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# Protection of mice against experimental infection wild *Brucella abortus* strain by vaccination via oral and intraperitoneal routes with *Brucella abortus* RB51

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**Abstract**

***The study*** was designed to detect the effect of an oral and intraperitoneal (I/P) immunization of mice with *B. Abortus* RB51 to protect mice against a wild strain of *B. abortus* I/P challenge infection. Three groups of mice were used in this study. The first group (1st G) immunized with $10^8$ *2 CFU of *B. Abortus* RB51 intraperitoneally (I/P). The second group (2nd G) immunized orally with $10^8$ *2 CFU dose (Ten minutes prior immunization, all mice were drenched with 0.2 ml of 10% sodium bicarbonate to neutralize gastric acidity). Whereas, the third group (3rdgroup) inoculated with 0.2 ml phosphate buffer saline (PBS) and acted as the control group. The results indicated that there was a significant difference ($P<0.05$) in antibody titer at 5th week in the I/P group and in the 3rd week in oral group, while there was no significance between the two route through all the periods. However, after the challenge, the antibody titer raised to 0.84±0.11 and 1.14±0.11 in the two route and control group in 3rd and 7th day post challenge respectively. The Ab titer reached 1.44±0.11 in the I/P route and remain at 1.14±0.11 in oral and control at 10th day/post-challenge. The oral inoculation gave a mild infection, which was cleared at 5th week after infection, and it induced a humoral response. However, I/P challenge gave moderate infection, which was cleared at 6th week after infection. Wild *B. Abortus* was isolated at a lowest level after the challenge from internal organs, in animals immunized I/P compared with the other two groups. In conclusion, I/P and oral immunization were able to give protection against the virulent wild strain *B. abortus* in mice. Besides, the probability of these mice in transmitting the vaccine to other animals was low and vaccine was safety in pregnant vaccinated mice.

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## Introduction

Bovine brucellosis is an important disease caused by *Brucella abortus*. The disease typically affects cattle often causing abortion of the first fetus following infection

(Nicoletti and Gilsdorf, 1997). Certain wild ruminants such as bison and elk are susceptible to brucellosis. There is a concern that these species could transmit *B. abortus* to cattle grazing in the area (Davis, 1990; Davis *et al*., 1990; Rhyan *et al*., 1994; Thorne *et al*., 1978, Williams *et al*., 1993). Moreover, the disease in these animals can be experimentally transmitted to cattle (Davis *et al*., 1990; Thorne *et al*., 1979). There is one problem associated with S19 vaccination. It induce antibodies, which are difficult to differentiate it from those caused by field strain infections (Cheville *et al*., 1998). It is generally agreed that administering an oral vaccine would be easier, and it would probably be less expensive, would cause less stress to the animals, wouldn't require an acclimation period, would likely lead to a greater proportion of the adult herd being vaccinated, and would more easily allow for booster vaccinations (Bowersock *et al.*, 1994). The SRB51 vaccine is effective in preventing brucellosis when given subcutaneously to cattle (Cheville *et al.*, 1996, Cheville *et al.*, 1993); intraperitoneal (Jimenez *et al*., 1994, Stevens *et al*., 1995a) and subcutaneously (Jimenez *et al*.,1994, stevens *et al*., 1995 a) to the mice. Most of the knowledge about the immunology and protective immunologic mechanisms associated with SRB51 vaccination has been derived from studies with mice. Mice have served as a useful animal model in testing the SRB51 vaccine because they exhibit immune responses to SRB51 and SRB51 antigens (Jimenez et al., 1994; Schurig *et al*., 1991; Stevens *et al*., 1995a, Stevens *et al.*, 1995b). These finding are similar to the responses, which occur in SRB51-vaccinated ruminants such as cattle (Stevens and Olsen, 1996; Stevens *et al.*, 1994, Stevens *et al.*, 1995b, Stevens *et al*., 1996) and goats (Roop *et al*., 1991; AL-Flahy, 2011). Administering the SRB51 vaccine parenterally to individual free-roaming wild ruminants in large herds would probably be highly impracticable. A less tedious method such as oral vaccination of entire herds with SRB51 in feed may be more beneficial in preventing brucellosis in these animals. Oral vaccination with SRB51 may be efficacious, since other vaccines such as S19 and *B. suis* 2 are effective when given orally to cattle (Nicoletti and Milward, 1983; Xin, 1986). Previous, preliminary studies also indicates that oral SRB51 vaccination of monogastric animals (feral pigs) induces partial protection from abortion following experimental infection with *B. suis* (Hagius *et al.*, 1995). In Iraq a little information are available on cell immune response following *B.abortus* RB51 vaccination. So, this study was designed to analyze and compare of an oral and I/P immunization of mice with *B. abortus* RB51 and its effect on the onset of protection against a challenge infection by I/P route with a wild strain of *B.abortus*.

