Protection against virulent *Brucella* spp. by gamma-irradiated *B. ovis* in BALB/c mice model

Ayman Al-Mariri, Laila Al-Hallab, Rasha Alabras, Heba Kherbik, Marwa Khawajkiah

Department of Molecular Biology and Biotechnology, Atomic Energy Commission of Syria, Damascus, Syria

Corresponding author: Ayman Al-Mariri, PhD
Department of Molecular Biology and Biotechnology, Atomic Energy Commission of Syria, Damascus, P.O. Box 6091, Syria
Tel: +963-11-6111926, Fax: +963-11-6112286
E-mail: ascientific37@aec.org.sy

Purpose: *Brucella* spp. is a zoonosis that causes undulant fever in humans and abortion in livestock worldwide. Lately, it was conveyed that vaccines developed by irradiation have induced a strong cellular and humoral immune response which have made these types of vaccines highly effective.

Materials and Methods: In this study, we aimed to use the gamma-irradiated *B. ovis* as a vaccine and to study the humoral immune response and cytokines production in order to evaluate it for protecting mice against *B. abortus* 544, *B. melitensis* 16M, and *B. ovis*.

Results: The humoral immune response in immunized mice with gamma-irradiated *B. ovis* showed a lasting for 8 weeks after immunization. Moreover, immunoglobulin G (IgG), IgG1, IgG2a, and IgG2b isotypes antibodies against *B. ovis* were observed after 4 and 8 weeks of the last immunization. It was noticed that the production of tumor necrosis factor-α, interferon-γ, and interleukin (IL)-10 continued after 4 and 8 weeks by splenocytes from immunized BALB/c mice, while no production of IL-4 or IL-5 was observed.

Conclusion: Our results indicate that the protection of BALB/c mice against *B. melitensis* 16M, *B. abortus* 544, and *B. ovis* was induced and the developed vaccine at our laboratory could stimulate similar protection to those induced by the traditional vaccine.

Keywords: *Brucella* spp., Cellular and humoral immune, Cytokines, Gamma rays, Vaccine

Introduction

The genus *Brucella* belongs to the family *Brucellaceae* and is placed in the alpha-2 subdivision of the class Proteobacteria. They are small, non-fermenting, anaerobic, non-motile, Gram-negative coccobacilli, and facultative intracellular bacteria that cause disease in a broad range of animal hosts. All strains share >94% homology; the genus has been divided into six species, that are recognized and differentiated according to an antigenic variation and primary hosts: *B. abortus* (cattle), *B. melitensis* (goats), *B. suis* (hogs), *B. ovis* (sheep), *B. neotomae* (wood rat), and *B. canis* (dogs). In several reports, there have been *Brucella* species isolated from marine mammals, mostly seals, and cetaceans and otters [1]. *B. ovis* has the distinction of being a rough isolate lacking the hydrophilic O-polysaccharide side chain of the lipopolysaccharide (LPS) at their outer cell membrane, different from the typically smooth forms of *Brucella* [2]. The dispersion of brucellosis has been essentially controlled in developed countries, but this disease still poses a threat in the Mediterranean region, parts of Asia, the Middle

East, Africa, and Latin America [3]. Major efforts have been devoted to the prevention and treatment of this disease. Although *Brucellae* are sensitive to many antibiotics, treatment is not practical and too expensive in most animal species [4]. On the other hand, the prevention of the disease by vaccination has played a key role in brucellosis eradication programs. However, brucellosis is still common in some countries where animal disease control programs have not reduced the disease spread among animals [5]. Killed vaccine candidates usually confer poor immunity, whereas live-attenuated vaccines of virulent strains typically provide adequate immunity against abortion but can lead to the release of the pathogenic organisms and possibly expose susceptible animals to infection [6]. Protective immunity against *Brucella* depends on the induction of effective and specific cell-mediated immune response (CMI) mediated by ; such as interferon (IFN)-γ, interleukin (IL)-2, and tumor necrosis factor (TNF)-β. Also, the production of TNF-α appears to be important in this response [7]. Several vaccines are available around the world to control brucellosis in cattle, sheep and goats. *B. abortus* strain 19 has been used to control *B. abortus* infections in cattle and *B. melitensis* strain Rev.1 to control brucellosis in goats and sheep [8]. These two vaccines have some disadvantages. They can cause abortions if used in pregnant animals and may result in persisting agglutinins that can interfere with various serological diagnostic tests. In addition, they are pathogenic to humans via aerosol exposure or self-inoculation [9]. Compared to inactivated or subcellular vaccines, live brucellosis vaccines generally provide more complete and lasting immunity. They can induce a long-lasting CMI response, and replicate within the host cells making them less expensive [10].

Throughout the years, the development of killed vaccines for protection against brucellosis has limited success and none of them have achieved the granted protection status by the live, attenuated vaccines. Live vaccines for brucellosis, in general, provide more complete and lasting immunity than killed or sub-cellular vaccines. This is due to their ability to provoke a necessary CMI response to clear intracellular bacterial infections such as *Brucella* [11]. An ideal vaccine should be stable, easy to produce and store, and provides long-lasting immunity. In addition, the vaccine should not induce immune responses that interfere with diagnostic tests and be non-pathogenic to vaccinated animals and humans handling the vaccine [12].

It was notified that irradiated vaccines have a strong T helper type 1 (Th1) type, humoral immune response, and protective immunity against virulent strain after just one immunization which makes these types of vaccines highly effective [13,14]. Many researchers evaluated the effectiveness of γ-irradiated *Brucella* strains to induce protection and challenge against virulent *Brucella* spp. [15,16].

This work focused on the comparison of γ-irradiated *B. ovis* (IRR-*B. ovis*) persistence of the mouse model and their ability to stimulate protective immunity compared to traditional vaccines (Rev.1 and S19).

Materials and Methods

Bacterial strains and growth conditions
Standard *Brucella* strains (*B. melitensis* 16M and *B. abortus*) and vaccinated strains (Rev.1 and S19) were obtained from the University of Namur (Namur, Belgium). *B. ovis* isolated from Awass sheep milk from Damascus countryside. *Brucella* was grown in 2YT medium (10 g of yeast extract, 10 g of tryptone, and 5 g of NaCl per liter) for 2–3 days at 37°C. All experiments with virulent *Brucella* were performed in a BLS-3 facility approved for the selected work agents.

