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Immunization with recombinant GntR plasmid confers protection against *Brucella* challenge in BALB/c mice

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ABSTRACT

It is essential to improve animal vaccine for brucellosis since conventional vaccines are residual virulent and poor protective effect, limit their applications. To solve these problems, the recombinant DNA vaccines were appeared, which could improve protective immunity and were attenuated to animals. In current research, the recombinant DNA vaccine (pVGntR) based on transcriptional regulator GntR of *Brucella abortus* (*B. abortus*) was constructed. The results show that pVGntR is significantly more protective than the conventional RB51 vaccine. Immunization with pVGntR increases the production of immunoglobulin G (IgG) and elicits elevated numbers of gamma interferon (IFN-γ) and interleukin-4 (IL-4). These results suggest that pVGntR is a highly efficacious vaccine candidate that confers protection against wild-type *B. abortus* challenge.

1. Introduction

Brucellosis, caused by *Brucella* spp., brings great economic burdens for developing countries [1]. *Brucella* spp. cause disease in animals and humans [2]. In animals, *Brucella* causes abortion and infertility [3]. In humans, *Brucella* causes orchiditis and fever [4]. It is important to prevent and control *Brucellosis*. However, most of the conventional vaccines have several drawbacks, such as residual virulence, low protective efficiency and so on [5]. Therefore, new vaccines with low virulence and high protective efficiency are needed to overcome these limitations.

B. abortus RB51 is the conventional live vaccine, which used to control of brucellosis in cattle [6]. RB51 is a rough mutant strain that was derived from smooth virulent strain of *B. abortus* 2308 (S2308) [7]. RB51 does not induce anti-lipopolysaccharide (LPS) antibody responses and it lacks virtually all of the LPS O-chains [8]. RB51 is effective in conferring protection against virulent strains of S2308 [9]. However, RB51 has the limitation of residual virulence [10], and it may also cause abortion in pregnant animals [11]. Furthermore, RB51 is pathogenic for humans and cannot eradicate [12]. Therefore, one potential approach to solve these problems is to develop an effective and safe DNA vaccine with good immunogenicity and vaccine efficacy.

Transcriptional regulator GntR plays important roles in *Brucella* [13]. GntR involves in virulence of *Brucella*, and is an important virulence factor. This protein serves as virulence factor in many pathogens. Some bacteria like *Mycobacterium tuberculosis* (*M. tuberculosis*) deleted this protein, and significantly decreased its capability to survive in murine models [14]. Given this protein is important for bacterial virulence, we hypothesize that S2308 GntR open reading frames (ORFs) is a potential candidates for developing new vaccines against brucellosis. The aim of this study is to use S2308 GntR ORFs to construct DNA vaccines and to detect its effect on the immune response in BALB/c mice.

2. Materials and methods

2.1. Ethics statement

All animal experiments were performed in strict accordance with the Experimental Animal Regulation Ordinances defined by the China National Science and Technology Commission. The protocol was approved by the Institutional Committee of Post-Graduate Studies and Research at Shihezi University and

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Shangqiu Normal University, China. Animals are provided with humane care and healthful conditions and all efforts were made to minimize animal suffering.

2.2. Bacterial strains, media and plasmids

S2308 virulent strain and B. abortus RB51 vaccine strain were obtained from the Center of Chinese Disease Prevention and Control (Beijing, China) and cultured in tryptic soy agar (TSA) or tryptic soy broth (TSB) (Difco, MI, USA) at 37 °C in 5% CO_2. *Escherichia coli* (*E. coli*) strains DH5α and BL21 (Thermo Fisher Scientific Inc., MA, USA) were grown on Luria-Bertani (LB) medium. If necessary, the culture media were supplemented with appropriate antibiotics (100 μg/mL ampicillin or 50 μg/mL kanamycin). Plasmid pVAX1 vector and pET-28a vector were purchased from Promega (Invitrogen, WI, USA).

2.3. Mice

Female six-week-old BALB/c mice were obtained from the Experimental Animal Center of the Academy of Military Medical Science (Beijing, China). The animals were provided with humane care and healthful conditions during their stay in the facility. Animals were maintained in barrier housing with filtered inflow air in a restricted-access room in pathogen-limited conditions. They were acclimatized for a minimum of 1 week before experimentation, and water and commercial food were provided *ad libitum*. All individuals who handled the animals received instructions in experimental methods and in the care, maintenance, and handling of mice. All experimental procedures and animal care were performed in compliance with institutional animal care regulations. All efforts were made to minimize animal suffering.