## Materials and methods

### RB51 vaccine

The vaccine was periodically renewed from freeze-dried stock to maintain a constant level of activity and plated on tryptose agar to determine the number of CFU.

### Wild *Brucella abortus*

Wild *Brucella abortus* isolated locally from aborted cow fetus, using selective media and biochemical tests (Quinn *et al*., 2004), this isolate was confirmed in the central public health laboratory.

**Animals**

BALB/c mice were obtained from Alkindy Company and used in the experiments at 12 to 14 weeks of age. They were fed a commercial diet, and water was provided. All mice were acclimatized for a minimum of 1 week prior to experimentation.

**RB51 Brucellin**

The RB51 brucellin was produced according to the method of (De Massis *et al*., 2005). The suspension was then stirred for 1 hour and diluted 1:10 (v/v) in phosphate buffered saline (pH 7.2) with 0.01% sodium merthiolate to the protein concentration of 2.89 mg/dl. The antigen was tested for sterility and safety before use according to (OIE, 2004).

**Immunization protocols**

Totally, 160 mice were used in this study, and divided into 3 groups. The 1st G was immunized I/P with live bacteria, then, the mice were given $10^8$ *2 CFU of *B. abortus* RB51 in 0.2 ml of sterile saline. The 2nd G was immunized orally with $10^8$ *2 CFU of *B. abortus* RB51 using a gastric lavage needle, meanwhile, ten minutes prior to oral inoculation , 0.2 ml of 10% sodium bicarbonate was given to these mice to neutralize gastric acidity. The 3rd G was inoculated with 0.2 ml phosphate buffer saline (PBS) and acted as control.

**Serology**

Five mice of each route and three mice from control were sacrificed after (1, 3 and 5) weeks of immunization, and blood was collected from heart and serum was separated in sterile test tubes. The titer of antibodies were determined by Passive haemagglutanation test according to method described previously (Renoux, 1980).

**Persistence of *B.abortus* in spleen**

To determine the persistence of *B.abortus*RB51in spleen of immunized group, mice were sacrificed weekly from (1-7) weeks after immunization (five mice from each group).Spleens were removed and homogenized in 1 ml of sterile saline. The aliquot of the resulting cell suspensions were plated to determine the number of CFU and assess the spleen colonization.

**Protective assay**

After 45 days of immunization, the other remaining immunized and control (non immunized) mice were challenged I/P with a wild strain of *B.abortus* $1\times10^5$ CFU then after (3,7,10) days, five mice of each route and three mice of control were sacrificed. The serum were separated to detect the titer of antibodies. In addition, *B.abortus* from internal organs of these mice was also isolated.

## Results

### Clinical signs

Through the time of the study, the post challenge with viable *B.abortus* revealed no clinical signs on the immunized experimental mice. They showed normal pregnancy with normal parturition, while non-immunized infected females showed abortion between fifth and twelve day post challenge.

### Serology

The results of the passive haemaggluttination test (PHT) showed that there was a significant difference ($P<0.05$) in Ab titer at $5^{th}$ week in I/P group, while there is no significance between the two route through all periods. After challenge the serum antibody titers showed significant difference at $10^{th}$ and $7^{th}$ than $3^{rd}$ day, in in I/P group ,while no significant difference in oral and control group, also there was no significant difference between I/P ,oral and control at 3rd days (Table. 1 and 2 ) .