Bacterial irradiation
B. ovis strain was grown in 2YT to mid-log phase, and aliquots of $5 \times 10^9 - 1 \times 10^{10}$ colony-forming unit (CFU)/mL were then stored at -80°C until use. Three weeks before immunization, an aliquot of the *B. ovis* was subjected to γ-irradiation using a ^{60}Co source gamma irradiator (Gammacell 220 irradiator; Issledovatel Gamma Irradiator, Techsnabexport Co. Ltd., Moscow, Russia). The inability of irradiated bacteria to replicate was confirmed by culturing on Tryptic Soy Agar (HiMedia, Mumbai, India) and incubating for at least 7 days. The irradiated bacteria were stored at 4°C until used for immunization.

Cell viability assay and metabolic measurements
Metabolic activity was measured using Alamar Blue (BioSource International, Camarillo, CA, USA), as described by the manufacturer's instructions. Briefly, the irradiated bacteria were incubated at 37°C in 96-well microplates and 10% of Alamar blue dye was added. Absorption was observed at wavelength 570 nm (reduction) and 600 nm (oxidation) overtime a period of 60 minutes. The percentage of reduction (equivalent to metabolic activity) was determined by subtracting absorption at wavelength 600 nm from the absorbance at a wavelength of 570 nm and multiplying by 100.

Immunization of mice for immune response

Specific pathogen-free female BALB/c mice (7 to 8 weeks old, purchased from Charles River Laboratories, L'Arbresle, France; then have been reproduced in the animal shed in Molecular Biology and Biotechnology Department, Syrian Atomic Energy Commission), were randomly distributed into five experimental groups of 20 mice each, received intraperitoneal (i.p.) injections as follows: group 1 was injected with 5×10^7 CFU of IRR-*B. ovis* groups 2 and 3 were injected with 1×10^5 CFU Rev.1 and S19 vaccines, respectively as conventional controls, group 4 received 1×10^5 of *B. ovis* as a positive control, and the last group received sterile saline (phosphate-buffered saline [PBS]) as a negative control, by using a 1 mL insulin syringe with a 28G needle. After 4 and 8 weeks from the last injection, five mice were randomly selected to sacrifice by cervical dislocation. Their spleens were taken out under sterile conditions to investigate the cellular immune response and sera were collected to determine the humoral immune response. The mice were kept in conventional animal facilities and supplied with water and food.

BALB/C mice survival

To assess the importance of γ-irradiation in inhibition bacterial replication, BALB/C mice (n = 4/group) were infected i.p. with *B. abortus* 544, *B. melitensis* 16M, *B. ovis* (2×10^5 CFU/100 μL), and IRR-*B. ovis* (2×10^7 CFU/100 μL). Mice survival was evaluated for 4 and 8 weeks post-infection.

Enzyme-linked immunosorbent assay ELISA

Specific murine immunoglobulin G (IgG), IgG1, IgG2a, and IgG2b isotypes were assayed by enzyme-linked immunosorbent assay (ELISA). The 96 wells of a polystyrene plate (ppt-Immuno plates Maxisorp), coated overnight at 4°C with an optimal concentration (25 μg) of the different antigens (sonicated whole-cell antigen of *B. melitensis* 16M, *B. abortus*, or *B. ovis* in 50 μL of PBS). Plates were washed twice with PBS and blocked with PBS 2.5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) for 2 hours at room temperature (RT). After three washes in PBS, 100 μL of serum 1/1,600 in dilution buffer (PBS, Tween 20, BSA) were performed and loaded in micro-wells and incubated at RT for 1 hour. Serum from unimmunized mice were used as a negative control. After five washes with washing buffer (PBS, Tween 20), biotinylated goat anti-mouse IgG, IgG1, IgG2a, and IgG2b antibodies (Amersham Life Sciences, Amersham, UK) were added at an optimal dilution and incubated for 1 hour at RT. Then five ad-

ditional washes were done with PBS-Tween, the plates were incubated for 1 hour with 50 μL of a 1:1,000 dilution of secondary antibody labeled with peroxidase (Amersham) at RT. Finally, the plates were washed 5 times and developed for 10 minutes in the dark with TMB (3,3,5,5-tetramethylbenzidine; Fermentas Life Sciences, EU). The reaction was then stopped by the addition of 2N H_2SO_4 to each well. The absorbance was measured at 450 nm (Thermo-lab Systems Reader, Helsinki, Finland). Titers were defined as the highest dilution of mouse serum that obtains an optical reading 3 times higher than the negative control.

Proliferation assays

The mice were sacrificed and their spleens were removed under aseptic conditions. Spleens were homogenized with 2 mL of tissue culture medium (RPMI 1640—fetal bovine serum; Eurobio, Les Ulis, France), and erythrocytes were lysed with ammonium chloride solution (0.8% NH_4Cl in water, 0.1 mM ethylenediaminetetraacetic acid, buffered with $KHCO_3$ to achieve a final pH of 7.2–7.6). A total count of 2×10^5 splenocytes per well were cultured at 37°C in a 96-well flat-bottom plate within a humidified atmosphere (5% CO_2 and 95% air) in the presence of concanavalin A (ConA, 3 μg/mL; Sigma-Aldrich). Bacterial lysate (3 μg/mL) was added or no additive in the culture medium for a total volume of 200 μL per well. After 72 hours of culturing, 10 μL of Cell Counting Kit-F working solution were added. After 30 minutes, the fluorescence intensity was determined for each well at 535 nm (excitation at 485 nm) using a fluorescence plate reader. Each sample was analyzed in triplicate. Data represent ±standard deviation error bars from the five mice. Cell proliferation was determined in triplicate for each antigen.

Cytokines production by spleen cells

As described for the lymphocyte proliferation assay; cell culture supernatants were collected after 72–96 hours of incubation with antigen or mitogen (ConA). Cytokines IFN-γ, TNF-α, IL-4, IL-5, and IL-10 production were analyzed by sandwich ELISA according to the protocol defined for the commercial kit (Mabtech, Stockholm, Sweden). The concentration of cytokines was calculated in the culture supernatants via a linear regression equation is derived from the absorbance values of the standards as specified by the manufacturer. Values between 10 and 40 pg/mL were considered negative for cytokines. All assays were performed in duplicate.

