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Short communication

# Partial protection induced by *Salmonella* based *Brucella* vaccine candidate in pregnant guinea pigs

Jonathan Lalsiamthara, Amal Senevirathne, John Hwa Lee *

*College of Veterinary Medicine, Chonbuk National University, Iksan Campus, 570-752 Iksan, Republic of Korea*

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ABSTRACT

Residual virulence is a major drawback in current *Brucella* vaccines. Live vaccines induce abortions in pregnant animals. Hence, a novel anti-*Brucella* vaccine was developed utilizing rough *Salmonella* delivering four *Brucella* antigens. Safety implications during pregnancy, humoral immune responses, and protective efficacy against wild type *Brucella* was investigated in guinea pig model. The vaccine did not induce abortions or severe complications in pregnant guinea pigs when administered $4 \times 10^8$ CFU via intraperitoneal route. Systemic IgG determination against antigen components reveals induction of immunity via the *Salmonella* delivery. Protection efficacy against abortions was 33.3% (2/6) when midterm sow challenged with virulent *Brucella* 544 strain while none was protected in control group. Lower *Brucella* recovery in spleen and liver and reduced histopathological burden were also noticed. Although abortion induced by *Brucella* challenge was not completely prevented, the vaccine candidate may perform better with optimization of vaccination such as inoculation dose optimization.



## 1. Introduction

Brucellosis is caused by the species of *Brucella* that affects a wide range of hosts including humans, livestock, feral and marine animals [1]. The disease is manifested in a diverse range of complications such as osteoarticular, gastrointestinal, pregnancy and neurological problems [2]. Currently, no ideal vaccines against human or animal brucellosis are available. Recently, we have reported the development of an anti-*Brucella* vaccine candidate, *Salmonella*-vectors delivering four highly conserved recombinant *Brucella* immunogens [3], viz. *Brucella* lumazine synthase (BLS), proline racemase subunit A (PrpA), lipoprotein outer membrane protein-19 (Omp19), and Cu-Zn superoxide dismutase (SOD). The recombinant strain was used in mixture with purified *Brucella* LPS, and the formulation was designated as RSrBL [3]. The single dose immunization with vaccine candidate was consistent with RB51 in mice challenge model [3].

Although the mice model is considered as a plausible animal model for chronic brucellosis, it fails to reproduce some clinical diseases caused by *Brucella* such as fever that often observed in human *Brucella* infection. Hence, here we report the impact of immunization using a pregnant guinea pig model due to the fact that guinea pigs exhibit the highest susceptibility to *Brucella* infections among other laboratory animals [4]. The *Brucella* pathogenesis in guinea pigs is consistent with human-*Brucella* pathology [5] reproducing the pulmonary, hepatic, spleen and reproductive organ-lesions and the hypersensitivity reactions observed in human by mimicking phases of the infection, including abortion [5,6]. Therefore, guinea pig model represents one of the best models for evaluating vaccine candidates [7–9].

In addition to immune response and safety implication of the anti-*Brucella* vaccine based on antigen-specific ELISA, general health, safety in pregnant sows, protection against challenge, post-mortem observations, prevention from abortion, and histopathological changes in guinea pig model are reported.

## 2. Materials and methods

### 2.1. Ethics and experimental animals

All animal experimental procedures were approved (CBNU2015-00085) by the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care and Korean Animal Protection Law, 2007; Article 13 (Experiments with animals). All Dunkin Hartley guinea pigs used were obtained from Koatech (Pyeongtaek, Gyeonggi-do, Korea) and maintained humanely. Water and antibiotic-free food were provided *ad libitum*. Animals were monitored twice daily for behavioral and physiological signs. At the completion of exper-

* Corresponding author.
*E-mail address:* johnhlee@jbnu.ac.kr (J.H. Lee).

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iments, animals were euthanized following standard procedures. All efforts were made to ensure humane handling of animals and to minimize animals suffering.

### 2.2. Mating, immunization and challenge

Specific pathogen-free male and female Dunkin Hartley guinea pigs were housed together at the ratio of 1:2 into three groups (A, B, and C). Each group consisted of 3 male and 6 female guinea pigs. Animals were allowed to mate and pregnancy was confirmed by ultrasonography and radiography. Group A served as a naïve control, group B was the non-immunized control received 400 µl phosphate buffered saline only via intraperitoneal route (IP). The group C was immunized with RSrBL vaccine by providing a total $4 \times 10^8$ CFU constituting each of JOL1878, JOL1879, JOL1880, and JOL1881 (Supplementary Table 1 [14,15]) in a volume of 400 µl PBS supplemented with 10 µg purified *Brucella* abortus lipopolysaccharide. Animals were closely monitored for possible vaccine-induced morbidity such as ruffled fur, weight loss, lack of movement and possible abortions in pregnant animals due to complications [7,10].

