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A safe non-toxic *Brucella abortus* ghosts induce immune responses and confer protection in BALB/c mice

Shuli Wang[a,1], Zhiqiang Li[a,1], Jinliang Zhang[a], Li Xi[a], Yanyan Cui[a], Wehihua Zhang[b], Junbo Zhang[c], Hui Zhang[d,*]

[a] College of Biology and Food, Shangqiu Normal University, Shangqiu 476000, Henan Province, People's Republic of China
[b] First People's Hospital of Shangqiu, Shangqiu 476000, Henan Province, People's Republic of China
[c] College of Biology, Agriculture and Forestry, Tongren University, Tongren 554300, Guizhou Province, People's Republic of China
[d] College of Animal Science and Technology, Shihezi University, Shihezi 832003, Xinjiang Province, People's Republic of China

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ABSTRACT

Brucellosis, which is caused by *Brucella* spp., is an important zoonotic infectious disease that can cause great hazard to public health and safety. However, the current vaccines have several drawbacks, including residual virulence for animals and humans. Bacterial ghost is the empty envelopes of bacteria, which emerge as a proper vaccine candidate. With the purpose of generating *B. abortus* ghosts and investigating the immunogenicity of bacterial ghosts as vaccine candidate, we used homologous recombination and bacterial ghost technologies to construct 2308Δ*gntR* ghost strain. Mice were injected with 2308Δ*gntR* ghost and the safety and immunogenicity of ghost were further evaluated. The mice inoculated with ghost showed no splenomegaly. The 2308Δ*gntR* ghost induced high protective immunity in BALB/c mice against challenge with S2308, and elicited an anti-*Brucella*-specific immunoglobulin G (IgG) response and induced the secretion of interferon gamma (IFN-γ) and interleukin-4 (IL-4). Additionally, 2308Δ*gntR* ghost demonstrated strong spleen $CD4^+$ and $CD8^+$ T cell responses. These results suggest that 2308Δ*gntR* ghost is a potential vaccine candidate and may represent a promising new approach for vaccination against *Brucella* infection.

1. Introduction

Brucella spp. are gram-negative and facultative intracellular bacterium that cause brucellosis in humans and animals (Boschiroli et al., 2001; Tadesse, 2016), resulting in heavy economic losses and human suffering (Bercovich, 2000). Brucellosis is caused by several species of the genus *Brucella* including *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. ovis*, *B. neotomae*, *B. pinnipedialis*, *B. ceti*, *B.microti* and *B. inopinata* (Ficht, 2010; Foster et al., 2007; Jimenez de Bagues et al., 2014; Pappas et al., 2005; Ross et al., 1996; Scholz et al., 2008, 2010). *B. melitensis*, *B. abortus*, *B. suis* and *B. canis* are the most pathogenic strains with the strongest pathogenicity (Doganay and Doganay, 2013; Franco et al., 2007). At present, vaccination is the most effective approach for preventing and controlling brucellosis (Goodwin and Pascual, 2016). Numerous *Brucella* vaccines have been developed and tested over the past several decades, but there are no safe, effective and alternatives that could differentiate the naturally infected from that of the actively immunized. Therefore, new avirulent and vaccines with high protective efficacy are needed.

The current live vaccine *B. abortus* RB51 is widely used in prevention of bovine brucellosis (Truong et al., 2015). RB51 is a rough mutant strain of which was derived from smooth S2308. RB51 does not induce anti-lipopolysaccharide antibody responses, enabling conventional serologic tests for diagnosing brucellosis in cattle (Stevens and Olsen, 1996). RB51 is safe in bison, because it did not induce abortions in pregnant bison (Elzer et al., 1998). However, RB51 is infectious to humans and can cause human brucellosis (Ashford et al., 2004). S19 is another live vaccine of which had been used to prevent the infection of *B. abortus*. Some studies have shown that S19 provided protection against cattle abortion (Moriyón et al., 2004). S19 is also effective at protecting animals against *B. abortus* infections. However, it cannot be distinguished between cattle naturally infected and vaccinated. In addition, S19 can cause abortion in the pregnant animals (Cardeña et al., 2009). Therefore, one potential approach to solve these problems is to develop a safe vaccine strain with good immunogenicity and protective efficacy.

* Corresponding author.
E-mail address: allanzhh@sohu.com (H. Zhang).
[1] Zhiqiang Li and Shuli Wang contributed equally to this paper.

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Bacterial ghost (BG) is an empty cell without contents of the cell, retaining only the outer membrane structure with the same as live cell and has high immunogenicity and the role of adjuvant. In the control of infectious disease research, the BG attracted the attention of researchers. BG is usually constructed by controlling the expression of PhiX174 lysis E gene which can integrate membranes of Gram-negative bacteria and form transmembrane lanes, resulting in the formation of channel on the cell surface and draining the content of bacteria (Jalava et al., 2002; Witte et al., 1992). BG as vaccine candidate is a new technology, which can develop safe and effective vaccines against Gram-negative bacteria, such as *E. coli* O157 (Cai et al., 2010; Mayr et al., 2012), *Edwardsiella tarda* (Lee et al., 2008; Wang and Lu, 2009), *Vibrio cholerae* (Kwon et al., 2009), *Aeromonas hydrophila* (Tu et al., 2010), *Salmonella* (Vinod et al., 2017) and *Pseudomonas aeruginosa* (Eko et al., 2003). As to *Brucella*, it was reported that *B. suis* S2 ghost induced better cellular immunity and humoral immunity than inactivated bacteria in mice (Liu et al., 2015). These results indicated that BG has a wide foreground in the prevention and control of brucellosis. However, these *Brucella* ghost stains were constructed by the transformation of suicide plasmid carrying lysis fragment. They have some drawbacks such as transfer of antibiotic resistance and loss of recombinant plasmid. Therefore, the 2308Δ*gntR* ghost strain constructed in this study has the characteristics of safety and genetic stability.

