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# FIELD STUDIES ON THE PREPARED INACTIVATED GAMMA-IRRADIATED *BRUCELLA ABORTUS* VACCINE IN CATTLE

By

**Hanan I. Mahmoud*, Noha A. Helmy**, and M. A. Makharita***

*Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo

**Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo

## ABSTRACT

This work aims to investigate the safety, immunogenicity, and protective immunity of gamma-irradiated *B. abortus* vaccine prepared from a local Egyptian field isolate (*B. abortus* biovar 9) in cattle calves as a potent alternative vaccine to protect cattle from brucella infection. It was found that the vaccine was safe for guinea pigs as a laboratory model and for cattle calves at a dose of $\simeq$ 5 x $10^{10}$ CFU/2ml.The immune response of cattle calves was evaluated for the irradiated *B. abortus* vaccine at a dose of $\simeq$ 3 x $10^{9}$ CFU / 2ml and 5 x $10^{10}$ CFU / 2ml. The humoral immune response was monitored by RBAT, BAPA, and ELISA test. It was found that, the irradiated vaccine at a dose of $\simeq$ 5 x $10^{10}$ CFU achieved protective immunity at $4^{th}$ week postvaccination (WPV) 0.7 . ELISA antibody titer reached peak titer at $12^{th}$ WPV (1.37) in INF-γ assay irradiated vaccine in dose of $\simeq$ 5 x $10^{10}$ CFU achieved a cut-off value $35^{th}$ day (5.3 SI) post vaccination and peak value was reached on the $60^{th}$ day post vaccination (5.5 SI) in blood samples of the vaccinated cattle calves Group 1 (G1). Therefore, it was concluded that irradiated *B. abortus* vaccine in a dose of $\simeq$ 5 x $10^{10}$ CFU is safe, immunogenic, and protective vaccine to protect cattle against brucellosis.

## INTRODUCTION

Brucellosis is a zoonotic disease caused by several species of *Brucella* such as *B. abortus*, *B. melitensis*, *B. ovis* and *B. suis*. It affects cattle, goat, sheep, and swine respectively, and it has a worldwide high prevalence and causes great economic losses **(Singh *et al.,* 2012).** Although potent live attenuated vaccines as *B. abortus* S19 and RB51 for cattle and *B. melitensis* Rev-1 for sheep and goat **(Arenas-Gamboa *et al,* 2009)** are available, but these vaccines retained unacceptable levels of virulence **(Magnani *et al.,* 2009).** They can also cause abortion in pregnant animals, shedding in milk, body excretions, and infertility in males **(Megid *et al.,* 2010).**

Several inactivated *Brucella* vaccines had been tested as alternative safer vaccine candidates for *B. abortus* strain 19 and *B. abortus* RB51 in mouse model **(Surendran *et al.,* 2010, Magnani *et al.,* 2009, Dabral *et al.,* 2014).** Nevertheless, it was found that gamma radiation is superior to conventional inactivation methods, such as heat and chemical treatments, due to its ability to penetrate effectively the pathogen and its target is nucleic acid causing less damage to surface antigenic proteins **(Seo, H. S., 2015).**

Clinical studies suggested that irradiated vaccines provide more potential immunogenicity than other inactivation methods.Moreover, the metabolically active form of irradiated vaccines was able to activate cytotoxic T-cells, which are important immune cells for treating intracellular pathogens **(Seo, H. S., 2015).**

The bacteria exposed to a minimum dose of radiation retained their de novo protein synthesis. Capabilities of this feature in case of intracellular bacterial pathogens can lead to elicitation of CMI responses to antigens that are expressed when the bacteria are inside the host cells. Gamma-irradiated *Brucella abortus* do not replicate but retain their metabolic activity **(Sanakkayala *et al.,*2005, Moustafa e*t al.,* 2012 ,Dabral *et al.,*2014)** which plays an important role in creating the proper antigenic and adjuvant properties required for efficient triggering of protective responses **(Magnani *et al.,* 2009).** Therefore, it could be a safer alternative to live vaccine for protection of cattle against brucella infection **(Dabral *et al.,* 2014).**This study aimed to evaluate the safety and effectiveness of gamma-irradiated *B. abortus* vaccine prepared from field isolate for cattle as a potent vaccine candidate that can protect cattle from brucellosis.

