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Short communication

Immunization of guinea pigs with *Salmonella* delivered anti-*Brucella* formulation reduces organs bacterial load and mitigates histopathological consequences of *Brucella abortus* 544 challenge

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ABSTRACT

With an objective to generate safe and effective anti-*Brucella* vaccine, an attenuated live *Salmonella* Typhimurium vector delivering heterologous *Brucella* immunogenic proteins SOD, Omp19, BLS, and PrpA formulated with purified *Brucella abortus* lipopolysaccharide was evaluated on a guinea pig model. This model represents high susceptibility to *Brucella* infections and showed similarities in reproducing human pathologies. On safety perspectives, the vaccine formulation induced no observable alterations on general health and histology of the vaccinated guinea pigs. Upon virulent strain 544 challenge, a protective index of 1.52 was observed based on differential splenic counts. Post-challenge histopathology revealed that *Brucella* induced microgranulomas and fatty degenerations were prominent in the organs of non-immunized animals as compared to immunized animals. With these findings, it is suggestive that this live *Brucella*-free vaccine formulation is safe and protective on a sensitive guinea pig model and may be suitable for further human-related vaccine trials.

1. Introduction

Brucellosis is one of the neglected bacterial zoonotic diseases caused by the species of *Brucella* that affects a wide range of hosts that includes humans, livestock, feral and marine animals (Nymo et al., 2011; Pappas et al., 2006, 2005). The disease is manifested as sub-acute or chronic, and clinical symptoms such as osteoarticular, gastrointestinal, pregnancy and neurological complications are frequently observed (Corbel, 2006). At the present time no ideal vaccines against human or animal brucellosis are available. This study reports evaluation of an anti-*Brucella* vaccine embodying a cocktail of recombinant attenuated *Salmonella* vaccine (RASV) delivering four highly conserved *Brucella* immunogens, the combined strains was used in conjunction with purified *Brucella* LPS.

This study reports preliminary investigation pertaining to protective efficacy and safety implications of the anti-*Brucella* vaccine in a guinea pig model. Guinea pigs shows highest susceptibility to *Brucella* infections among laboratory animals and they also shows infection similarities in reproducing human pathology (García-Carrillo, 1990). The animal reproduce the pulmonary, hepatic, spleen and genital lesions and the hypersensitivity reactions observed in humans, and match the different phases of the infection caused by *Brucella* in natural hosts, including abortion (Braude, 1951; Braude and Spink, 1951; García-Carrillo, 1990). Hence, the guinea pig represents one of the best models for several immunological and vaccine studies (Grilló et al., 2012; OIE, 2009; Oliveira et al., 1996).

To these objectives, we reported here the evaluations of anti-*Brucella* vaccine based on post mortem observations, challenge bacterial-organ recovery, and histopathological changes in guinea pig model.

2. Materials & 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 guinea pigs used in the study were procured from Koatech-Lab Animals, Gyeongi-do, Pyeongtaeg-si, South Korea. Animals were housed and maintained humanely, and were provided water and antibiotic-free food *ad libitum*. Immediately after immunizations, hourly monitoring was conducted. However, the frequency was reduced to thrice daily beyond 7 days post immunization. Animal experiments involving live *Brucella* organism was performed under biosafety level-2

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Table 1
Bacterial strains, plasmids and primers used in the study.

Strain/plasmid /primer	Description	Reference
JOL401	*Salmonella* Typhimurium wild type, SPI-1 *invAE*$^+$ *hilA*$^+$ *avr*$^+$; SPI-2, amino acid permease$^+$; SPI-3, *mgtC*$^+$; SPI4, ABC transporter; SPI5, *pipB*$^+$; source antigen preparation	(Woong et al., 2012)
JOL912	JOL401 *Δlon, ΔcpxR, Δasd;* bacterial delivery vector	Lab stock
JOL1800	JOL912 *ΔrfaL;* O-antigen deficient rough strain, bacterial delivery vector	Lab stock
JOL1878	JOL1800 delivering heterologous *Brucella abortus* antigen, SOD	Lab stock
JOL1879	JOL1800 delivering heterologous *Brucella abortus* antigen, Omp19	Lab stock
JOL1880	JOL1800 delivering heterologous *Brucella abortus* antigen, BLS	Lab stock
JOL1881	JOL1800 delivering heterologous *Brucella abortus* antigen, PrpA	Lab stock
Strain 544	*Brucella abortus* strain 544, virulent challenge strain	Lab stock
Plasmids		
pJHL65	*asd* $^+$ plasmid, pBR ori, β-lactamase signal sequence-based periplasmic secretion plasmid, 6xHis, antigen expressing plasmid vector	(Lalsiamthara and Lee, 2017b)
Primers		
Brucella E11	5′-GACGAACGGAATTTTTCCAATCCC	(Bricker and Halling, 1995)
Brucella E12	5′-TGCCGATCACTTAAGGGCCTTCAT	

requirements. Biosafety level-3 protocol was followed for handling and manipulation of the *Brucella abortus* under the supervision and monitoring of Ministry of Health & Welfare, South Korea.