Protection assay

Protection experiments were performed after 4 weeks from the last injection. Every 10 remaining mice from all groups was challenged by the i.p. route with approximately 1×10^5 CFU equivalents of (*B. melitensis* 16M, *B. abortus* 544 or *B. ovis*) in 100 µL sterile PBS by using 1 mL insulin syringe with a 28G needle. Briefly, 4 and 8 weeks later, the infected mice were sacrificed by cervical dislocation, and the spleens were removed aseptically. Each spleen was homogenized with 2 mL Triton-PBS (0.1%), and to determine the number of *Brucella* CFU per spleen, 10 µL of 10-fold serial dilution were plated in triplicate on 2YT agar.

Ethics statement

Animals were handled in strict accordance with good animal practice as defined by the relevant local animal welfare bodies.

Statistical analysis

Significances of differences were determined by using the Student t-test as appropriate. A p-value of <0.05 was considered significant. Log units of protection were obtained by subtracting mean counts of the vaccinated group from the mean of the corresponding control group. A mean value for each spleen count was obtained by averaging the triplicate values after log conversion.

Results

Bacterial irradiation

Inactivation by γ-irradiation was evaluated as an alternative method to produce a metabolically active and replication-incompetent *B. ovis*. The effect of irradiation on the survival of bacteria was analyzed. There is a decrease in metabolic activity observed with increasing irradiation doses (1.5, 3, and 4

kGy) (Fig. 1). Thus, there is a proportional correlation between the decrease in metabolic activity and the increase in the irradiation time and the bacterial count. This result confirms that γ-irradiated *Brucella* and 3 kGy of gamma irradiation were debilitating and not completely inhibitory.

BALB/C mice survival

It was noticed that immunization with IRR-*B. ovis* induced a significantly higher degree of bacterial inactivation, compared with *B. melitensis* 16M, *B. abortus*, or *B. ovis* (5.62, 5.21 $\log_{10}$), (4.97, 4.73 $\log_{10}$), and (3.36, 4.29 $\log_{10}$), respectively, 4 and 8 weeks post-infection (Table 1).

In this table, important differences in $\log_{10}$ CFU counts were noticed among mice injected with gamma IRR-*B. ovis* (0.85 $\log_{10}$ in 4 weeks) and (0.5 $\log_{10}$ in 8 weeks) post-infection. This means that γ-irradiation at 3 kGy provided an effective way to decrease the $\log_{10}$ CFU counts since its impaired microbial replication by DNA fragmentation. Therefore, we selected these bacteria in our study since IRR-*B. ovis* does not replicate but still has metabolic activity.

Determine the humoral immune response

Antibody levels against bacterial extract (*B. abortus* 544, *B. melitensis* 16M, and *B. ovis*) were tittered by endpoint titration of the pooled serum samples of each vaccinated group using ELISA.

Serum samples were collected at 4 and 8 weeks after the initial immunizations and analyzed in comparison with the control group (Fig. 2). After 4 and 8 weeks post-vaccination, the vaccinated mice with IRR-*B. ovis* have been developed significantly higher levels of IgG specific to the total antigen of *Brucella* spp. than mice inoculated with saline. Assays with IgG1, IgG2a, and IgG2b specific conjugates revealed that antibodies of all isotypes were presented in significantly higher levels than in saline inoculated mice. The immune response
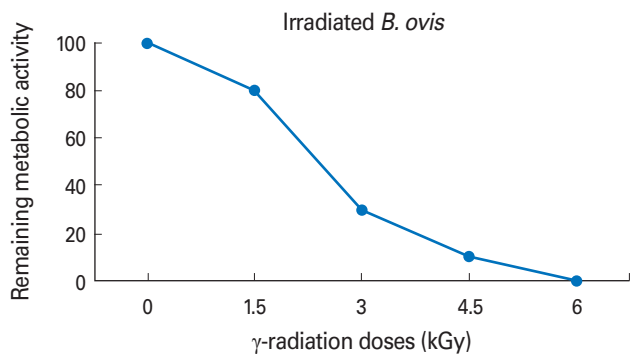

Fig. 1. Effect of γ-radiation doses (kGy) on *Brucella ovis* metabolic activity (%).

Table 1. Significant differences in $\log_{10}$ CFU counts of *Brucella* strains and IRR-*B. ovis* in infected mice

Brucella strains	4 Weeks	8 Weeks
B. melitensis 16M	5.62±0.35[a]	5.21±0.29[a]
B. abortus 544	4.97±0.28[a]	4.73±0.32[a]
B. ovis	4.29±0.17[a]	3.36±0.45[a]
IRR-*B. ovis*	0.85±0.12	0.5±0.1