GntR family is an important virulence regulator. GntR regulators are known to control many fundamental cellular processes (Casali et al., 2006; Haine et al., 2005). It has been reported that *B. melitensis* 16 MΔ*gntR* mutant was attenuated in mice (Haine et al., 2005). Therefore, GntR plays an important role in pathogenetic process of *Brucella*.

Since the BG is inactivated, the inactivated S2308 may be effective. But the difficulties of differentiating of infection from vaccine immunization, which limit their applications. Therefore, the development of a vaccine that could differentiate infection from immunization. The *gntR* can be used as a molecular tag in distinguishing natural infection and BGs immunization.

In the present study, we combined the advantage of homologous recombination and bacterial ghost technologies, integrated the lysis gene compartment into the genome of *B. abortus*. After SacB gene counter-selection, the 2308Δ*gntR* ghost strain was selected. Its growth kinetic and lysis kinetic were subsequently measured, and optimal lysis condition were determined. The 2308Δ*gntR* ghost strain showed satisfying safety and immunogenicity. Furthermore, due to the deletion of *gntR* gene, the 2308Δ*gntR* ghost strain could differentiate natural infection from vaccination. In general, we developed a nontoxic, efficient and differentiated vaccine candidate.

2. Materials and methods

2.1. Ethics statement

The study was performed in strict accordance with the experimental practices and standards approved by the Animal Welfare and Research Ethics Committee at Shihezi University (Permit No. SHZU-MO-0139) and Shangqiu Normal University (Permit No. SQNU-MO-0163). All animal handling procedures were approved by the Committee on the Ethics of Animal Experiments of Shihezi University (Reference nr. 07/A1501/83 and Approval ID: 2017072582-1) and Shangqiu Normal University (Reference nr. 07/S1205/66 and Approval ID: 2017052832-1). All efforts were made to minimise suffering during animal handling and experimentation. The challenged mice were kept in animal biosafety level 3 (ABSL-3) facility.

2.2. Bacterial strains, plasmids and media

S2308 and vaccine strain RB51 were obtained from the Center of Chinese Disease Prevention and Control (Beijing, China). S2308 and RB51 strains were cultured in tryptic soy agar (TSA) or tryptic soy broth (TSB) (Difco, MI, USA) at 37 ˚C in 5% CO_2. S2308 ghost strain was grown at 28 ˚C. *E. coli* strain DH5α was grown on Luria-Bertani (LB) medium (Difco, MI, USA) at 37 ˚C. Plasmid pGEM-7Zf$^+$ and pBV-220 were purchased from Promega (Madison, 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. 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.

2.4. Construction of suicide plasmid and temperature-sensitive shuttle lysis plasmid

The S2308 ghost strain was constructed performed according to previously published methods (Qian et al., 2017) with some modifications. Briefly, the sequence upstream of *gntR* was amplified from S2308 genome using the primer pair GntR-N-F and GntR-N-R (Table 1). The sequence downstream of *gntR* was amplified from S2308 genome using the primer pair GntR-C-F and GntR-C-R (Table 1). The two arms of S2308 *gntR* were ligated to pGEM-7Zf$^+$ via *Sph* I, *Xho* I and *BamH* I sites, and to generate the suicide plasmid pGEM-7Zf$^+$-*gntR*. SacB-F and SacB-R primers (Table 1) were designed for amplification of the *B. subtilis* SacB DNA fragment, which is a selectable marker gene. The *BamH* I-*BamH* I insert from a plasmid containing the PCR amplified DNA was subcloned into plasmid pGEM-7Zf$^+$-*gntR* to generate plasmid pGEM-7Zf$^+$-*gntR*-SacB.

Table 1
Primers used in this work.

Primer name	Sequence (restriction enzyme) (5′-3′)	Locus (gene)
GntR-N-F	GCATGCATCAACTTCGTCAGCCCG (*Sph*I)	*gntR* upstream
GntR-N-R	CTCGAGTATGACATCATGATGTCATTACGT (*Xho*I)	
GntR-C-F	CTCGAGAAGCCATGACCGGGGTGC (*Xho*I)	*gntR* downstream
GntR-C-R	GGATCCCTCAGATTTTGAACCTGAAGCCGA (*BamH*I)	
SacB-F	GGATCCGGGCTGGAAGAAGCAGAC (*BamH*I)	*SacB*
SacB-R	GGATCCGCTTATTTGTTAACTGTTAATTGT (*BamH*I)	
E-F	GAATTCATGGTACGCTGGACTTTGTGGGATAC (*EcoR*I)	lysis gene E
E-R	CTCGAGTGTCCGACGTGATAA (*Xho*I)	
λ E-F	CTCGAGCCATTCTTCATAATTCAATCC (*Xho*I)	temperature regulatory system
λ E-R	CTCGAGAAATAAACAAAGAGTTTGTAGAAAC (*Xho*I)	
GntR-F	ATGAATGTTGAATCGGATCAT	*gntR*
GntR-R	CTACCTTGTCCGACGTGATAA	

The lysis gene E was amplified from the genome of phage phiX174 using the primer pair E-F and E-R (Table 1). The amplified DNA fragments were ligated to the pBV-220 vector containing temperature sensitive fragment λ*PL/PR-cI857* via *EcoR* I and *Xho* I sites to generate pBV-E, as previously described (Liu et al., 2012). The temperature regulatory system, λ*PL/PR-cI857* fragment (λE), was amplified from the pBV-E plasmid by PCR using the primer pair λ E-F and λ E-R (Table 1). The amplified DNA product was cloned into pGEM-7Zf$^+$-*gntR*-SacB through *Xho*I restriction site to construct suicide plasmid pGEM-7Zf$^+$-*gntR*-SacB-λE.