## MATERIAL AND METHODS

**Bacterial strains:**

***Brucella abortus* biovar 9:**

A field isolate supplied by the Animal Health Research Institute was used for the preparation of irradiated vaccine and it was.

**Animals:**

**a. Calves.**

Twenty cross bred cattle calves of either sex, their age ranged between 6 - 12 monthes and seronegative for brucella antibodies, were used for safety and vaccination experiment.

**b. Guinea pigs.**

Five Brucella-free adult Guinea pigs and their sera were tested to be free from anti-brucella antibodies for safety test.

**Antigens for diagnostic purposes;**

The antigens were obtained from the Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo (VSVRI). They are:

* Rose Bengal antigen (RBA).

* Buffered acidified plate antigen (BAPA).

It was used for monitoring humoral immune response in the sera of vaccinated calves.

**Preparation of inactivated gamma-irradiated *B. abortus* vaccine:**

At the Atomic Research Institute, the preparation was carried out according to **Moustafa *et al.* (2012).** Briefly, *B. abortus* field isolate was reconstituted in sterile phosphate buffer saline (PBS) then grown onto tryptic soy broth at 37°C. The culture suspension was then subdivided into two cultures adjusted into two doses as $\simeq$ a concentration of $5 \times 10^{10}$ CFU/2ml and the other as $\simeq 3 \times 10^{9}$ CFU/2ml and both the two were exposed to 350 Krads of gamma irradiation using 60Co Source Irradiator (Gamma Cell 220 Irradiator). The inability of the irradiated bacteria to replicate was confirmed by plating them on tryptic soy agar (TSA) and having them incubated at 37°C for at least seven days. The irradiated bacteria were then stored at 4°C until use for immunization.

**Quality control tests on the prepared vaccine:**

**Sterility test:**

The test was carried out according to the Office of International Epizootics [OIE] (2013). The prepared gamma irradiated *B. abortus* vaccine was tested for sterility on different media for aerobic, anaerobic bacterial and fungal contaminants.

**Safety test:**

The test was carried out according to the Office of International **Epizootics [OIE] (2013).**

**Safety in Guinea pigs (Kamaraj *et al.*, 2009)**

Five Brucella-free guinea pigs were injected intramuscularly at a dose of the vaccine containing $5 \times 10^{9}$ CFU then kept under observation for seven days.

The vaccine passes the safety test if the inoculated animals do not exhibit adverse effects or mortalities.

**Safety in cattle: (Kamaraj *et al.*, 2009 and Office of International Epizootics, 2013).**

Three cattle calves their sera were free from brucella antibodies, were inoculated with double the recommended dose for subcutaneous route of inoculation. The injected animals were kept for seven days under observation for local or systemic reaction.

**Immunization of cattle calves:**

Seventeen cattle calves, 6-12 months of age, seronegative for brucella antibodies subdivided as follows:

**Group 1 (G1):**

Seven calves were inoculated with irradiated B. abortus vaccine at a dose $\simeq 5 \times 10^{10}$ CFU/2ml subcutaneous route of inoculation (S/C).

**Group 2 (G2):**

Seven calves were inoculated with irradiated B. abortus vaccine in a dose $\simeq 3 \times 10^{9}$ CFU/2ml subcutaneous route of inoculation (S/C).

**Group 3 (G3):**

Three calves were kept as negative control unvaccinated, they were inoculated with 2ml sterile phosphate buffer saline solution (PBS) S/C route.

Both calves in Group 1 (G1) and Group 2 (G2) were inoculated with booster dose of irradiated B. abortus vaccine S/C route of inoculation 28 days from the first dose.

**Collection of blood and serum samples:**

Blood samples were collected from all calf groups (G1, G2, and G3) from the jugular vein.

**A. Samples for monitoring humoral immune response.**

Blood samples were collected in sterile test tubes. Serum samples were then separated from blood samples, and divided into aliquots then stored at -20$^{o}$C until further use for studying the humoral immune response in vaccinated animals.

**B. Samples for INF-γ assay.**

Heparinized test tubes were used for obtaining blood samples for monitoring of cell-mediated immunity on day zero pre-vaccination and on the 21$^{st}$, 35$^{th}$, 60$^{th}$ and 90$^{th}$ post-vaccination days.