Values are presented as mean±standard deviation.
CFU, colony-forming unit; IRR-*B. ovis*, γ-irradiated *B. ovis*.
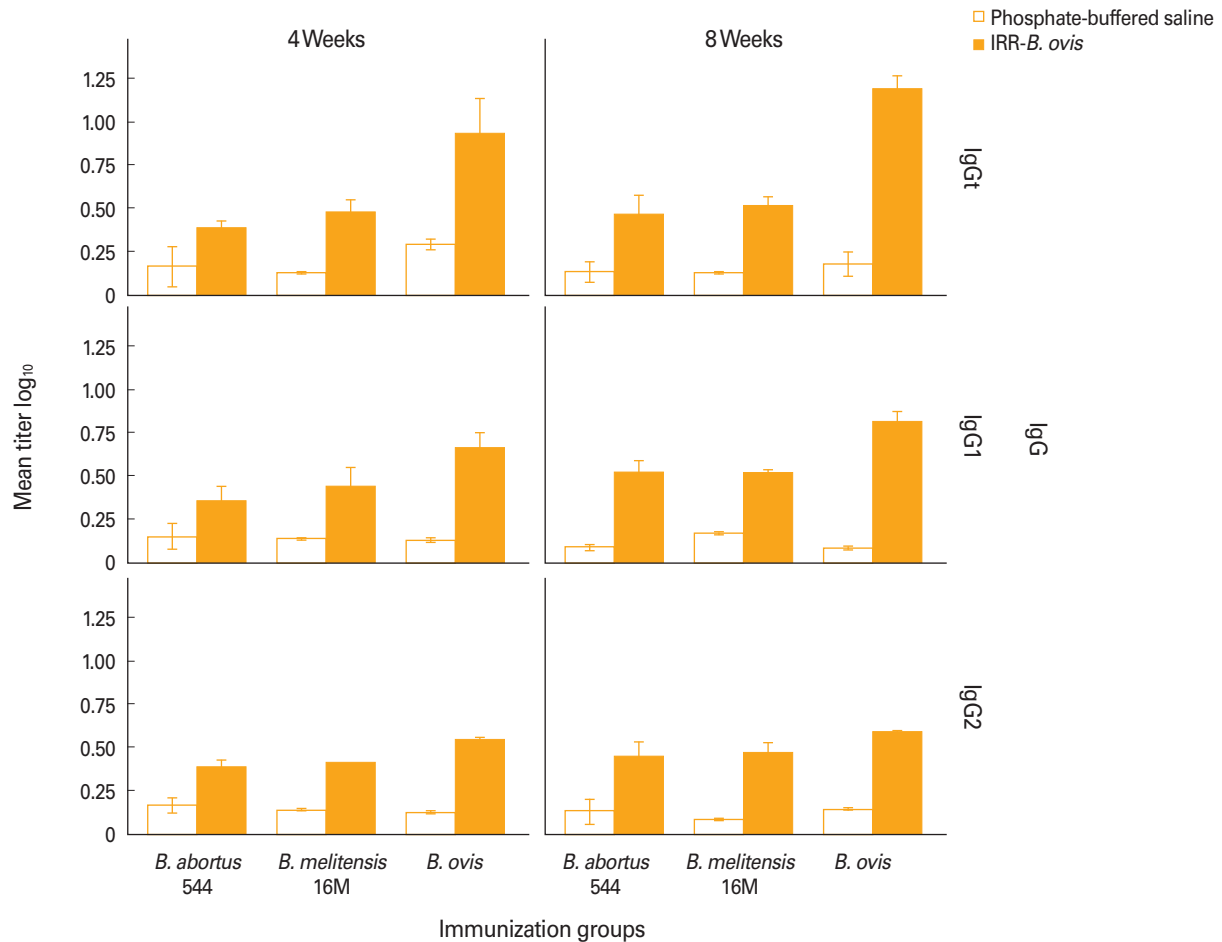[a]p<0.05 (significant) compared to the IRR-*B. ovis* group.

Fig. 2. Mean serum level of immunoglobulin G (IgG): IgGt, IgG1, and IgG2a against *Brucella abortus* 544, *B. melitensis* 16M, or *B. ovis* in mice groups immunized with γ-irradiated *B. ovis* (IRR-*B. ovis*) 4 and 8 weeks after the last vaccination.

was maximal against *B. ovis* extract as an antigen (Fig. 2).

The IgG levels against bacterial extract were higher at 8 weeks than at 4 weeks. There weren't almost any obvious differences between the level of antibody isotypes (IgG1, IgG2a, and IgG2b) after 4 or 8 weeks. We also obtained nearly the same result of IgG2a and IgG2b (unpublished results).

Cellular immune response: cytokines production

Fig. 3 shows that the splenocytes of immunized mice with IRR-*B. ovis* produce TNF-α after 4 and 8 weeks against *B. ovis*, *B. melitensis* 16M and *B. abortus* 544 with a value ranged between 210 to 405 pg/mL, respectively.

Production of IFN-γ after 4 weeks against the same antigens was (2,650, 2,125, and 1,990 pg/mL) respectively, whereas it was (2,875, 2,275, and 2,105 pg/mL) after 8 weeks, respectively.

Fig. 3 shows that the production of IL-10 after 8 weeks was higher than 4 weeks after immunization with IRR-*B. ovis*. For example, IL-10 production at mice group BALB/c that immu-

nized with IRR-*B. ovis* was 1,670, 1,230, and 1,050 pg/mL against *B. ovis*, *B. melitensis* 16M, and *B. abortus* after 8 weeks, respectively. Whereas, it was 1,350, 985, and 890 pg/mL against *B. ovis*, *B. melitensis* 16M, and *B. abortus* 544 after 4 weeks, respectively.

At our study's condition, there wasn't any production of IL-4, or IL-5 by splenocytes for injected BALB/c mice with IRR-*B. ovis* even after 4 weeks or 8 weeks of immunization (data not shown).

Lymphocytes proliferation by BALB/c mice splenocytes upon stimulation with different antigens