2.5. Selection of 2308ΔgntR ghost strain

The suicide plasmid pGEM-7Zf$^+$-*gntR*-SacB-λE was introduced into electro-competent cells of S2308 via electroporation as previously described (Lai et al., 1990). Potential 2308Δ*gntR* ghost strain was selected in the presence of 100 μg/mL ampicillin for the first screening and 5 % (w/v) sucrose for the second screening at 28 °C. The deletion of *gntR* gene in S2308 was further verified by PCR amplification and DNA sequencing analysis as described previously (Qian et al., 2017), and the ghost strain is referred to as 2308Δ*gntR* ghost. GntR-F and GntR-R primers were designed for the detection of the ghost strain.

2.6. Growth curve and production of 2308ΔgntR ghost

The growth curve analysis of 2308Δ*gntR* ghost strain was performed according to previously published methods (Qian et al., 2017). The growth curve and inactivation were measure by OD_{600} value and colony forming units (CFU). Briefly, S2308 and 2308Δ*gntR* ghost were incubated into 100 mL TSB medium and cultured in a rotary shaker (250 rpm) at 28 °C. When the culture reached OD_{600} of 0.6, the culture temperature was converted to 42 °C to induce the expression of lysis protein E. Growth curves (OD_{600} value) under shifted temperature of both S2308 and 2308Δ*gntR* ghost were measured. The lysis was monitored by examining the OD_{600} value and viable cell counts through CFU every 8 h. The TSB medium alone served as a control. The resulting cell ghosts were sedimented by centrifugation at 12,000 × *g* for 10 min at 4 °C, then washed three times with sterile phosphate-buffered saline (PBS, pH 7.2). Pellets were resuspended in saline at the original culture volume and stored at −80 °C prior to use. S2308 and 2308Δ*gntR* ghost were collected at 56 h under 28 °C and 42 °C, respectively. Then, S2308 and 2308Δ*gntR* ghost were fixed for ultracryotomy and were subsequently observed by transmission electron microscopy (H-600, Hitachi, Japan) operating at 80 kV.

2.7. Safety evaluation of 2308ΔgntR ghost in mice

Safety evaluation of ghost was performed as previously described (Liu et al., 2015). Briefly, six-week-old female BALB/c mice were randomly divided into four groups (n = 25 per group) and inoculated intraperitoneally (i.p.) with 200 μL PBS containing 1 × 10^6 CFU of S2308, RB51, S19, formalin-killed S2308 (FK2308) and 2308Δ*gntR* ghost or PBS. Infected mice were held in microisolator cages in biosafety level 3 facilities. To evaluate the safety of these antigens, mice were evaluated by enumerating the bacteria in the spleens at different time points post-infection. At 2, 4, 6, 8 and 10 weeks post-inoculation, mice (n = 5/time point per group) were euthanized and spleens were removed aseptically. The spleens were collected, weighed and homogenized in 1 mL PBS containing 0.1 % (v/v) Triton X-100, ten-fold serially diluted, and then homogenized suspension were plated on TSA plates. Plates were incubated at 37 °C, and the number of CFU was counted after three days. All assays were performed three times with similar results.

2.8. Immunization of mice

Six-week-old female BALB/c mice were randomly divided into four groups (n = 10 per group) and i.p. with 200 μL PBS containing 1 × 10^6 CFU of RB51, S19, FK2308, 2308Δ*gntR* ghost or PBS. The mice of unvaccinated control group were given only PBS in all the experiments performed.

2.9. Protection induced by 2308ΔgntR ghost in BALB/c mice

At 15 weeks post-vaccination, mice (n = 10 per group) were challenged i.p. with 1 × 10^6 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. The numbers of CFU recovered from spleens were determined as described above. The mean value for each spleen count was obtained after logarithmic conversion. Log units of protection were obtained by subtracting the mean log CFU for the vaccinated group from the mean log CFU for the unvaccinated control group, as previously described (Adone et al., 2005; Grilló et al., 2006). The experiments were repeated twice with similar results.

2.10. Evaluation of antibody production

Serum samples were obtained from peripheral blood of immunized BALB/c mice (n = 5 per group) at 2, 4, 6, 8 and 10 weeks post-immunization and sera were stored at −80 °C until tested (Zhang et al., 2011). The IgG antibody in serum samples obtained from immunized mice were determined by ELISA, as described previously (Goel and Bhatnagar, 2012; Zhang et al., 2013). The IgG levels were measured using an ELISA Quantikine Mouse Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, heat-killed and sonicated S2308 whole-cell antigen was used to coat 96-well plates at a concentration of 25 μg protein/well. Following overnight incubation at 4 °C, plates were washed three times with 100 mL PBST buffer (PBS containing 0.05 % Tween-20) and blocked with 200 mL blocking buffer (10 % heat-inactivated fetal bovine serum in PBS, pH 7.4) for 1 h at 37 °C. Mice serum samples diluted 1:300 in the dilution buffer were added to wells in triplicate and incubated for 1 h at 37 °C. Then plates were washed three times with 100 mL PBST buffer, 100 μL of rabbit anti-mouse IgG-horseradish peroxidase conjugate (SBA, Birmingham, Al, USA) at a dilution of 1:3000 was added, and then the plates were incubated 37 °C for 1 h. After washed three times with 100 mL PBST buffer, 100 μL per well of O-phenylenediamine dihydrochloride peroxidase substrate (Sigma, Germany) solution was added and incubated at 37 °C in darkness for 15 min. The reaction was stopped by the addition of 50 μL of H_2SO_4 and the absorbance measured at 450 nm. All assays were performed in triplicate and the IgG levels in the serum samples were calculated using a linear regression equation obtained from the absorbance values of standards, according to the manufacturer's protocol.