**Monitoring of humoral immune response of vaccinated cattle calves and potency of the irradiated *B. abortus* vaccine**

**Agglutination test:**

*** Rose Bengal antigen (RBA) (Alton *et al.,* 1988).** used for monitoring of humoral antibody response in sera of vaccinated calves groups in comparison with sera of negative control unvaccinated group by mixing equal volumes of serum sample and rose bengal antigen (RBA) (30µL)

*** Buffered acidified plate antigen (BAPA) (Alton *et al.,* 1988).** Sensitive agglutination test for rapid screening of humoral antibody response in sera of vaccinated calves groups by mixing 0.08 ml of (BAPA) with 0.03 ml of tested serum samples.

**Enzyme-linked immunosorbent assay (ELISA):**

It was used for detection of anti-brucella IgG antibodies **(Mohan *et al.,* 2016).**

Serum samples of vaccinated cattle were tested using brucellosis serum ELISA test kit (IDEXX) using inactivated antigen of *B. abortus* at optical density (OD) 450 nm wavelengths.

**Monitoring of cell-mediated immune (CMI) response of vaccinated cattle:**

**Interferon Gamma assay (IFN-γ) (Singh *et al.*, 2012).**

CMI response was assessed on day zero pre-vaccination and on the 21$^{st}$, 35$^{th}$, 60$^{th}$ and 90$^{th}$ days post vaccination, employing Interferon gamma (IFN-γ) assay by stimulating peripheral whole blood samples using killed *B. abortus* antigen. The interferon gamma response was assayed using Bovigam Kit from Prioni, USA. From each sample, 1 ml was duplicated and stimulated with heat-killed *B. abortus* 544 antigen for both vaccinated groups and saline-inoculated control group in 24 well cell culture plates (Nunc). Whole blood samples were also stimulated with pokeweed mitogen and PBS as positive and negative controls respectively. The 24 well plates were incubated in a humidified chamber at 37$^{o}$C with 5% $CO_2$ for approximately 24 hours.Plasma was collected then stored at-70$^{o}$C until processed. The results were expressed as stimulation index (SI).

## RESULTS

**Results of safety test:**

**(Table 1):** The irradiated *B. abortus* vaccine was safe for G. pigs and calves. There were no abnormal clinical signs, and no mortality or lesion at the site of inoculation during observation periods.

| Host used for safety test | Observations | Result |
|---|---|---|
| G. pigs | * No morbidity<br>* No mortality<br>* No lesions at site of inoculation | Safe |
| Cattle calves | * No adverse effect observed<br>* No abnormal clinical signs<br>* No lesions at site of inoculation | Safe |

**Results of humoral immune response in vaccinated calves with irradiated *B. abortus* vaccine:**

**Table (2):** Results of agglutination tests

| Weeks post vaccination (wpv) | Serological tests | | | | | |
|---|---|---|---|---|---|---|
| | Rose Bengal antigen test (RBAT) | | | Buffered acidified plate antigen test BAPAT) | | |
| | Irradiated *B. abortus* vaccinated calves | | Negative Control (G3) | Irradiated *B. abortus* vaccinated calves | | Negative Control (G3) |
| | (G1) | (G2) | | (G1) | (G2) | |
| 2 | + | + | - | ++ | + | - |
| 4 | ++ | ++ | - | +++ | ++ | - |
| 6 | +++ | +++ | - | +++ | +++ | - |
| 8 | ++++ | ++++ | - | ++++ | ++++ | - |
| 10 | ++++ | ++++ | - | ++++ | ++++ | - |
| 12 | +++ | +++ | - | ++++ | +++ | - |
| 16 | +++ | +++ | - | +++ | +++ | - |
| 18 | ++ | ++ | - | ++ | ++ | - |
| 20 | + | + | - | + | + | - |

**G1: Group 1 of calves vaccinated with irradiated *B. abortus* vaccine in dose ≃ 5 x $10^{10}$ CFU**

**G2: Group 2 of calves vaccinated with irradiated *B. abortus* vaccine in dose ≃ 3 x $10^{9}$ CFU**

**G3: Group 3 control unvaccinated calves, inoculated sterile (PBS).**

**CFU: Colony Forming Unit**

**- [No Agglutination**

**+ Fine rims formation**

**++ Slight Agglutination**

**+++ Moderate Agglutination**

**++++ Severe agglutination with clumps formation**

**Table (3):** Results of indirect enzyme-linked immunosorbent assay on sera of calves vaccinated with gamma irradiated *B. abortus* vaccine and potency.