Vaccines that are used in our study induce a specific response by T-cell proliferation against *B. ovis*, *B. melitensis* 16M, and *B. abortus* 4 and 8 weeks after injection. Fig. 4 demonstrates that lymphocytes from immunized mice splenocytes have recognized the specific antigens at fluorescence intensity between 0.85 to 1.22 nm.
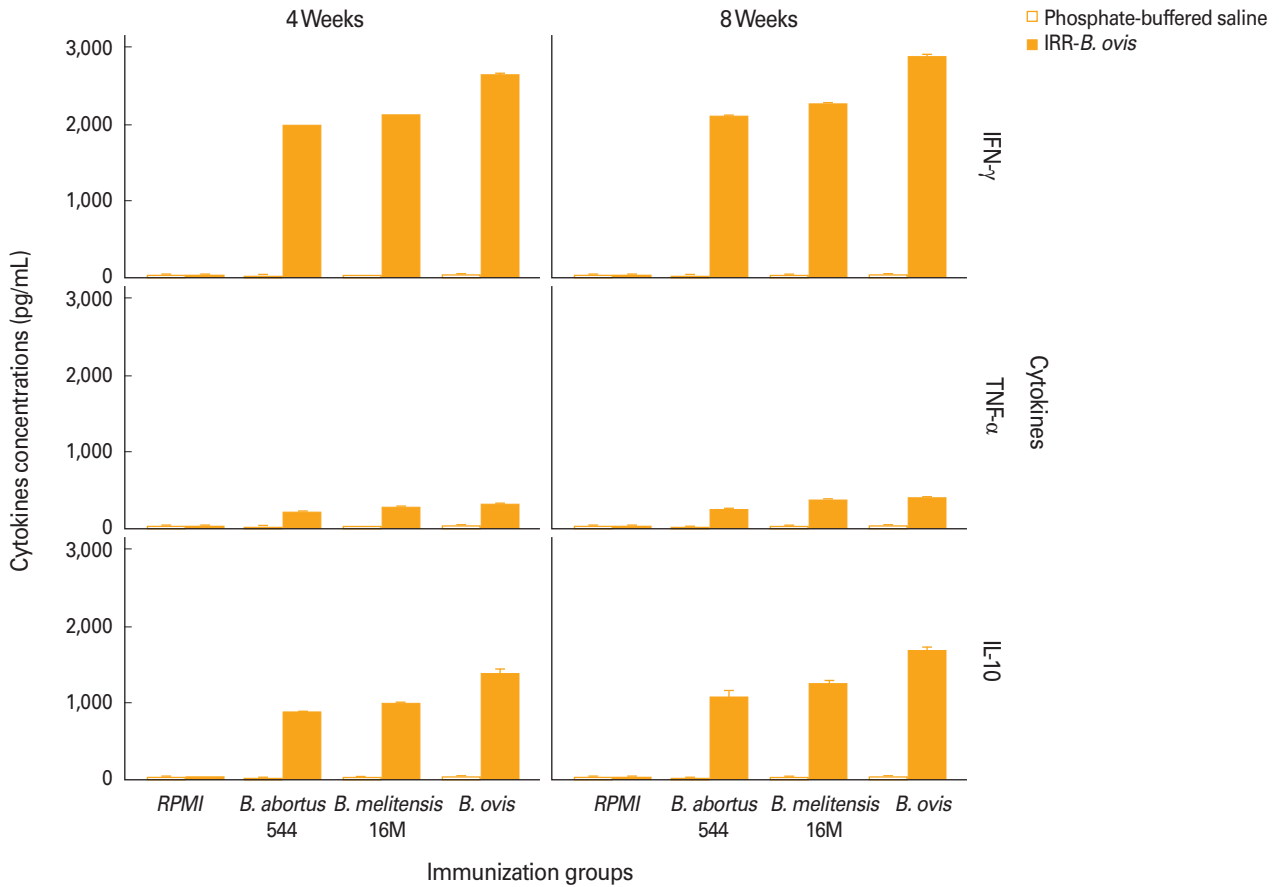
Fig. 3. Interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-10 production *in vitro* by splenocytes of immunized BALB/c mice after 4 and 8 weeks after last vaccination. IRR-*B. ovis*, γ-irradiated *Brucella ovis*.
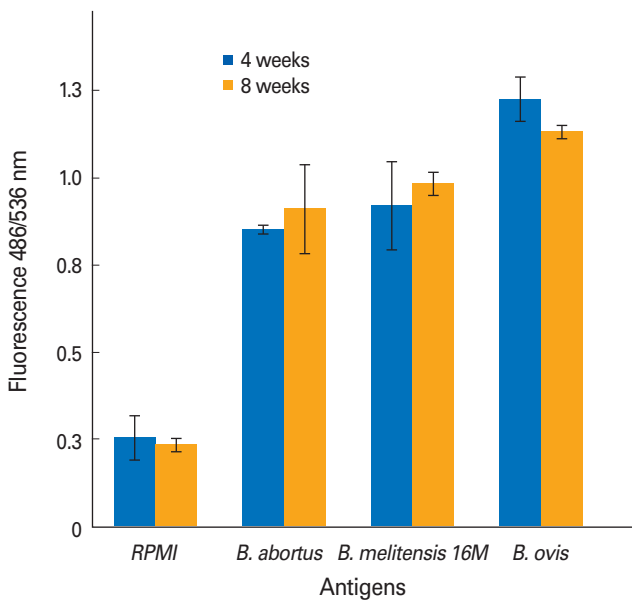

Fig. 4. Lymphocyte proliferation assay by splenocytes from immunized BALB/c mice after 4 and 8 weeks after their last vaccination with phosphate-buffered saline or IRR-*B. ovis* (γ-irradiated *Brucella ovis*).

Studying the protection induced by vaccine

To examine the protective activity of the induced immune response, the remaining immunized mice were tested by *B. ovis*, *B. melitensis* 16M, or *B. abortus* 544. In this study, the protection was defined as a significant reduction in the number of bacteria in the spleen of immunized mice compared to the control group. The vaccine efficiency was evaluated as the $\log_{10}$ reduction in bacterial burdens.

The $\log_{10}$ of protection in BALB/c immunized mice with IRR-*B. ovis* against *B. melitensis* 16M increased from 2.63 to 3.34 whereas the $\log_{10}$ of protection against *B. ovis* increased from 2.62 to 3.34 after 4 and 8 weeks, respectively. Moreover, it was increased from 2.71 after 4 weeks to 3.83 after 8 weeks for the immunized mice with conventional vaccine Rev.1 against *B. melitensis* 16M; whereas it was increased from 2.71 to 3.5 against *B. ovis*, respectively (Tables 2, 3). This means that the developed vaccine at our laboratory could stimulate similar protection to that induced by the classical vaccine Rev.1.

The results listed in Table 4 showed that immunized mice

Table 2. Protection of BALB/c mice against *Brucella melitensis* 16M conferred by immunization with Rev.1 or IRR-*B. ovis* compared to the PBS (n=5)

Mice groups	Vaccine	Log$_{10}$ reduction in *B. melitensis* 16M burdens		Units of protection	
		4 Weeks	8 Weeks	4 Weeks	8 Weeks
1	PBS	5.88±0.53	5.99±0.47	-	-
2	IRR-*B. ovis*	3.25±0.16	2.65±0.13	2.63[a]	3.34[a]
3	Rev.1	3.17±0.19	2.61±0.38	2.71[b]	3.38[b]

Values are presented as mean±standard deviation, unless otherwise stated. Mice were challenged intraperitoneally with $1×10^5$ CFU of strain *B. melitensis* 16M after 4 weeks of the last immunization.
IRR-*B. ovis*, γ-irradiated *B. ovis*; PBS, phosphate-buffered saline; CFU, colony-forming unit.
[a]$p<0.05$, [b]$p<0.005$ (significant) compared to the control group.