2.11. Analysis of T lymphocyte subpopulations by flow cytometry

Single-cell lymphocyte suspensions were prepared aseptically from mice spleens as previously described (Wang and Lu, 2009; Wang et al., 2011) with some modifications. Briefly, 10 weeks 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 by homogenization. The cells were suspended in complete RPMI 1640 medium (Gibco Life Technologies, Rockville, MD, USA) supplemented with 2 mM L-glutamine (Solarbio Science and Technology, Beijing, China) and 10 % (v/v) heat-inactivated FBS. 100 μL of the cell suspension was incubated with 1 μL anti-mouse FITC/CD4$^+$ and PE/CD8$^+$ clonal antibodies (eBioscience, San Diego, CA, USA) for 15 min at

room temperature. The cells were washed twice with PBS, and then resuspended in 500 μL PBS. The T lymphocyte cells were analyzed by flow cytometry.

2.12. Cytokine detection

Cytokine detection assays were performed as previously described (Wang et al., 2011). Splenocytes were obtained according to the above methods. Splenocytes (5×10^5 cells/well) were cultured in 96-well plates; the cultures were stimulated by adding 25 μg RB51, S19 or heat-killed S2308 lysate/well, 0.5 μg ConA (positive control), or medium alone (negative control), respectively. The cells were incubated at 37 °C with 5% CO_2 for 72 h. 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).

2.13. Statistical analysis

Bacterial survival in mice was expressed as the mean Log CFU ± the standard deviation (SD). Statistical analysis was performed with Student's unpaired *t*-test. T lymphocyte subpopulations were expressed as the mean percentages for analysis ± SD. The differences between groups were analyzed by analysis of variance (ANOVA) followed by Tukey's honestly significant difference post-test, by comparing all the groups to one another. 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. Results expressed as percentages were analyzed by the Fisher test. The differences between groups were analyzed by analysis of variance using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). P values of < 0.05 were considered statistically significant.

3. Results

3.1. Construction of 2308ΔgntR ghost

The 2308Δ*gntR* ghost was successfully obtained. A 735 bp DNA fragment was amplified from S2308 with primers GntR-F and GntR-R, but nothing was amplified from 2308Δ*gntR* ghost with the same primers, indicating that 2308Δ*gntR* ghost was correctly constructed (Fig. 1A and B). The cultures were collected at 56 h and observed through transmission electron microscope. S2308 bacteria were solid black ellipsoids or spheres under 28 °C or 42 °C conditions (Fig. 1C and D). The ghosts were solid black ellipsoids or spheres under 28 °C condition (Fig. 1E), but the ghosts were hollowed envelopes under 42 °C conditions (Fig. 1F).

3.2. Growth curve of 2308ΔgntR ghost strain

The effects of *gntR* deletion on S2308 growth were examined. 2308Δ*gntR* ghost and S2308 were cultured at the same condition. At 28 °C, the 2308Δ*gntR* ghost demonstrated a similar growth curve to S2308 (Fig. 2A and 2B), which showed that the deletion of *gntR* did not affect the growth of *B. abortus*. To detect the effect of lysis E gene expression, the culture temperature was shifted from 28 °C to 42 °C. At 42 °C, the S2308 kept growing, but the cell viability of 2308Δ*gntR* ghost was significantly reduced at 56 h (Fig. 2C and 2D). These results indicated that 2308Δ*gntR* ghost strain was successfully constructed and the expression of lysis E gene was correct.

3.3. Safety analysis of mice immunized with 2308ΔgntR ghost

To determine the safety of 2308Δ*gntR* ghost *in vivo*, BALB/c mice were i.p. infected with 1×10^6 CFU of S2308, RB51, S19, 2308Δ*gntR* ghost and FK2308 or PBS (negative control). As to spleen bacteria count, S2308, RB51 and S19 groups showed significantly increase compared to 2308Δ*gntR* ghost, consistent with spleen weight, showing presence of live bacteria in S2308, RB51 and S19 groups (Fig. 3A). Mice inoculated with S2308, RB51 and S19 presented with spleen weight significantly greater than following inoculation with 2308Δ*gntR* ghost, FK2308 or PBS (Fig. 3B). Weight gain of mice given 2308Δ*gntR* ghost, FK2308 or PBS was normal. These results showed the safety of 2308Δ*gntR* ghost as it exhibits no live bacteria residence in spleen.

3.4. Vaccination with 2308ΔgntR ghost confers protection in mice

To determine the protection efficiency of 2308Δ*gntR* ghost, mice were vaccinated i.p. with 1×10^6 CFU of RB51, S19, FK2308, 2308Δ*gntR* ghost or PBS. At 15 weeks post-vaccination, mice were challenged with 1×10^6 CFU of virulent strain S2308. The mice immunized with 2308Δ*gntR* ghost had significantly fewer splenic *Brucella* than unimmunized animals at 2 (3.18 log units) and 4 (4.04 log units) weeks post-challenge ($P < 0.01$) (Table 2). As expected, the mice immunized with strain RB51, S19 or FK2308 had significantly fewer splenic *Brucella* than unimmunized animals (Table 2). We also observed similar protection in BALB/c mice immunized with ghost as in mice immunized with vaccine RB51 and S19 strains (Table 2). In general, 2308Δ*gntR* ghost performs better than FK2308, showing a similar protection ability to RB51 and S19. These results indicate that 2308Δ*gntR* ghost can induce immune protection efficacy against challenge with wild-type virulence S2308 strain.

3.5. 2308ΔgntR ghost induced humoral immune response

The presence of *Brucella*-specific IgG antibodies were detected by iELISA. Serum samples from mice inoculated with RB51, S19, FK2308, 2308Δ*gntR* ghost or PBS were collected at 2, 4, 6, 8 and 10 weeks post-immunization. We found that mice immunized with the 2308Δ*gntR* ghost produced significant levels of IgG antibodies (Fig. 4). Furthermore, the IgG levels peaked at 6 weeks post-vaccination (Fig. 4). But the levels of IgG antibodies in the serum samples of mice immunized with PBS were not detected (Fig. 4). In addition, we found that although the IgG levels of 2308Δ*gntR* ghost group dropped from 6 week, it still maintained at a relatively high level compared to RB51, S19 and FK2308 groups (Fig. 4). These results indicated that 2308Δ*gntR* ghost is an effective vaccine providing competent IgG response and long-lasting specific IgG levels.