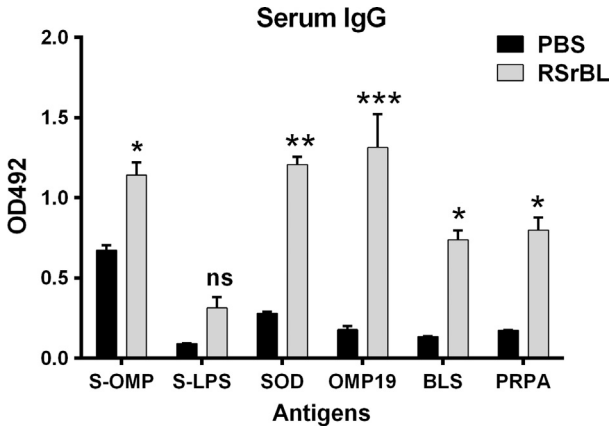


**Fig. 1.** Humoral immune response of guinea pigs against *Salmonella* and *Brucella* antigens. The serum IgG responses elicited by the immunization was investigated using purified antigens in indirect ELISA format. All immunized mice groups except control group showed a significant rise in IgG response against respective antigens ($P \leq 0.05$). Antibody reactivities are expressed as means $OD_{492}$ ± standard errors of the mean. The asterisks indicate significant differences $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ ns indicates non-significant. S-OMP; *Salmonella* outer membrane proteins, S-LPS; purified *Salmonella* lipopolysaccharide, RSrBL; a cocktail of rough *Salmonella* vectors delivering *Brucella* antigens, SOD; Cu-Zn superoxide dismutase, OMP19; lipoprotein outer membrane protein-19, BLS; *Brucella* lumazine synthase, PRPA; proline racemase subunit A.

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The stages of pregnancies differed ± 1 week as chemical synchronization were not performed. On completion of mid-term pregnancy (4–5 weeks gestation), group B and C (n = 6) were challenged intraperitoneally with $2 \times 10^7$ CFU of wild type *Brucella abortus* strain 544. Challenge-induced illness and abortion were recorded. At 6 weeks post challenge, animals were euthanized and subjected to postmortem examination. Spleen, liver, lung and uterus specimens were harvested for histopathological studies to evaluate the *Brucella* induced pathogenesis and the degree of protection derived by immunization. Bacterial organ recovery was performed as per protocol described previously with minor modifications [11]. Briefly, pieces of spleen and liver were weighed and homogenized in 2 ml PBS. The homogenized inoculum was further serially diluted and a volume of 200 µl was spread-plated on *Brucella* agar plates and incubated at 37 °C in a 5% $CO_2$ incubator. After 5 days of incubation, the colonies were confirmed for *Brucella* organism using specific PCR primers. The CFU was expressed as mean log10 bacterial CFU per gram.