Table 4. Protection of BALB/c mice against *Brucella abortus* conferred by immunization with S19 or IRR-*B. ovis* compared to the PBS (n=5)

Mice groups	Vaccine	Log$_{10}$ CFU in spleen of *B. abortus* 544		Units of protection	
		4 Weeks	8 Weeks	4 Weeks	8 Weeks
1	PBS	5.73±0.42	5.86±0.39	-	-
2	IRR-*B. ovis*	3.66±0.18	2.64±0.2	2.07[b]	3.22[b]
3	S19	3.3±0.29	2.45±0.33	2.43[a]	3.43[a]

Values are presented as mean±standard deviation, unless otherwise stated. Mice were challenged intraperitoneally with $1×10^5$ CFU of strain *B. abortus* after 4 weeks of the last immunization.
IRR-*B. ovis*, γ-irradiated *B. ovis*; PBS, phosphate-buffered saline; CFU, colony-forming unit.
[a]$p<0.05$, [b]$p<0.005$ (significant) compared to the control group.

Table 3. Protection of BALB/c mice against *Brucella ovis* conferred by immunization with Rev.1 or IRR-*B.ovis* compared to the PBS (n=5)

Mice groups	Vaccine	Log$_{10}$ reduction in *B. ovis* burdens		Units of protection	
		4 Weeks	8 Weeks	4 Weeks	8 Weeks
1	PBS	5.90±0.53	5.99±0.51	-	-
2	IRR-*B. ovis*	3.28±0.15	2.65±0.19	2.62[a]	3.34[a]
3	Rev.1	3.19±0.14	2.41±0.48	2.71[a]	3.58[a]

Values are presented as mean±standard deviation, unless otherwise stated. Mice were challenged intraperitoneal with $1×10^5$ CFU of strain *B. ovis* after 4 weeks of the last immunization.
IRR-*B. ovis*, γ-irradiated *B. ovis*; PBS, phosphate-buffered saline; CFU, colony-forming unit.
[a]$p<0.0001$ (significant) compared to the control group.

with IRR-*B. ovis* vaccine induced protection against *B. abortus* 544. The log$_{10}$ of bacteria count were 2.07, 3.22 after 4 and 8 weeks of injection, respectively. Whereas, the log$_{10}$ were 2.43 and 3.43 at immunized mice with the conventional vaccine (S19) after 4 and 8 weeks, respectively.

Discussion

Brucellosis is a zoonotic disease that causes enormous economic losses and human suffering [11]. The development of an effective vaccine to control brucellosis has proven to be a challenge over the years. An effective vaccine must be safe and provides sustained protection with the elimination of the challenge infection.

One of the earliest methods used in the manufacture of stable and safe vaccines is the use of chemical and physical treatments to give inactivated forms of pathogens.

Despite the success of these vaccine types in eliciting specific humoral and cellular immune responses to pathogen-associated immunogens, the demand for the development of fast, safe, and effective vaccine manufacturing strategies remains great [17]. Radiation sterilization has been used to develop different types of vaccines because it can destroy chemical contaminants and penetrate pathogens to destroy nucleic acids without damaging the pathogen surface antigens [18]. Recent successful clinical experiments of irradiated vaccines against pathogens and tumors led to a re-evaluation of radiation technology as an alternative process to produce vaccines [14,19-21]. Irradiation of vaccines has been carried out in the past to vaccinate against fungal, parasitic, and bacterial diseases with various degrees of success. With the aim of generating a *Brucella* vaccine that is effective but completely attenuated by abrogating its replication capacity, we irradiated *B. ovis* vaccine strain that is a naturally rough species, expressing R-LPS (R-form lipopolysaccharide) as a major surface antigen.

Attenuated vaccines that were required for chronic infection became ideal for live vaccine development. To be effective, live attenuated vaccines against brucellosis must persist long enough to elicit protective immunity but should be cleared as quickly as possible to avoid unnecessary side effects [22]. In order to determine whether greater persistence correlates with greater protection, the ability of IRR-*B. ovis* to protect vaccinated mice against a challenge infection of wild type *B. ovis*, *B. melitensis* 16M, and *B. abortus* 544 were examined at different time points following vaccination. Evidence indicates that Th1 cells' response promotes resistance to intracellular pathogens [23]. Th1 cells characteristically secrete IFN-γ, IL-

2, and TNF-β. Th2 cells usually produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13. The study of T cell-mediated responses against IRR-*B. ovis* helps to identify predictive correlates of adaptive immunity and to establish whether optimal immunity is a function of the survival and/or the intracellular trafficking and processing of the organism.

Thus, cytokine profiles (TNF-α, IFN-γ, IL-4, IL-5, and IL-10) that induced by IRR-*B. ovis* were analyzed to determine the immune response type induced by our vaccine. IRR-*B. ovis* stimulated IFN-γ production after 4 and 8 weeks of the last immunization; it also stimulated production of TNF-α but in slight quantities (Fig. 3). It is also expected to induce the production of IL-10 4 and 8 weeks from the last time of BALB/c mice immunization. In the previous studies, there has been shown that IL-10 stimulates inflammatory reactions [24]. IL-10 may play a role in inducing protection against Brucella spp. infection [25].

Our results showed that IRR-*B. ovis* did not induce IL-4 or IL-5 production under our study conditions. Cytokine TNF-α is released and served to control *Brucella* infection early in the process. TNF-α, however, appears to act via an INF-γ independent pathway [26]. In addition, the acquired immunity was characterized by lymphocyte proliferative response and antibody titers. The lymphocyte proliferation of mice with bacterial extract (*B. ovis*, *B. melitensis* 16M, or *B. abortus* 544) was observed 4 and 8 weeks of injection (Fig. 4). Our results showed that there were antibody responses compared to saline control mice after 4 and 8 weeks from immunization with IRR-*B. ovis* against *B. ovis*, *B. melitensis* 16M, and *B. abortus* which were IgGt, IgG2a, IgG2b, and IgG1 isotypes antibodies (Fig. 2).

The vaccine possibility of *B. ovis* was mentioned by Sancho et al. [27] where the vaccinated mice with a mutant *B. ovis* developed anti-*B. ovis* antibodies IgG1, IgG2a, and IgG2b subclasses in their serum which was compatible with our results. Also, this isotype profile is consistent with what has been previously reported for serological responses to *Brucella* spp. in general and for strain RB51 in particular [28,29]. Total IgG antibody titer in sera of vaccinated mice after 4 weeks of immunization with IRR-*B. ovis* exposed that there is a significant increase in the IgG antibody and this is in agreement with Zorgi et al. [13] who registered those vaccines developed by irradiation have been found to be strong inducers for cellular and humoral immune responses that make this type of vaccine highly effective.

