vaccines combined together and injected in one site S/C may lead to hyper activity of the same draining L.N. due to BCG as immune modulator and increased in the cell mediated immunity, this phenomenon was observed by Miller *et al.*, (1973).

In this study BCG used as a vaccine vector to induce protection against *Brucella* challenge. The cross-protection strategy has been used in the development of new vaccination against Brucellosis with good protective effect.

Usage of BCG in combined vaccines was studied by many investigators, Mazantini *et al.*, (2004) developed a combined *Mycobacterium bovis* BCG vaccine with diphtheria, pertusis and tetanus by administration sub immunizing dose of the diphtheria-tetanus toxoid vaccine. This combination induced 75% protection in mice challenged with 100 minimum lethal doses (MLD) of tetanus toxin this combination produced of a more effective immune response

against both diseases. Also, Osman, *et al.*, (1991) indicated the safety and efficiency of simultaneous inoculation of cattle with rinderpest and BCG vaccines without impairing the response of individual vaccines.

From the above mentioned results, it may be concluded that vaccination of guinea pigs with Rev-1 and BCG vaccines in one shot (S/C) is effective in minimizing the pathogenic effect of *Brucella melitensis* virulent strain (16 M) and gives 90% protection.

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### استخدام بين لقاح ال بي سي جي لرفع كفاءة لقاح الريف - 1 للاغنام .

تم في البحث حقن لقاح البروسيل ريف واحد تحت الجلد في حيوانات التجارب (أرانب هندي) مع استخدام لقاح ال بي سي جي كمحفز مناعي خلوي الى 8 مجموعات : خلط اللقاحين في حقنة واحدة و حقن اللقاحين منفردين متزامنين و حقن لقاح البروسيل ريف واحد بعد لقاح ال بي سي جي ب 7 أيام. و حقن لقاح البروسيل ريف واحد بعد لقاح ال بي سي جي ب 14 يوم و حقن لقاح البروسيل ريف واحد بعد لقاح ال بي سي جي ب 21 يوم و حقن لقاح ال بي سي جي منفرداً. و حقن لقاح البروسيل ريف واحد منفرداً و مجموعة حاکمة. و تم الحصول على نسب الحماية الآتية (90% ، 80% ، 80% ، 60% ، 70% ، 0% ، و 70% و 0% على التوالي). و يتضح من النتائج انه عند استخدام لقاح البروسيل ريف-1 مخلوطاً مع لقاح ال بي سي جي حيث كانت نسبة الحماية عالية و تم رفع كفاءة لقاح البروسيل ريف و بذلك يمكن استخدام طريقة خلط اللقاحين معا كبديل لاستخدام لقاح البروسيل ريف منفرداً في مكافحة مرض الأجهاض المعدي في الحيوانات