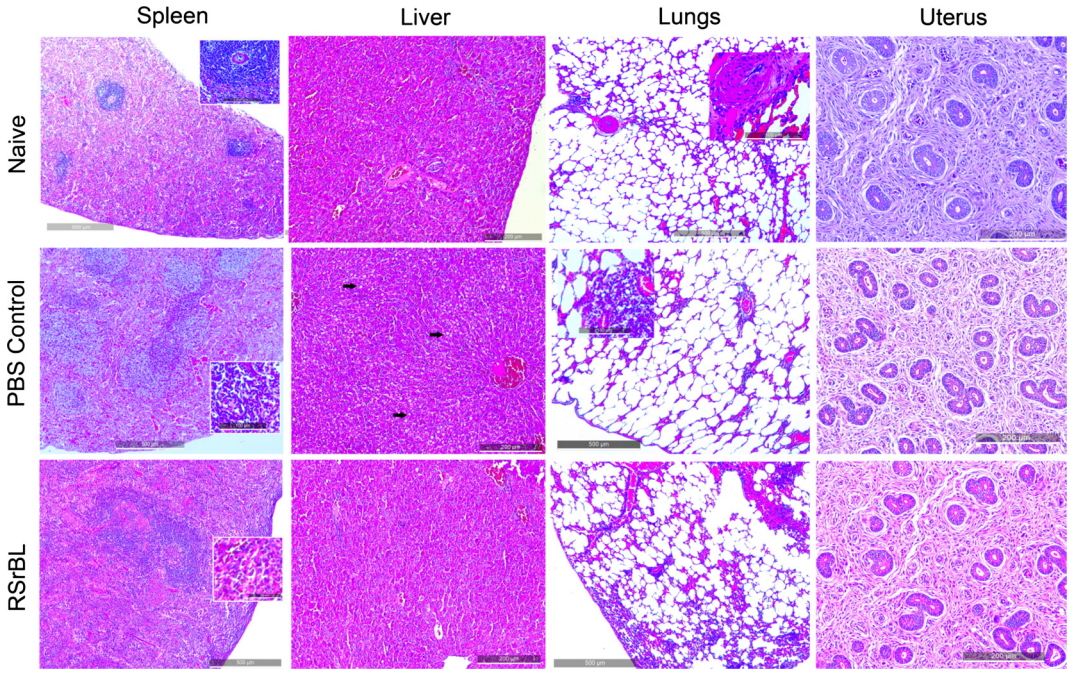


**Fig. 2.** Histopathological assessment post-*Brucella* challenge. Haematoxylin & eosin stained tissue sections were investigated. Spleen; Signs of fatty degeneration was observed in both challenged groups with prominent expansion in white pulp regions in PBS control groups. Liver; Fatty degeneration and focal lesions were observed in liver of PBS control group (black arrows) where moderation is observed in immunized group. Lungs; Both challenged groups show signs of inflammation compared to naïve group with much higher reduction in tissue density was observed in PBS control group. Uterus; Disturbed tissue integrity and reduced size of uterus glands were evident in both challenged groups with higher severity in PBS control group. Signs of microgranuloma formation and glands filled with exudates were also prominent in PBS control group.

### 2.3. Antigen-specific humoral immune response

The *Brucella* vaccine-induced IgG was measured by the specific IgG induction as determined using an indirect ELISA. Blood samples were collected from anterior vena cava at 4 weeks post-immunization and serum samples were harvested. Briefly, 400 ng/well of purified *Brucella* antigens (SOD, OMP19, BLS, PRPA), 500 ng/well of *Brucella* LPS, or 450 ng/well of *Salmonella* Typhimurium total outer membrane protein was used to coat ELISA wells (Microlon, Greiner). Primary serum was diluted at 1:50 with PBS and secondary horseradish peroxidase (HRP)-conjugated goat anti-guinea pig IgG (SouthernBiotech, USA) antibodies were used at 1:5000 dilutions. The colorimetric reaction was initiated by adding OPD (Sigma-Aldrich, USA) and after 6 min post-development signal was measured (TECAN, Austria) at 492 nm (620 nm reference. The IgG reactivity was expressed as the mean OD value ± standard error of the mean (SEM).

### 2.4. Statistical analysis

One-way analysis of variance (ANOVA) and Student's *t*-test (Welch corrected) was used to determine statistically significant differences, with a P value $\leq 0.05$. Tukey's test was applied for multiple comparisons and post hoc analysis. Analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

## 3. Results

### 3.1. Humoral immune response to immunization

The humoral response induced against the vaccine component was determined. A significant difference in IgG reactivity was observed (Fig. 1) in immunized Guinea pigs as compared to PBS control group ($P \leq 0.05$). A significantly high induction of IgG levels were observed against *Salmonella* delivered-Omp19 immunization followed by *Salmonella* delivered SOD. *Salmonella* outer membrane proteins also induced a significantly high IgG levels in immunized animals compared to the PBS control group. Importantly, no significant IgG induction was observed against *Salmonella* LPS.

### 3.2. Safety, histopathology, and protective efficacy of candidate vaccine in pregnant guinea pigs

The safety implication of RSrBL was studied using pregnant guinea pigs. No vaccine-induced apparent clinical signs were observed, which indicated the gross safety of the vaccine. Candidate vaccine did not show signs of pregnancy complications or abortion.

Upon completion of 4–5 weeks of pregnancy, the pregnant sows of group B and C were challenged with virulent *Brucella abortus* 544. The animals were closely monitored for any signs of complications. Body temperature was monitored using infra-red thermal imaging (Fluke VT04 Visual IR Thermometer, US), elevated temperatures ranging from 39.6 to 41.5 °C were observed in both PBS and vaccinated groups (Supplementary Fig. 1A). In group B, six out of six females have aborted pregnancy on 3rd, 7th, 10th (2 sows), 17th, and 20th days post challenge. In group C, four out of six females have abortion on 5th, 10th, 12th, and 15th days (Supplementary Fig. 1B). The animals were subjected to postmortem inspection of viscera (Supplementary Fig. 1C), where no significant hepatomegaly was observed in PBS versus vaccinated groups. Darker appearance of liver was observed in challenge animals. Comparing both challenged groups, the discoloration of viscera in non-immunized challenge group was more prominent than the immunized group.