**Table.1.** Shows means of Antibody titers against *B.abortus* RB51 by PHT at 1st, 3rd and 5th week post immunization.

| Route | Killing time/ Week ($Log_{10}$) | | |
|---|---|---|---|
| | $1^{st}$ | $3^{rd}$ | $5^{th}$ |
| I.P | B 0.54±0.11$^{a}$ | AB 0.84± 0.11 $^{a}$ | A 1.14± 0.11$^{a}$ |
| Orally | A 0.84± 0.11 $^{a}$ | A 1.14± 0.11$^{a}$ | A 0.84± 0.11 $^{a}$ |

Means with different small letter in the same column differ significantly ($P<0.05$)
Means with different capital letter in the same row differ significantly

**Table.2.** Means of Antibody titer of mice by PHT at 3rd, 7th and 10th day post Challenge

| Route | Killing time/ day ($Log_{10}$) | | |
|---|---|---|---|
| | $3^{rd}$ | $7^{th}$ | $10^{th}$ |
| I.P | B 0.84± 0.11 $^{a}$ | AB 1.14± 0.11$^{a}$ | A 1.14± 0.11$^{a}$ |
| Orally | A 0.84± 0.11 $^{a}$ | A 1.14± 0.11$^{a}$ | A 1.14± 0.11$^{a}$ |
| Control | A 0.84± 0.11 $^{a}$ | A 1.14± 0.11$^{a}$ | A 1.14± 0.11$^{ab}$ |

Means with different small letter in the same column differ significantly ($P<0.05$)
Means with different capital letter in the same row differ significantly

### Persistence of *B.abortus* RB51 in spleen

The mice were immunized I/P, exhibited $3\times10^{5}$ CFU in $3^{rd}$ week, and the isolation persistent until $5^{th}$ week. However, no bacteria were isolated from the spleens at $6^{th}$ week. In contrast, the number decline to ($6.7\times10^{5}$) in orally immunized mice and the isolation

were persistence until 4th week. In addition, no bacteria were isolated from the spleens at 5th week (Table.3.).

**Table.3.** Shows the persistence of *B.abortus* RB51 in spleens of the immunized mice.

| Week | IP | Orally |
|---|---|---|
| 1st | +($3\times10^5$) | +($6.7\times10^5$) |
| 2nd | + | + |
| 4th | + | + |
| 5th | + | - |
| 6th | - | - |
| 7th | - | - |

**Post challenge wild *B.abortus* isolation**

The control group revealed almost negative to moderate wild *B.abortus* isolation. In both immunized challenged groups, wild *B. abortus* were mildly to moderately isolate from the liver, spleen, and kidney at the 3rd day post challenge. However, the heart and brain isolation was negative to very mild.
(Table. 4A).

**Table. 4A.** Shows the isolation of wild *B.abortus* from immunized mice 3days post challenge.

| Route of immunization | No. of mice | Organs | | | | | |
|---|---|---|---|---|---|---|---|
| | | Spleen | liver | Heart | Brain | Lung | Kidney |
| orally | 1 | +++ | ++ | - | - | + | + |
| | 2 | ++ | ++ | - | - | ++ | + |
| | 3 | ++ | - | - | - | + | + |
| | 4 | + | + | + | - | + | + |
| | 5 | + | + | - | - | - | - |
| I/p | 1 | ++ | ++ | - | - | + | - |
| | 2 | ++ | ++ | - | - | ++ | + |
| | 3 | +++ | ++ | - | - | ++ | + |
| | 4 | ++ | + | - | - | + | + |
| | 5 | + | - | - | - | - | - |
| control | 1 | +++ | +++ | + | - | + | ++ |
| | 2 | +++ | +++ | - | - | + | ++ |
| | 3 | +++ | +++ | - | - | + | ++ |
| | 4 | +++ | +++ | - | - | + | ++ |
| | 5 | +++ | +++ | - | - | - | ++ |