3.6. Measurement of cytokine secretion in vitro

To determine 2308Δ*gntR* ghost induced cell-mediated immune response, the Th1-type cytokine IFN-γ and the Th2-type cytokine IL-4 levels in the splenocytes of immunized mice. The splenocytes of immunized mice were stimulated with heat-killed S2308, ConA (positive control) or complete RPMI 1640 medium (negative control). When stimulated with heat-killed S2308, spleen cells from 2308Δ*gntR* ghost, RB51, S19 and FK2308 produced significantly higher amounts of IFN-γ and IL-4 relative to that of PBS-dosed mice ($P < 0.01$) (Fig. 5). Furthermore, ConA stimulation induced the production of IFN-γ and IL-4 in splenocytes from all groups, whereas no cytokine production was induced by RPMI 1640 medium, and no cytokine production was induced by PBS stimulation in any of the groups (Fig. 5). However, there was no statistically significant difference in production of IFN-γ and IL-4 between 2308Δ*gntR* ghost and RB51, S19 or FK2308. These results indicated that immunization with 2308Δ*gntR* ghost could induce Th1 and Th2 cellular immune responses.

3.7. Measurement of splenocyte subsets in vitro

Compared with mice i.p. with PBS, levels of $CD4^+$ and $CD8^+$ cells

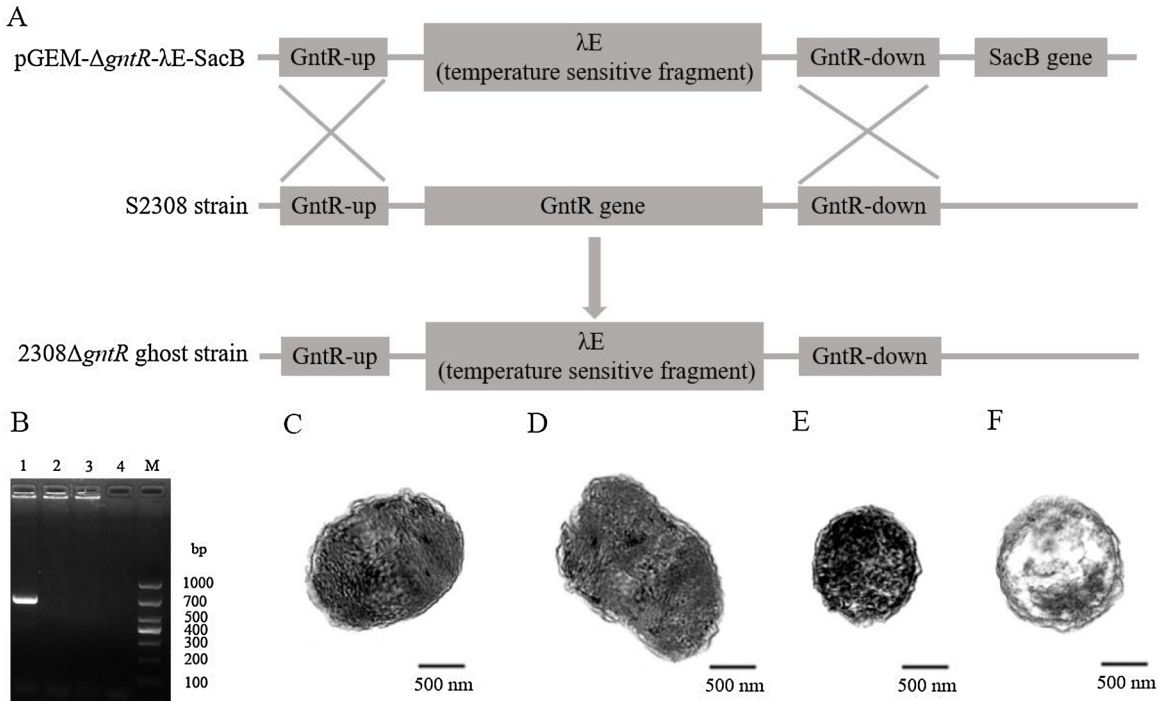

Fig. 1. Construction and observation of the 2308Δ*gntR* ghost strain. (A) The *gntR* gene on S2308 was replaced by temperature sensitive fragment, and *SacB* gene was depleted through selection. (B) Amplification of S2308 strain and 2308Δ*gntR* ghost strain *gntR* genomic regions with primers GntR-F and GntR-R. The correct recombinant ghost strain cannot amplify anything. Lanes: 1, the parental strain S2308; 2 and 3, 2308Δ*gntR* ghost strain; 4, negative control; M, DNA marker. (C) and (D) Morphologic analysis results of S2308 at 28 °C and 42 °C by transmission electron microscopy. S2308 with a solid black ellipsoids or spheres at 28 °C and 42 °C. (E) and (F) Morphologic analysis results of 2308Δ*gntR* ghost at 28 °C and 42 °C by transmission electron microscopy. The ghosts were solid black ellipsoids or spheres at 28 °C, while they were hollowed envelopes at 42 °C. The ghosts showing similar outer structure to S2308 parent strain, yet no content inside.

were significantly increased in the spleens of mice i.p. with 2308Δ*gntR* ghost, RB51, S19 or FK2308 ($P < 0.01$) (Table 3). But there was no significant difference between 2308Δ*gntR* ghost and RB51, S19 or FK2308 groups ($P > 0.05$) (Table 3). The $CD4^+/CD8^+$ ratios of mice vaccinated with 2308Δ*gntR* ghost or RB51 were significantly higher than with PBS ($P < 0.01$) (Table 3).