The presence of antigen-specific IgG1, IgG2a, and IgG2b

antibodies in the serum of the vaccinated mice suggests that the IRR-*B. ovis* vaccine induced a mix of Th1 and Th2 immune responses. In general, a Th1 type of immune response is considered desirable for protection against intracellular bacterial infections, such as brucellosis.

However, based on antigen-specific splenocytes secreting cytokines, the induced Th1 response appears to be more prominent due to the significantly higher concentration of IFN-γ and TNF-α in supernatants of cultures stimulated with the bacterial lysates, and this deal with the effect of IRR-*B. ovis* vaccine. Also, the secretion of Th2 cytokines as IL-4 and IL-5 wasn't noticed while IL-10 was observed in the supernatants splenocytes immunized mice. Several studies revealed that *Brucella* infection in mice induces secretion of TNF-α and IFN-γ but less quantity of IL-10; without inducing secretion of IL-4 and IL-5 [30-32]. TNF-α and IFN-γ have been shown to be one of critical protective cytokines for the control of brucellosis and other diseases caused by intracellular pathogens, whereas IL-4 and IL-5 a Th2 cytokine, is associated with decreased protection [33]. This fact is suitable with the hypothesis that a protective immune response to *Brucella* infection requires the production of pro-inflammatory cytokines such as IFN-γ and TNF-α [10,34].

Another cytokine associated with the Th2 profile is IL-10, high levels of this cytokine were detected after 8 weeks from immunization rather than that detected after 4 weeks in the culture supernatants of splenocytes from our vaccinated mice upon stimulation with *B. ovis*, *B. melitensis* 16M, and *B. abortus*. It has been shown that vaccination with strains (RB51, S19, Rev.1) or infection with virulent *Brucella* also induces the production of IL-10 [35-38]. In addition, the presence of IL-10 has been associated with increased susceptibility to *Brucella* spp. infection [30,39].

In our study, the vaccinated mice with IRR-*B. ovis* were protected against *B. ovis*, *B. melitensis* 16M, and *B. abortus* infections (Tables 2–4). Therefore, the role of IL-10, in this case, might be to limit the elicitation of an exacerbated immune response. Velikovsky et al. [35] have also reported the production of IL-10 when they evaluated protective vaccination with purified lumazine synthase with different adjuvant formulations and challenge with *B. abortus*. These results suggest that although IL-10 may decrease the protective response against *Brucella* challenge, to some extent, it appears to play an important role in protective immunity against *Brucella* infection by limiting the "intensity" of the response geared by IFN-γ [40]. Seo [14] reported that irradiated bacterial vac-

cines retained their metabolic activity and generated protection against extracellular and intracellular bacterial infection. Also, Magnani et al. [20] found that vaccinated mice with irradiated *Brucella* reduced colonization of pathogen. Similarly, Moustafa et al. [25] demonstrated that vaccination with γ-irradiated *B. neotomae* induced protection against *B. abortus* 2308, *B. melitensis* 16M, and *B. suis* 1330 challenge in BALB/c mice.

Taken together, the results suggest that IRR-*B. ovis* can be used as an effective vaccine against brucellosis caused by *B. melitensis*, *B. abortus*, and *B. ovis*. This type of vaccine that is prepared at our laboratory is a safe, potent, and immunogenic vaccine. Also using it as a vaccine makes the vaccinated animals can be distinguished from infected ones by *B. melitensis* or *B. abortus*. The non-virulence of *B. ovis* for humans makes it a safer vaccine candidate for human and animal brucellosis, and it will be tested on sheep at a later time.

ORCID

Ayman Al-Mariri *https://orcid.org/0000-0002-9586-6573*
Laila Al-Hallab *https://orcid.org/0000-0002-4110-9622*
Rasha Alabras *https://orcid.org/0000-0002-9832-6820*
Heba Kherbik *https://orcid.org/0000-0003- 2488-3357*
Marwa Khawajkiah *https://orcid.org/0000-0001-7357-3879*

References

1. Bricker BJ, Ewalt DR, MacMillan AP, Foster G, Brew S. Molecular characterization of Brucella strains isolated from marine mammals. J Clin Microbiol 2000;38:1258-62.
2. Godfroid J, Garin-Bastuji B, Saegerman C, Blasco JM. Brucellosis in terrestrial wildlife. Rev Sci Tech 2013;32:27-42.
3. Wareth G, Abdeen A, Ali H, Bardenstein S, et al. Brucellosis in the Mediterranean countries: history, prevalence, distribution, current situation and attempts at surveillance and control. Paris: World Organisation for Animal (OIE); 2019.
4. Wareth G, Hikal A, Refai M, Melzer F, Roesler U, Neubauer H. Animal brucellosis in Egypt. J Infect Dev Ctries 2014;8: 1365-73.
5. Byndloss MX, Tsolis RM. Brucella spp. virulence factors and immunity. Annu Rev Anim Biosci 2016;4:111-27.
6. Dorneles EM, de Faria AP, Pauletti RB, et al. Genetic stability of Brucella abortus S19 and RB51 vaccine strains by multiple locus variable number tandem repeat analysis (MLVA16). Vaccine 2013;31:4856-9.
7. Dadelahi AS, Lacey CA, Chambers CA, Ponzilacqua-Silva B, Skyberg JA. B cells inhibit CD4+ T cell-mediated immunity to brucella infection in a major histocompatibility complex class II-dependent manner. Infect Immun 2020;88: e00075-20.
8. Goodwin ZI, Pascual DW. Brucellosis vaccines for livestock. Vet Immunol Immunopathol 2016;181:51-8.
9. Gonzalez D, Grillo MJ, De Miguel MJ, et al. Brucellosis vaccines: assessment of Brucella melitensis lipopolysaccharide rough mutants defective in core and O-polysaccharide synthesis and export. PLoS One 2008;3:e2760.
10. Ficht TA, Kahl-McDonagh MM, Arenas-Gamboa AM, Rice-Ficht AC. Brucellosis: the case for live, attenuated vaccines. Vaccine 2009;27(Suppl 4):D40-3.
11. Schurig GG, Sriranganathan N, Corbel MJ. Brucellosis vaccines: past, present and future. Vet Microbiol 2002;90:479-96.
12. Clapp B, Skyberg JA, Yang X, Thornburg T, Walters N, Pascual DW. Protective live oral brucellosis vaccines stimulate Th1 and th17 cell responses. Infect Immun 2011;79: 4165-74.
13. Zorgi NE, Costa A, Galisteo AJ Jr, do Nascimento N, de Andrade HF Jr. Humoral responses and immune protection in mice immunized with irradiated T. gondii tachyzoites and challenged with three genetically distinct strains of T. gondii. Immunol Lett 2011;138:187-96.
14. Seo HS. Application of radiation technology in vaccines development. Clin Exp Vaccine Res 2015;4:145-58.
15. Sanakkayala N, Sokolovska A, Gulani J, et al. Induction of antigen-specific Th1-type immune responses by gamma-irradiated recombinant Brucella abortus RB51. Clin Diagn Lab Immunol 2005;12:1429-36.
16. Dabral N, Martha-Moreno-Lafont, Sriranganathan N, Vemulapalli R. Oral immunization of mice with gamma-irradiated Brucella neotomae induces protection against intraperitoneal and intranasal challenge with virulent B. abortus 2308. PLoS One 2014;9:e107180.
17. Jorge S, Dellagostin OA. The development of veterinary vaccines: a review of traditional methods and modern biotechnology approaches. Biotechnol Res Innov 2017;1:6-13.
18. Abuhanoglu G, Ozer AY. Radiation sterilization of new drug delivery systems. Interv Med Appl Sci 2014;6:51-60.
19. Datta SK, Okamoto S, Hayashi T, et al. Vaccination with irradiated Listeria induces protective T cell immunity. Im-