2.4. Construction of recombinant plasmid

The recombinant GntR plasmid was constructed as previously described [15]. Briefly, The S2308 GntR ORF (BAB2_1138) was cloned into the pVAX1 vector. The GntR ORF sequence (735 bp) was amplified from S2308 genome by polymerase chain reaction (PCR) using the primer pair GntR-F and GntR-R (Table 1). The productions of PCR were ligated to pVAX1 vector via *BamH*I and *Xho*I sites using T4 DNA ligase (Takara, Japan), and to generate the recombinant plasmid pVGntR. The recombinant plasmid pVGntR was used for DNA immunization.

2.5. Immunization of mice

The immunization of mice was carried out as previously described [16]. Briefly, 6-week-old female BALB/c mice were subcutaneous injection (s.c.) with 100 μL phosphate buffer saline (PBS) containing 100 μg of the pVGntR constructs (DNA vaccine), divided into multi-point injections. The reference vaccine group was inoculated intraperitoneally (i.p.) with 200 μL PBS containing 1×10^8 CFU of RB51. The negative control group was injected s.c. with 100 μL PBS. All groups (except RB51 group) were immunized three times at 10-day intervals.

Table 1
Primers used in this study.

Primer name	Sequence (5′-3′)
GntR-F	*GGATCC*ATGAATGTTGAATCGGATCAT
GntR-R	*CTCGAGC*TACCTTGTCCGACGTGATAA

2.6. Production of recombinant protein

The recombinant GntR plasmid and protein were constructed and purified as described previously [17,18]. Briefly, the GntR ORF sequence (735 bp) was amplified from S2308 genome by PCR using the primer pair GntR-F and GntR-R (Table 1). Then the amplified DNA fragment was cloned in pET-28a vector and expressed in *E. coli* BL21 (DE3) and purified using Ni-NTA column according to manufacturer's instruction. The expression of the recombinant protein was analyzed by 12% SDS-PAGE.

2.7. Evaluation of antibody production

Serum samples were obtained from peripheral blood of immunized BALB/c mice (n = 5 per group) at 15, 30, 45 and 60 days post-immunization. The immune serum IgG, IgG1 and IgG2a levels were measured by indirect enzyme-linked immunosorbent assay (iELISA) using an ELISA Quantikine Mouse Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions, as described previously [15,19]. All assays were done in triplicate.

2.8. Cytokine detection

To determine the levels of IFN-γ and IL-4, cell suspensions from the spleens of immunized BALB/c mice were prepared, and the cytokine detection assays were performed as previously described [20]. Briefly, 45 days post-immunization, BALB/c mice (n = 5 per group) were euthanized and their spleens were removed aseptically. Single cell suspensions were obtained from the spleens. The cells were suspended in complete RPMI 1640 medium (Gibco Life Technologies, Rockville, MD, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco Life Technologies, Rockville, MD, USA). Splenocytes (4×10^6 cells/well) were cultured in 24-well plates containing 5 μg GntR recombinant protein/well, 0.5 μg ConA (positive control), or medium alone (negative control), respectively. The cells were incubated for 72 h at 37 °C with 5% CO_2. Subsequently, the clear culture supernatants were collected and stored at −20 °C until tested. IFN-γ and IL-4 levels in the supernatants were measured using an ELISA Quantikine Mouse kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. All assays were performed in triplicate.

2.9. Protection induced by recombinant plasmid in BALB/c mice

BALB/c mice were vaccinated as described above. At 60 days post-vaccination, mice (n = 10 per group) were challenged i.p. with 1×10^4 CFU of virulent strain S2308. The mice (n = 5/time point per group) were euthanized at weeks 2 and 4 post-challenge, and spleens were removed aseptically, collected homogenized in 1 mL PBS containing 0.1% (v/v) Triton X-100, ten-fold serially diluted, and then plated on TSA plates. Plates were incubated at 37 °C, and the number of CFU per spleen was counted after three days. The mean value for each spleen count was obtained after logarithmic conversion. Results are reported as units of protection represented by the difference between mean ± standard deviation (SD) of Log CFU/spleen of the PBS control group with respect to mean ± SD of Log CFU/spleen values of experimental groups.

2.10. Statistical analysis

Antibody response was expressed as the mean OD_{450} ± SD. Cytokine production was expressed as the mean cytokine concentration ± SD. The protective efficiency at different time points was expressed as the mean Log CFU ± SD. Data were