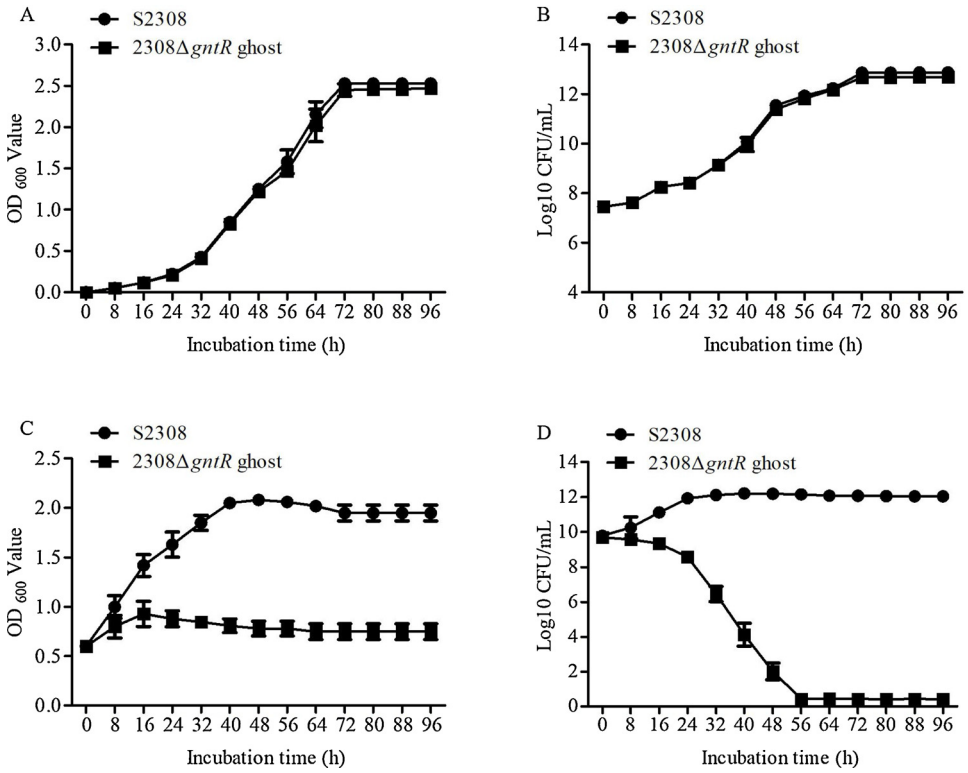

Fig. 2. Growth and lysis kinetics of 2308Δ*gntR* ghost and S2308 strains. Growth and lysis were assessed by the measurement of the OD_{600} and the number of CFU. (A) and (B) Growth and lysis kinetics under 28 °C to 96 h. (C) Lysis kinetics under shifted temperature from the point OD_{600} value reached 0.6. (D) The cell viability of 2308Δ*gntR* ghost was significantly reduced under 42 °C for 56 h.

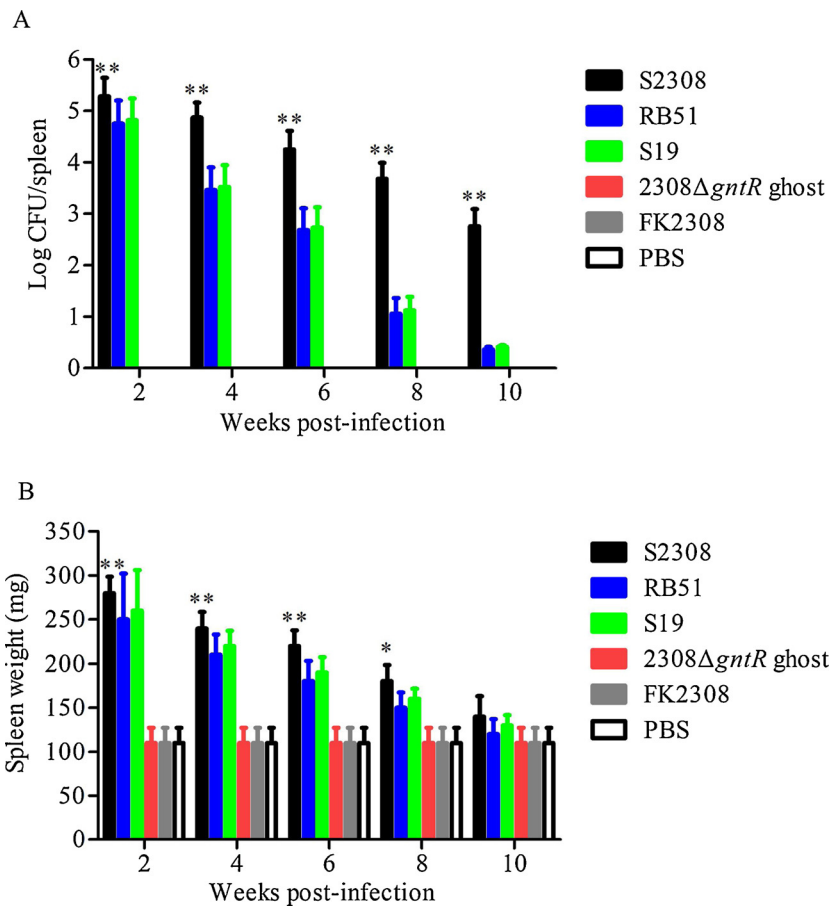

Fig. 3. Clearance of different *Brucella* strains after infection. BALB/c mice (n = 25 per group) were infected with 200 μL PBS containing 1 × 10^6 CFU/mouse of S2308, RB51, S19, FK2308 and 2308Δ*gntR* ghost or 200 μL PBS as a negative control. At 2, 4, 6, 8 and 10 weeks post-infection, spleens were harvested and individual spleens were assessed for colonization (A) and weight (B). Values are the means from individual mice ± SD (n = 5 per time point). Differences in splenic weight and colonization were determined *via* the unpaired *t*-test between the 2308Δ*gntR* ghost and S2308 (* $P < 0.05$, ** $P < 0.01$).