| Weeks post vaccination (wpv) | ELISA antibody titers expressed as optical density (OD) | | |
|---|---|---|---|
| | Group 1 (G1) | Group 2 (G2) | Group 3 (G3) |
| 2 | 0.40 | 0.30 | 0.029 |
| 4 | 0.70 | 0.65 | 0.029 |
| 6 | 1.08 | 1.02 | 0.031 |
| 8 | 1.14 | 1.10 | 0.033 |
| 10 | 1.36 | 1.29 | 0.031 |
| 12 | 1.37 | 1.35 | 0.031 |
| 14 | 1.37 | 1.35 | 0.030 |
| 16 | 1.24 | 1.21 | 0.030 |
| 18 | 1.20 | 1.17 | 0.029 |
| 20 | 1.16 | 1.12 | 0.028 |

**The cut-off for a positive antibody response for the vaccine was ELISA antibodies titer (OD) value = 0.70 (Singh *et al.*, 2012).**

**Absorbance of wavelength 450 nm**

**G1: Group 1 of calves vaccinated with irradiated *B. abortus* vaccine in dose $\simeq$ 5 x $10^{10}$ CFU**

**G2: Group 2 of calves vaccinated with irradiated *B. abortus* vaccine in dose $\simeq$ 3 x $10^{9}$ CFU**

**G3: Group 3 control unvaccinated calves, inoculated sterile (PBS).**

**CFU: Colony Forming Unit**

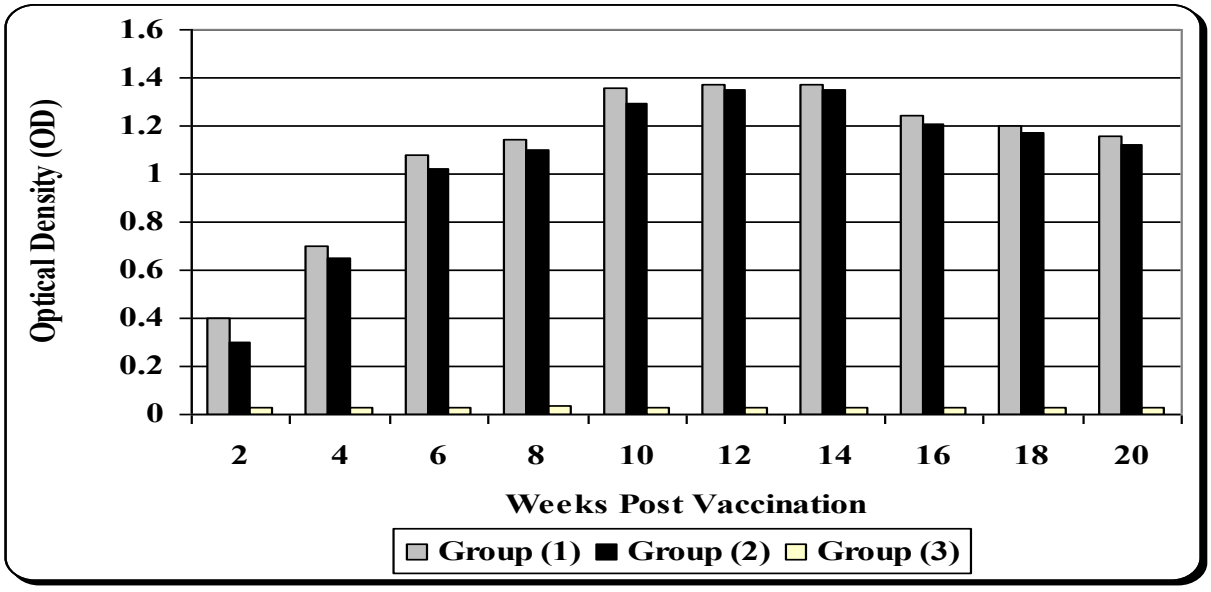


**Fig. (1):** Illustrates ELISA antibodies titers, humoral immune response in sera of cattle calves vaccinated with gamma irradiated *B. abortus* vaccine.