At 7th day post challenge, negative to moderate wild *B.abortus* isolation were seen in oral immunized challenged group. However, I/P vaccinated infected group revealed a negative to mild isolation. In addition, *B.abortus* was recovered in very mild to heavy from spleen, liver, kidney and lung and negative to very mild from the heart and brain of all groups (Table. 4B). At the 10th day post challenge, negative to moderate wild *B. abortus* were isolated from spleen, liver, kidney and lung of the oral immunized challenge group. However, the I/P immunized infected group revealed negative isolation from the liver, heart and brain while, it was very mild in spleen, lung and kidney. The

control group revealed heavy wild *B.abortus* isolation from spleen, liver, kidney and lung and negative to mild in heart and brain.

**Table. 4B**. Shows the isolation of wild *B.abortus* from immunized mice 7 days post challenge.

| Rout of immunization | No. of mice | Organs | | | | | |
|---|---|---|---|---|---|---|---|
| | | Spleen | liver | Heart | Brain | Lung | Kidney |
| orally | 1 | + | + | + | - | - | ++ |
| | 2 | + | - | - | - | - | +++ |
| | 3 | + | - | - | - | + | ++ |
| | 4 | + | - | + | - | + | + |
| | 5 | - | - | - | - | - | + |
| I/p | 1 | + | - | - | - | - | ++ |
| | 2 | + | + | + | - | + | +++ |
| | 3 | ++ | - | - | - | - | ++ |
| | 4 | + | - | - | - | - | + |
| | 5 | + | - | - | - | + | + |
| control | 1 | +++ | ++ | + | - | ++ | ++ |
| | 2 | +++ | ++ | + | - | ++ | +++ |
| | 3 | +++ | ++ | - | - | +++ | +++ |
| | 4 | ++ | + | - | - | - | ++ |
| | 5 | ++ | + | - | - | - | ++ |

**Table. 4C.** Shows the isolation of wild *B.abortus* from immunized mice 10 days post challenge

| Route of immunization | No. of mice | Organs | | | | | |
|---|---|---|---|---|---|---|---|
| | | Spleen | liver | Heart | Brain | Lung | Kidney |
| Orally | 1 | + | + | + | - | + | ++ |
| | 2 | + | ++ | - | - | + | ++ |
| | 3 | + | - | - | - | - | + |
| | 4 | + | - | - | - | - | + |
| | 5 | - | - | - | - | - | + |
| I/p | 1 | + | - | - | - | + | + |
| | 2 | + | - | - | - | - | + |
| | 3 | - | - | - | - | - | + |
| | 4 | - | - | - | - | - | + |
| | 5 | - | - | - | - | + | + |
| control | 1 | +++ | + | + | - | ++ | +++ |
| | 2 | +++ | ++ | + | - | ++ | ++ |
| | 3 | ++ | ++ | - | - | +++ | ++ |
| | 4 | ++ | ++ | - | - | + | ++ |
| | 5 | ++ | + | - | - | + | + |

(-)= negative (0) colony, (+) = very mild (1-5) colony, (++) = mild (6-10) colony, (+++) = moderate (11-15) colony, (++++) = heavy (16 and more) colony

## Discussion

*B. abortus* RB51 is a stable, rough mutant of strain 2308. It has ability to induce protection against challenge with smooth virulent *Brucella spp.* in cattle, swine, mice, and goats (Cheville, 1993; Hagius *et al*., 1995; Jimenez *et al*., 1994; Winter *et al*., 1996). In this study, the results of PHT showed that the level of Ab titer from the 1$^{st}$ week elevated to the peak at the 5$^{th}$ week in I/P RB51 immunized mice, while oral immunized group showed elevated and then declined titer at 5$^{th}$ week. The detection of anti *B. abortus* antibodies as response to vaccination by RB51 strain was verified by PHT using RB51 antigen (Al-Zhaidy, 2005; Al-Flahy, 2011). However, Abs titer to RB51 antigen was also detected by Stevens *et al*., (1996) using Dot ELISA test.