munity 2006;25:143-52.

20. Magnani DM, Harms JS, Durward MA, Splitter GA. Nondividing but metabolically active gamma-irradiated Brucella melitensis is protective against virulent B. melitensis challenge in mice. Infect Immun 2009;77:5181-9.

21. Surendran N, Hiltbold EM, Heid B, et al. Heat-killed and γ-irradiated Brucella strain RB51 stimulates enhanced dendritic cell activation, but not function compared with the virulent smooth strain 2308. FEMS Immunol Med Microbiol 2010;60:147-55.

22. Lalsiamthara J, Lee JH. Development and trial of vaccines against Brucella. J Vet Sci 2017;18(S1):281-90.

23. Lee N, Kim WU. Microbiota in T-cell homeostasis and inflammatory diseases. Exp Mol Med 2017;49:e340.

24. Sharif MN, Tassiulas I, Hu Y, Mecklenbrauker I, Tarakhovsky A, Ivashkiv LB. IFN-alpha priming results in a gain of pro-inflammatory function by IL-10: implications for systemic lupus erythematosus pathogenesis. J Immunol 2004;172:6476-81.

25. Moustafa D, Garg VK, Jain N, Sriranganathan N, Vemulapalli R. Immunization of mice with gamma-irradiated Brucella neotomae and its recombinant strains induces protection against virulent B. abortus, B. melitensis, and B. suis challenge. Vaccine 2011;29:784-94.

26. Salim T, Sershen CL, May EE. Investigating the role of TNF-α and IFN-γ activation on the dynamics of iNOS gene expression in LPS stimulated macrophages. PLoS One 2016;11:e0153289.

27. Sancho P, Tejedor C, Sidhu-Munoz RS, Fernandez-Lago L, Vizcaino N. Evaluation in mice of Brucella ovis attenuated mutants for use as live vaccines against B. ovis infection. Vet Res 2014;45:61.

28. Vemulapalli R, He Y, Cravero S, Sriranganathan N, Boyle SM, Schurig GG. Overexpression of protective antigen as a novel approach to enhance vaccine efficacy of Brucella abortus strain RB51. Infect Immun 2000;68:3286-9.

29. Zhang J, Guo F, Chen C, et al. Brucella melitensis 16MΔhfq attenuation confers protection against wild-type challenge in BALB/c mice. Microbiol Immunol 2013;57:502-10.

30. Murphy EA, Sathiyaseelan J, Parent MA, Zou B, Baldwin CL. Interferon-gamma is crucial for surviving a Brucella abortus infection in both resistant C57BL/6 and susceptible BALB/c mice. Immunology 2001;103:511-8.

31. Pasquevich KA, Garcia Samartino C, Coria LM, et al. The protein moiety of Brucella abortus outer membrane protein 16 is a new bacterial pathogen-associated molecular pattern that activates dendritic cells in vivo, induces a Th1 immune response, and is a promising self-adjuvanting vaccine against systemic and oral acquired brucellosis. J Immunol 2010;184:5200-12.

32. Pollak CN, Wanke MM, Estein SM, et al. Immunization with Brucella VirB proteins reduces organ colonization in mice through a Th1-type immune response and elicits a similar immune response in dogs. Clin Vaccine Immunol 2015;22:274-81.

33. Silva AP, Macedo AA, Silva TM, et al. Protection provided by an encapsulated live attenuated ΔabcBA strain of Brucella ovis against experimental challenge in a murine model. Clin Vaccine Immunol 2015;22:789-97.

34. Murphy EA, Parent M, Sathiyaseelan J, Jiang X, Baldwin CL. Immune control of Brucella abortus 2308 infections in BALB/c mice. FEMS Immunol Med Microbiol 2001;32:85-8.

35. Velikovsky CA, Goldbaum FA, Cassataro J, et al. Brucella lumazine synthase elicits a mixed Th1-Th2 immune response and reduces infection in mice challenged with Brucella abortus 544 independently of the adjuvant formulation used. Infect Immun 2003;71:5750-5.

36. Grillo MJ, Blasco JM, Gorvel JP, Moriyon I, Moreno E. What have we learned from brucellosis in the mouse model? Vet Res 2012;43:29.

37. Corsetti PP, de Almeida LA, Carvalho NB, et al. Lack of endogenous IL-10 enhances production of proinflammatory cytokines and leads to Brucella abortus clearance in mice. PLoS One 2013;8:e74729.

38. Hop HT, Simborio HL, Reyes AW, et al. Immunogenicity and protective effect of recombinant Brucella abortus Ndk (rNdk) against a virulent strain B. abortus 544 infection in BALB/c mice. FEMS Microbiol Lett 2015;362:1-6

39. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. J Immunol 2008;180:5771-7.

40. Avila-Calderon ED, Lopez-Merino A, Sriranganathan N, Boyle SM, Contreras-Rodriguez A. A history of the development of Brucella vaccines. Biomed Res Int 2013;2013:743509.