Table 2
Protection conferred by 2308Δ*gntR* ghost, RB51, S19 or FK2308 against S2308 in BALB/c mice.

Vaccine or control	Protection criteria			
	Log CFU/spleen (mean ± SD)[a]		Units of protection[b]	
	2	4	2	4
2308Δ*gntR* ghost	4.25 ± 0.37[c]	2.18 ± 0.47[c]	3.18	4.04
RB51	4.83 ± 0.69[c]	2.55 ± 0.67[c]	2.60	4.07
S19	4.91 ± 0.67[c]	2.62 ± 0.69[c]	2.52	4.00
FK2308	5.12 ± 0.96[c]	3.11 ± 0.94[c]	2.31	3.51
PBS	7.43 ± 0.52	6.62 ± 0.51	—	—

[a] Statistical significance.
[b] Log units of protection = average of log CFU in spleens of control unvaccinated mice minus the average of log CFU in spleens of vaccinated mice.
[c] $P < 0.01$ with respect to unvaccinated controls.

4. Discussion

Brucellosis is a widespread zoonosis, which brings great economic burdens for developing countries. Currently, live attenuated vaccines are the most frequently means for prevention and control the spread of brucellosis. However, the presently licensed vaccines have many drawbacks, such as residual virulence and induction of splenomegaly (Ashford et al., 2004; Strausbaugh and Berkelman, 2003). Furthermore, the difficulties of differentiating of infection from vaccine immunization, which is essential for eradication programs, limit their applications. Therefore, the development of better vaccine is imminent.

The ideal *Brucella* vaccine must have no virulent for the host and induce higher protection (Ugalde et al., 2003). As to protection ability, our previous study has confirmed 2308Δ*gntR* mutant could provide high protection levels against S2308 challenge in mice (Li et al., 2017). In this study, we detected the protection of the 2308Δ*gntR* ghost in BALB/c. We observed similar protection in BALB/c mice immunized with ghost as in mice immunized with vaccine RB51 and S19 strains. These results showed that the 2308Δ*gntR* ghost conferred protection against challenge with S2308.

To evaluate the humoral and cell-mediated immune responses, we detected productions of Th1-type cytokine (IFN-γ) and Th2-type cytokine (IL-4) in splenocytes from 2308Δ*gntR* ghost immunized mice. IFN-γ is involved in against intracellular pathogens, which belongs to Th1-type cells immune response (Golding et al., 2001). IL-4 is involved in against extracellular pathogens, which belongs to Th2-type cells immune response (Allen and Maizels, 1997). In present study, we found that 2308Δ*gntR* ghost induces slightly higher levels of IFN-γ and IL-4 than *B. abortus* vaccine RB51 or S19. In addition, we also found that IgG titers induced by vaccination with 2308Δ*gntR* ghost were higher than that of RB51 or S19. All together, our data suggested that infection with 2308Δ*gntR* ghost induced humoral immune and cytokine responses.

It was shown that both $CD4^+$ and $CD8^+$ T cells contributed to the control of *Brucella* growth in the BALB/c mice (Araya and Winter, 1990). It has been reported that both $CD4^+$ and $CD8^+$ T cells involved in the control of *Brucella* infection in ruminant species (Weynants et al., 1998). Recently, it was shown that the overall percentage of $CD3^+$, $CD4^+$ and $CD8^+$ T cell subpopulations increased markedly in response to immunization with *B. suis* live vaccine S2 or *B. suis* ghosts (Liu et al., 2015). In our study, we observed that mice vaccinated with 2308Δ*gntR* ghost could induce higher $CD4^+/CD8^+$ ratios.

The 2308Δ*gntR* ghost constructed in this study exhibited a stable and non-drug resistant character. In addition to guaranteed safety, the 2308Δ*gntR* ghost is lack of *gntR* gene which can be used as a molecular tag to differentiate between natural and vaccinated infection. Therefore, the 2308Δ*gntR* ghost can provide a feasible strategy for

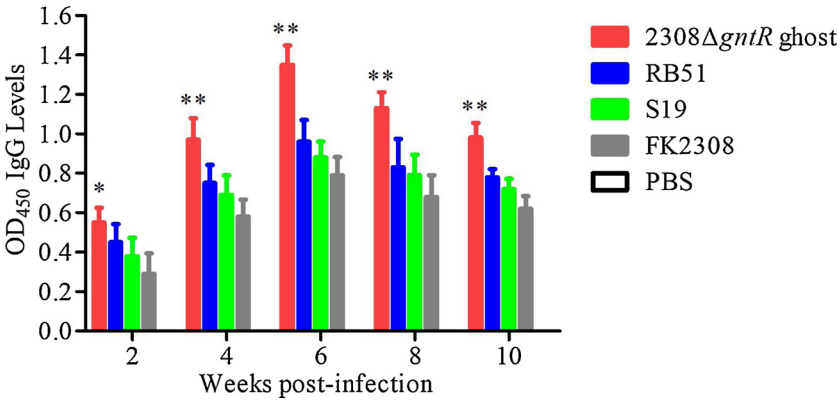

Fig. 4. Anti-*Brucella* antibodies in sera from mice immunized with 2308Δ*gntR* ghost, RB51, S19, FK2308 and PBS. BALB/c mice (n = 5 per group) were immunized with 200 μL PBS containing 1 × 10^6 CFU of 2308Δ*gntR* ghost, RB51, S19 and FK2308. The control group received 200 μL PBS. At 2, 4, 6, 8 and 10 weeks post-immunization, serum samples (n = 5 per time point) were collected and IgG specific antibody responses were determined by iELISA. The values are expressed as means ± SD of the absorbance at OD_{450}. Differences in IgG specific antibody was determined *via* the unpaired *t*-test between the 2308Δ*gntR* ghost and PBS (* $P < 0.05$, ** $P < 0.01$).

serological diagnosis.