**Table (4):** Results of interferon gamma assay (INF-γ) in calves vaccinated with irradiated *B. abortus* vaccine.

| Days post vaccination | Interferon gamma (INF-γ) value expressed as stimulation index (SI) | | |
|---|---|---|---|
| | Group 1 (G1) | Group 2 (G2) | Group 3 (G3) |
| 0 | - | - | - |
| 21 | 4.50 | 4.02 | 0.03 |
| 35 | 5.30 | 4.90 | 0.03 |
| 60 | 5.50 | 5.30 | 0.04 |
| 90 | 4.98 | 4.70 | 0.03 |

**G1: Group 1 of calves vaccinated with irradiated *B. abortus* vaccine in dose ≃ 5 x $10^{10}$ CFU**

**G2: Group 2 of calves vaccinated with irradiated *B. abortus* vaccine in dose ≃ 3 x $10^{9}$ CFU**

**G3: Group 3 of negative control calves inoculated with sterile PBS**

**CFU: Colony Forming Unit**

**INF-γ was measured at wavelength 450 nm**

$$\text{Stimulation Index (SI)} = \frac{\text{Mean OD value of INF-}\gamma\text{ from blood cells stimulated specific antigen}}{\text{Mean OD value of INF-}\gamma\text{ from blood cells stimulated with PBS control}}$$

The cut-off (SI) value = 4.85 **(Singh *et al.*, 2012).**

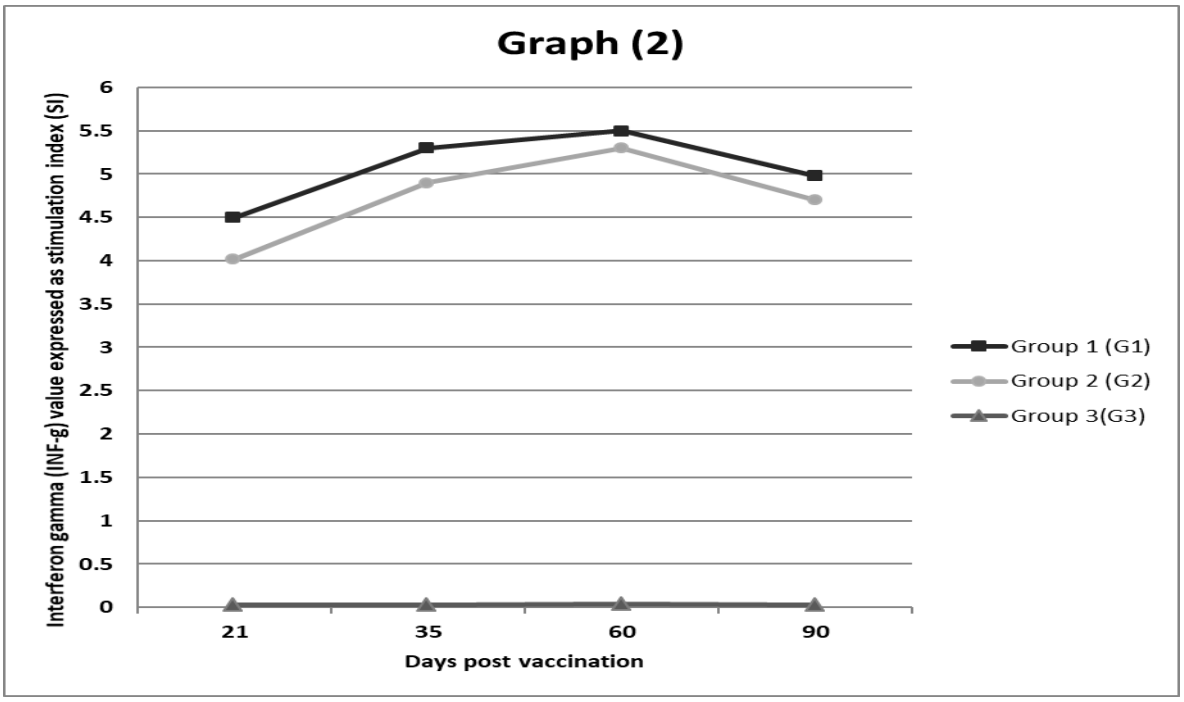


**Fig. (2):** Illustrates Interferon gamma values (INF-γ) in cattle calves vaccinated with gamma irradiated *B.abortus* vaccine.