2.2. Bacterial strains, plasmids, media, and growth condition

The bacterial strains, plasmids, and primers used are described and listed in Table 1. All *Salmonella* strains were routinely grown in Luria-Bertani (LB) medium or brilliant green agar medium. *Brucella abortus* strain was grown in BD *Brucella* medium in 5% CO_2 atmosphere. For the construction of the candidate vaccines, the open reading frames of *Brucella* genes were amplified from the *Brucella* strain 544 and cloned into the delivery plasmid pJHL-65 via the incorporated *Eco*RI and *Hind*III restriction sites (Lalsiamthara and Lee, 2017a). The delivery plasmid contained the *asd* gene of ST, which has assisted plasmid-host complementation of the *asd* gene negative-auxotrophic *Salmonella* vector. The gene of interest was also fused with a *bla* secretion signal and a 6 × His tag sequence and the fused cassette was constitutively expressed under the Ptrc promoter. The plasmid was used to electro-transformed the ST delivery strain JOL1800. The expression of *Brucella* antigen was confirmed from the culture of JOL strains using a previously described protocol with minor modifications (Lalsiamthara et al., 2016). Expressed protein was detected via the incorporated histidine tags using primary mouse IgG1 anti-His-tag antibody (Anti-HIS_6, AprilBio, South Korea) and secondary anti-mouse IgG1 antibody-HRPO conjugate (Sigma-Aldrich, USA) antibodies, diluted at 1:2500 (1% BSA-PBS) and 1:5000 (PBS) respectively. Specific reactive bands were developed using DAB substrate conversion. Purified *Brucella* LPS was extracted from smooth *Brucella abortus* strain 544 using a phenol-based commercial LPS extraction kit (iNtRON biotechnology, South Korea). For vaccine formulation, purified LPS resuspended in 10 mM Tris-HCl buffer (pH 8.0) was directly added to pre-specified *Salmonella*-PBS suspension, at 10 μg/dose concentration.

2.3. Immunization, virulent-wild type challenge and bacterial recovery

Specific pathogens free, female Dunkin Hartley guinea pigs (n = 8) were grouped equally into two groups. At the 6th week of age, the first group of guinea pigs that served as a non-immunized control received 400 μL of phosphate buffered saline (PBS) only, via intraperitoneal (IP) route. The second group were immunized intraperitoneally with a total 1×10^8 colony forming units (CFU) each of JOL1878, JOL1879, JOL1880, and JOL1881 (Table 1), in a volume of 400 μL of PBS containing 10 μg purified *Brucella abortus* lipopolysaccharide. The animals were closely monitored for possible vaccine induced illness. At 30 days post immunization, the animals were challenge with 2×10^7 CFU of wild type *Brucella abortus* strain 544. At 30 days post challenge, the animals were euthanized and subjected to post mortem examination. Organs samples were harvested for histopathological studies as well as organ- bacterial recovery. Additionally, two un-challenged immunized animals were maintained for vaccine-related histopathological studies. Bacterial-organ recovery was performed as per protocol described previously with minor modifications (Lalsiamthara et al., 2015). Briefly, sections of spleens and livers 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 which were incubated at 37 °C in a CO_2 incubator. After 5 days of incubation, the colonies were confirmed for *Brucella* organism using specific primers (Table 1).

2.4. Histopathological assessment

Histopathological assessment was performed on tissues sections derived from organs of the guinea pigs (n = 2) following immunization as well as post-virulent challenge as described in section 2.3. Lungs, liver, spleen, and uterus were isolated. The samples were processed for tissue sectioning and hematoxylin-eosin staining. Additionally, organs of a naïve guinea and two post-vaccinated animals were also processed and examined for comparisons and were maintained as a normal control. The histopathological slides were studied and observed for any alterations induced due to vaccination or due to virulent challenge using stereo-microscope (Leica Microsystems, Germany).

2.5. Statistical analysis

Statistical analyses were used wherever applicable. Student's T-test with Welch's correction was used to analyze the differences among the group means. Differences were considered statistically significant at a *P*-value ≤ 0.05. *Brucella* cell counts of each group were mathematically transformed to conform to statistical fitting or normal model, using the formula: y = log(x/log x) as described previously (Bosseray and Plommet, 1990). Protective index (PI) was calculated as: PI = y value of test vaccine subtracted from y value of PBS.

3. Results and discussion

Small laboratory animals served as an important tool for investigating the pathophysiology of brucellosis, since experimentation in hosts such as ruminants, humans and primates are limited due to economical and ethical concerns (Grilló et al., 2012). Chicken embryo represents foremost experimental models (Buddingh and Womack, 1947). Rabbit mostly served as an animal for producing antibodies