In conclusion, in the present study, we described the production of 2308Δ*gntR* ghost vaccine candidate through the combination of homologous recombination and bacterial ghost technologies and evaluated its immunogenicity in BALB/c mice. The data presented indicate that 2308Δ*gntR* ghost confers protection against challenge by virulent strain S2308. In addition, 2308Δ*gntR* ghost was capable of effectively eliciting an anti-*Brucella*-specific antibody response and induced secretion of IFN-γ and IL-4. Furthermore, 2308Δ*gntR* ghost enhanced splenic T lymphocyte activity. The 2308Δ*gntR* ghost is lack of GntR protein which can be used as a molecular tag to differentiate immunized animals clinically. These results suggested that 2308Δ*gntR* ghost may be developed as a new vaccine candidate against *Brucella* infection. However, the efficacy and safety of 2308Δ*gntR* ghost in livestock demand further investigation.

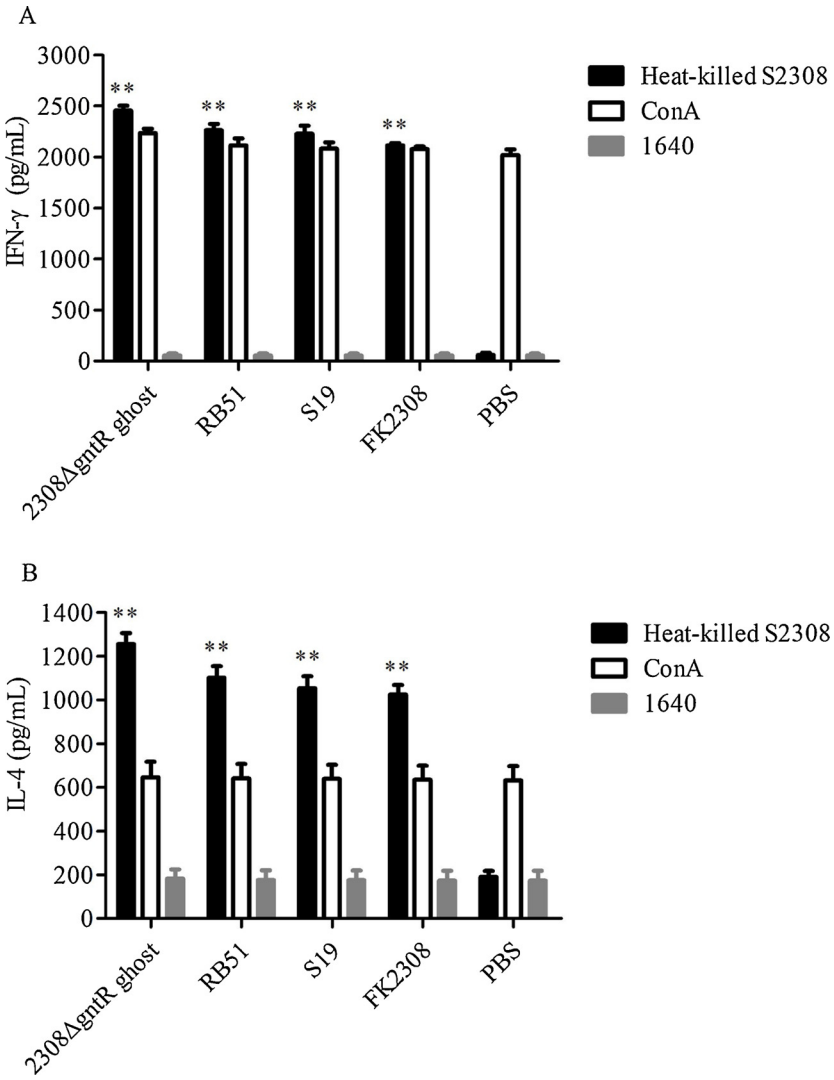

Fig. 5. Production of cytokines in stimulated splenocytes from mice immunized with 2308Δ*gntR* ghost, RB51, S19, FK2308 and PBS. BALB/c mice (n = 5 per group) immunized with 200 μL PBS containing 1 × 10^6 CFU of 2308Δ*gntR* ghost, RB51, S19 and FK2308. The control group received 200 μL PBS. 10 weeks post-infection, mice were euthanized and splenocytes were isolated and stimulated with heat-killed S2308, ConA or RPMI 1640 medium. Splenocytes culture supernatants were harvested after 72 h of culture. IFN-γ (A) and IL-4 (B) concentrations in the supernatant were measured by iELISA. The values are mean cytokine concentration ± SD. The significant differences from same stimulus in PBS-immunized mice are indicated by ** ($P < 0.01$).

Table 3
T lymphocyte subpopulations in the spleens of immunized mice (%)[a] (mean ± SD).

Group	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
2308Δ*gntR* ghost	45.36 ± 3.27[b]	19.21 ± 1.92	2.36 ± 0.22[b]
RB51	40.96 ± 2.59[b]	18.45 ± 1.77	2.22 ± 0.14[b]
S19	38.85 ± 3.28[b]	17.91 ± 1.44	2.16 ± 0.22[b]
FK2308	37.67 ± 4.25[b]	17.73 ± 0.99	2.12 ± 0.19[b]
PBS	24.38 ± 1.23	14.62 ± 1.93	1.66 ± 0.09

[a] 10 weeks post-immunization, spleens were homogenized and detected.
[b] Compared with PBS group, $P < 0.01$.

Declaration of Competing Interest

The authors have declared that no competing interests.

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