The present study indicates the persistence of RB51 in the spleen of I/P immunized group at 5th week, in contrast to the oral route, the persistent was at 4th week. This result is compatible with Schrage *et al*., (1991) study, who revealed that laboratory mice generally clear the RB51 strain within 4th week following I/P vaccination. However, Stevens *et al.*, (1994) revealed that live bacteria and bacterial antigens of RB51 persisted in spleens at 4th week after infection, and no bacteria was recognized at 6th week from the spleens although the mice were infected with approximately 100 times more RB51 than *B. abortus* 2308. In addition, the I/P immunized mice exhibited decline in the number of bacteria between 2-8 week and no bacteria at 12 week (Steven and Olsen, 1996) , while in oral immunized group, the bacterial cultured from spleens was revealed at 2-12 week. Pasquali *et al.,* (2001) found I/p immunization of mice with live *B. abortus* RB51 resulted in a pattern of bacterial growth after 6 days. The peak numbers were seen at 18 days after vaccination, followed by a progressive decline. So, the previous studies explained that the Ab titer in I/P route was higher accompanied with longer persistence of RB51 in the spleen than in oral route.

The optimal protection against wild *B. abortus* in mice required I/P vaccination of strain RB51 in numbers exceeding $10^8$ CFU (Zhan *et al.,* 1993; Jimenez, 1994). When mice were inoculated I/P with $10^8$ *2 CFU of RB51, there was a steady decline in numbers by first week post immunization, bacterial counts averaged well below to $3\times10^5$ and no bacteria cultured at 6th week. Similar result, was seen in this study, where the count decline at 1st week and no Bacteria cultured at 5th week post immunization. Immunization by oral or I/P route with strain RB51 showed high titer with the homologous antigen. The antibodies titer increased and reach to 32,16 at 10th days in I/P and oral route respectively post challenge , this increasing in the anti RB51 antibodies were attributed to challenge with *B.abortus.*

The results of bacterial isolation showed that wild *B.abortus* was isolated from some internal organs of both immunized and control animals post challenge. And it was variable in their intensity according to the route of immunization and the organ from which it was isolated. The lowest bacterial level was reported in animals immunized intraperitoneal compared with the animals immunized orally and control groups, but no bacterial isolates were recovered from heart and brain of all examined animals. These results were supported by Al-Oubaidy,(2008), who mentioned that *Brucella* has predilection sites, which are in the liver and spleen, this may be due to presence of large number of macrophages or presence of specific receptor site on the cells of spleen and liver. In addition to precocious production of IFN-γ, which supported by Pasquali *et al*., (2001), who clarified that RB51 induced protection as a result of precocious production of IFN-γ that may prime macrophages, resulting in the inhibition of the establishment of persistent infection after challenge infection, thus, protection may be more dependent upon the timing of the cytokine response rather than on absolute level of cytokine expression. Vaccination of mice induces humoral immune response and proliferation in spleen cell population ,protection induced by RB51 is based upon cell-mediated immunity and antibodies play a minor role in protection (Jimenez *et al*.,1994; Stevens *et al*.,1994),early production of gamma interferon seems to have the prominent role in the inducing an immunologically based protection (Pasquali *et al*., 2001). Some workers suggested that vaccination with RB51 and challenge with wild virulent *B. abortus* will induce number of significant effects upon cytokine expression, IL-12, IFN-γ, IL-4, and