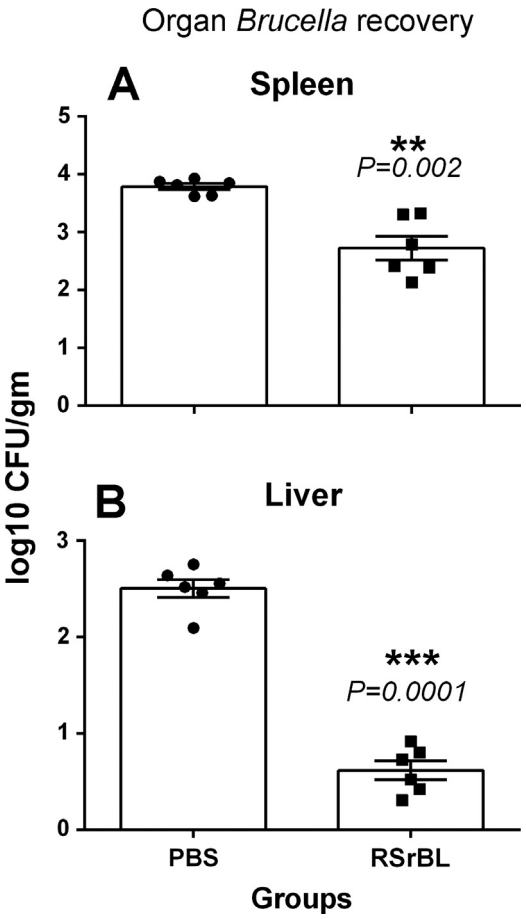


**Fig. 3.** Organ *Brucella* recovery. Female pregnant sows were challenged with virulent strain S544 during mid-term of pregnancy, the animals were euthanized 2 weeks post-abortion or delivery. The splenic bacterial load was determined, bacterial CFU was log10 transformed and average CFU was ascertained. (A) Splenic bacterial recovery post-*Brucella* challenge. (B) Hepatic bacterial recovery post-*Brucella* challenge.

Organs samples from post challenged animals were collected and subjected to histopathological analysis. Signs of fatty degeneration were evident in spleen, liver and uterus tissue sections in both challenged groups with higher number of incidents in PBS control group. An increased number of focal lesions liver tissues and expanded white pulp in spleen sections were also observed in PBS control group. In lung tissue sections, both challenged groups show signs of inflammation as compared to the naïve group (Fig. 2). Protective efficacy of the vaccine candidate was determined based on organ-bacterial recovery. The splenic *Brucella* number varied from 3.63 to 3.93 and 2.13 to 3.32 log10 CFU/g for the PBS and RSrBL groups (Fig. 3A) whereas for the liver, 2.09 to 2.75 and 0.3 to 0.91 log10 CFU/g for PBS and RSrBL groups were observed (Fig. 3B). The protection index (PI = mean PBS $\log_{10}$ CFU count – mean immunized log10 CFU count) for spleen and liver was 1.06 and 1.89, respectively.

## 4. Discussion

Brucellosis remains an important disease due to the absence of an ideal vaccine and zoonotic implications. Spontaneous abortion in pregnant animals is a hallmark of *Brucella* abortus infection. Development of a protective vaccine would be a huge leap in curbing *Brucella* infection. Conventionally, mice model is used to evaluate the protective efficacy of vaccine candidates against *Brucella* challenge. However, for pregnancy-related studies, guinea pig

served a better model [11]. This study investigated the induction of immune response and safety-related issues of a rough *Salmonella* delivery *Brucella* antigen candidate vaccine in guinea pig model. Successful induction of antibody against the delivered antigens was detected upon immunization. Specific serum IgG antibodies were detected against recombinant *Brucella* antigens vis. SOD, BLS, OMP19 and PRPA (Fig. 1).

The safety assessment of the candidate vaccine showed none of the immunized pregnant females exhibited abortion due to immunization with the candidate vaccine. The transient elevated temperatures observed12 hours post-immunization may relate to general body response to *Salmonella* antigens. This data shows a promising aspect compared to Strain 19 which causes abortion in a small fraction of vaccinated pregnant animals [12]. In the protective efficacy study, 2 pregnant sows out of total 6 were protected against abortions (approximately 33.3%) in the vaccinated group whereas none were protected in control sows. In this study, no clear association of lower organ bacterial count and prevention of abortion were observed. Abortion due to *Brucella* infection is a complex and poorly understood process which is mainly attributed to the inflammation of gravid placenta [13]. Ideally, the vaccination must prevent almost zero bacterial counts in order to avoid invasion of the reproductive organs especially, the highly *Brucella*-supportive erythritol rich placenta. In conclusion, this vaccine formulation may be safer in pregnant animals and may be an alternative to live attenuated *Brucella* vaccines such as *Brucella* abortus S19.

## 5. Conflict of interest statement

The authors declare no conflict of interest.

## 6. Contributions

J.L. & J.H.L. conceived and designed the experiments. J.L. performed the experiments. J.L. & J.H.L. analyzed the data and wrote the manuscript. A.S. also involved in paper writing and formatting. All authors discussed the results and commented on the final manuscript.

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