## DISCUSSION

Vaccination is a determinant strategy for Brucellosis control and eradication programs. Therefore, it has been the target of numerous studies over years. Although the officially approved and successful *B. abortus* S19 and RB51 vaccines for control of the disease in cattle are available **(Dorneles *et al.,* 2015),** but they have many drawbacks, such as pathogenicity for human, potential to cause abortion in pregnant animals, shedding in milk of vaccinated cows, and infertility in bulls **(Corner and Alton, 1981)**. Therefore, there is still a need to develop better bovine vaccine in terms of safety **(Dorneles *et al.,* 2015)** that can confer protection levels comparable to those conferred by S19 but without its disadvantages **(Uglade *et al.,* 2003).** Inactivated vaccines are more stable and safer than live attenuated vaccines **(Seo, H. S., 2015).** Gamma irradiation prevented the replication of bacterial cells with retained metabolic activity. A step that enables them to produce higher immune response thus protection against intracellular and extracellular bacteria **(Sanakkayala *et al.,* 2005, Gaidamakova *et al.,* 2012)** with preserved *B. abortus* adjuvant and antigenic properties that are destroyed in other inactivation **methods (Magnani *et al.,* 2009)** parenteral immunization of mice with gamma-irradiated *B. abortus* RB51 and *B. neotomae* induces, protection against

challenge with virulent Brucella spp. **(Sanakkayala *et al.,* 2005 and Moustafa *et al.,* 2012).** In this study, we evaluated safety, immunogenicity, and cell-mediated immune response of cattle to two different doses of gamma-irradiated Egyptian field isolate of *B. abortus.* (Table 1), shows that, the prepared irradiated *B. abortus* vaccine was safe for guinea pigs, as a laboratory model and for cattle as no lesions were observed at the site of inoculation. No morbidity or mortality were recorded during the observation period in the vaccinated animals post inoculation and this was in agreement with **Kamaraj *et al.* (2009)** and the Office of International **Epizootics (2013).** Both T-cells and anti-smooth LPS antibodies play a role in mediating protection against brucellosis (**Gonzalez, *et al.,* 2008, and Yingst and Hoover, 2003). Darbal *et al.* (2014)** illustrated that antibodies to the O_polysacharide (O_ps) of the lipopolysaccharide play an important role in the protection against infection by B. abortus, *B. melitensis,* and *B. suis.* (Table 2) shows that, the gamma-irradiated Brucella abortus vaccine was capable of eliciting good antibodies titer in sera of vaccinated calves in both Group 1 (G1) and Group 2 (G2) when their sera were monitored using Rose bengal antigen (RBAT) and buffered acidified plate antigen (BAPAT) when compared with results in sera of negative control unvaccinated calves Group 3 (G3). Sera of calves in both G1 and G2 gave positive agglutinating antibodies titer after the second post-vaccination week (wpv). The titer was increased during the fourth week (wpv). Its peak was achieved after the booster during the fourth week giving peak titers and reaching a plateau during the eighth week until the tenth (wpv) then declined in agglutinating titers decreased from the sixteenth week (wpv) until the twentieth week (wpv). The obtained results were in accordance with those of enzyme-linked immunosorbent assay (ELISA) antibodies titer in (Table 3) and Fig. (1) in a manner that sera of vaccinated calves in G1 and G2 showed slight rise in ELISA antibodies titer by the second (wpv) and sera of calves in G1 achieved the protective cut-off titer as (0.7) **(Singh *et al.,* 2012)** by the fourth (wpv) in sera of calves of G1. Therefore, the dose of $\simeq 5 \times 10^{10}$ CFU used in immunization of calves of G1 could be considered superior to $\simeq 3 \times 10^{9}$ CFU that was used in vaccination of calves of G2. Then, both ELISA antibodies titer showed an increase after booster inoculation at the fourth (wpv) until achieving the peak. It was achieved in both G1and G2 at 12th (wpv) as 1.37 in G1 and 1.35 in G2 then reached a plateau till the 16th (wpv) followed by a gradual decrease till the 20th (wpv) as 1.16 in G1 and 1.12 in G2. This is in accordance with **Magnani *et al.* (2009),** who stated that gamma-irradiated *B. abortus* vaccine plays an important role in creating the proper antigenic and adjuvant properties