IL-10 that inhibit the establishment of persistent infection after challenge (Pasquali *et al.*, 2001). The results of previous studies showed that immunized animals had lower bacterial isolation than unimmunized animal in different internal organs, this could be due to the early releasing of IFN-γ and IL-10 in the spleen cells that lead to activation of macrophage and starting of phagocytosis. These results were supported by (Pasquali *et al*, 2001), who revealed that IFN-γ and IL-10 were detected during the early phase of infection as early as 3rd day after challenge infection in vaccinated mice, which was higher than in unvaccinated animal and will protect as early as 3 days after challenge infection. They revealed that IFN-γ was found to be positively correlated with clearance of the infection, while they showed that no difference in the level of IFN-γ and IL-10 throughout the experiment (10 days post challenge) and they showed that the temporal relationships between *Brucella*, macrophage and IFN-γ may determine the outcome of infection, which was supported the results obtained through bacterial isolation of internal organs and was differ throughout the 10 days post challenge. However, this study showed that the isolation of *B.abortus* was lower in the RB51 immunized group in compare with unimmunized group, suggesting that RB51 vaccine in mice had played an important role for intracellular killing of challenged bacteria in various organs like spleen, liver, kidney and lung through cell-mediated immunity. This results is in agreement with Pasquali *et al.*, (2001), who observed that mice vaccinated with RB51 strain and challenged with *B.abortus* were protected from reinfection, this may be attributed, after vaccination, both Th1 and Th2 cytokine pattern and early production of gamma interferon were showed to have the prominent role in induction an immunologically based protection.This study was also shown that the isolation of bacteria from I/P immunized mice was lower than oral route, this may be due to higher Abs induction caused by I/P vaccination. This result is in agreement with previous observations reported by Jimenez de Bagues *et al.*, (1994). They reported that I/P route was more effective than oral route in protection against infection. In another study, Dunkley *et al.*, (1994) showed that Th1 cell act together with B cell to induce Abs producing cells that opsonize smooth *Brucella* and induce phagocytosis, complement activation and bacterial killing. The vaccination stimulates the effector cells and induced memory cell, then challenge dose caused proliferation and differentiation of memory cells and formation of Th1 that produce many of cytokines like IFN-γ that caused activation and proliferation of macrophages and destruction of *Brucella*. The mice which were vaccinated I/P with SRB51 and challenged with S2308, had spleen cells which proliferated better and produced higher levels of IFN-γ in response to S2308 than did orally SRB51vaccinated mice which had been challenged with S2308because these mice produced smaller amounts of IFN-γ in response to S2308 infection than did mice vaccinated I/P. It is reported that SRB51and SRB51 are not highly invasive when given orally to mice and that the bacteria apparently stimulate immune responses primarily in the lymph nodes because it drain in the oral cavity. Previous studies reported that mice vaccinated orally with *B. abortus* RB51 were protected at a lower degree than mice vaccinated intraperitoneally against a challenge infection with a virulent strain inoculated intraperitoneally (steven,1996). The results of the current study revealed that the I/P immunization was better than the oral rout. This results is supported with previous observation reported by Pasquali *etal*, (2003), who found that Oral inoculation of *B. abortus* RB51 was able to give protection to mice infected with the virulent strain *B.*

*abortus* 2308 by the oral route but not to mice challenge intraperitoneally. The current results of this study confirm these findings but indicate also that oral vaccination in mice induces a mild infection, which is able to confer protection at a level comparable to that of intraperitoneal vaccination against intraperitoneal infection challenge, and this is due to gastric acidity neutralization and induced a systemic infection with *B. abortus* RB51. Although strain RB51 vaccine has been shown to cause a mild level of placentitis without fetal death in mice after inoculation of high doses (Tobias 1992), and has been shown to produce in vitro cytotoxic effects on bovine chorioallantoic membrane explants (Samartino and Enright, 1992), it has not been shown to induce fetal death or abortions in mice, goats, and cattle (Buhrman, 1989; Cheville *et al*., 1992; Enright *et al* ,1994; Roop*et al* ,1991; Schurig*et al*.,1991; Tobias *et al*., 1992). These previous studies are supported our results which indicated the safety of RB51 in pregnant vaccinated mice and parturition normally.

In conclusion, this study revealed that vaccination of mice with *B. abortus* RB51 via I/P route was better than oral route. The oral vaccination was induced a mild infection and was able to confer protection, but it was less activity than I/P vaccination when challenge via intraperitoneal infection because of the gastric acidity neutralization and induced a systemic infection with *B. abortus* RB51.

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