required for efficient triggering of protective responses. It is also in accordance with **Sanakkayala *et al.* (2005), Moustafa *et al.* (2012), and Darbal *et al.* (2014),** who mentioned that irradiated *B. abortus* vaccine generates higher humoral immune responses and protection against extra cellular and intracellular bacteria. Cell-mediated immunity (CMI) is known to play an important role in brucellosis **(Mohan *et al.,* 2016)**. Therefore, in this study, we monitored the level of CMI response in vaccinated calves by using gamma interferon assay (IFN-γ) which is an important cytokine that plays a critical role in the CMI response of the host to *B. abortus* **(Feng *et al.,* 2017)** from (Table 4) and Fig.(2) It was found that irradiated *B. abortus* vaccine was able to elicit a good IFN-γ response in both G1 and G2 calf groups on the 21$^{st}$, 35$^{th}$, and 60$^{th}$ post-vaccination days, which is 4.5, 5.3, 5.5 respectively in G1, and 4.02, 4.9, and 4.7 in G2 expressed as stimulation index (SI). After that, the amount of INF-γ began to decrease by day 90 post vaccination; it was 4.98 in G1 and 4.7 in G2. Both G1 and G2 achieved protective cut-off value by the 35$^{th}$ day post- vaccination, which is 5.3 for G1 and 4.9 for G2, with higher value in G1. This was in agreement with **Singh *et al.* (2012)** who reported that cut-off value IFN-γ for B. abortus vaccine is 4.85. From our data, it was found that gamma-irradiated B. abortus vaccine prepared from the Egyptian field isolate was safe, potent and immunogenic for calves in dose of $\simeq$ 5 x 10$^{10}$ cfu so we conclude that this vaccine can be used as safer alternative vaccine candidate for cattle to protect them against brucellosis.

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# دراسة حقلية على لقاح البروسيلا أبورتس المثبط بأشعة جاما في الماشية

*** د. حنان إبراهيم محمود محمد**، د. نهى حلمي*، أ.د. محمد علي مخاريطة**

* المعمل المركزي للرقابة على المستحضرات الحيوية البيطرية – العباسية – القاهرة

** معهد بحوث الأمصال واللقاحات البيطرية – العباسية – القاهرة

## الملخص العربي

تهدف هذه الدراسة إلى بحث درجة الامان والقوة المناعية والصد الذين ينتجون عن تحصين عجول الماشية بلقاح البروسيلا أبورتس المثبط بأشعة جاما والذي تم تحضيره من العترة المحلية المصرية biovar.9 المعزولة حقلياً والتي تؤهله للاستخدام كلقاح آمن وفعال لحماية الماشية من عدوى البروسيلا. وجدت الدراسة أنه عند استخدام لقاح البروسيلا أبورتس المثبط بأشعة جاما بجرعة حوالي $10^{10}$ x 5 CFU ، حقق اللقاح حماية مناعية في عجول الماشية على الاسبوع الرابع بعد الحقن (0.7) كمستوى للأجسام المناعية لاختبار ELISA. وقد وصل مستوى الأجسام المناعية على الاسبوع الثاني عشر بعد الحقن (1.37). وفي اختبارات INF-γ عند حقن لقاح البروسيلا أبورتس المثبط بأشعة جاما في عجول الماشية بجرعة $10^{10}$ x 5، وجدت الدراسة أن العجول المحقونة بنسبة 100% حققت قيمة الصد العيارية (S.I. 5.3) على اليوم الخامس والثلاثين بعد الحقن وأعلى قيمة لـ INF-γ (S.I. 5.5) عند اليوم الستين بعد الحقن. نستخلص من هذه الدراسة أن لقاح البروسيلا أبورتس المثبط بأشعة جاما بجرعة حوالي $10^{10}$ x 5 يمكن استخدامه حقلياً كلقاح آمن ذي قوة صد مناعية لحماية الماشية من مرض البروسيلا.