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A safe and molecular-tagged *Brucella canis* ghosts confer protection against virulent challenge in mice

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ABSTRACT

Canine Brucellosis, caused by *Brucella canis*, is a persistent infectious reproductive disease in dogs. The absence of effective treatment to the intracellular pathogen and the irreversible consequence of infection makes the need of a specific vaccine urgent. Bacterial ghosts (BGs) are the empty envelopes of bacteria with no genome content inside, which emerge as a proper vaccine candidate due to its intact outer antigen. It is generally derived from a genetically engineered strain, through the expression of *Bacteriophage* phiX174 lysis *E* gene upon induction. In this study, we combined the homologous recombination (HR) and bacterial ghost technologies, generating a genetically stable *B. canis* ghost strain which bears no drug resistance gene. When the ghost strain grows to OD_{600} of 0.6, 100% inactivation can be achieved under 42 °C in 60 h. The resultant BGs showed guaranteed safety and comparable immunogenicity to a live vaccine. The bacterial *B0419* protein was depleted during HR process, which is subsequently proved to work as a molecular tag in distinguishing natural infection and BGs immunization through ELISA. Additionally, the BGs also conferred protection against *B. canis* RM6/66 and *B. melitensis* 16 M. Therefore, the application of current BGs as a vaccine candidate and the corresponding serological diagnostic approach may provide better *B. canis* prevention strategy.

1. Introduction

Brucella species are a group of Gram-negative facultative intracellular bacteria, the cause of brucellosis in humans and animals (Corbel, 2009). On the basis of host preference and biochemical characters, there currently are ten species of the genus, including *Brucella melitensis*, *Brucella abortus*, *Brucella suis*, *Brucella canis*, *Brucella ovis*, *Brucella neotomae*, *Brucella ceti*, *Brucella pinnipedialis*, *Brucella microt*, *Brucella inopinata* (Corbel, 2009; Foster et al., 2007; Scholz et al., 2008, 2009; Jiménez de Bagüés et al., 2014). Four of them are pathogenic to human: *B. melitensis*, *B. suis*, *B. abortus* and *B. canis* in descending order of pathogenicity. In the past, most studies have focused on *B. melitensis*, *B. suis* and *B. abortus*, while little research was done about *B. canis*. As *B. canis* infection in dogs and human tends to increase these years (Wang et al., 2012; Dentinger et al., 2015; Gao et al., 2012; Kim et al., 2012; Lucero and Corazza, 2010; Nomura et al., 2010), and no commercial vaccine against the pathogen is licensed, the development of *B. canis* vaccine is urgent.

In China, there are three widely used live attenuated vaccines, *B. abortus* A19, *B. melitensis* M5 and *B. suis* S2, which have provided sufficient protection in live stocks. However, culling infected individuals, a critical step in pathogen purification, remains infeasible due to the absence of proper and convenient diagnostic method to distinguish vaccinated and infected animals. To solve the problem, several engineered mutant stains were generated as vaccine candidates, based on the development homologous recombination (HR) technology. Through vaccination with an engineered mutant strain lacking a certain protein and the application of the corresponding serological test, natural infection can be differentiated from immunization (Campos et al., 2002). Nevertheless, these mutants are mostly antibiotic resistant because the production process involves the screen through antibiotic resistance gene. Thus, a modified construction strategy was developed,

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which employs antibiotic-resistant positive selection and *SacB* counter-selection, generating mutant strains without antibiotic resistant ability (Campos et al., 2002). With this method, several non-resistant *Brucella* deletion mutants with potential use in diagnosis of brucellosis have recently been studied (Wang et al., 2014a; Tan et al., 2012). Although these non-resistant live mutant strains can effectively elicit immune responses, their inherent safety risk of bacterial replication and pathogenicity in young, immunocompromised or pregnant individuals still limits their use as vaccines (McDonald et al., 2006; Blasco and Díaz, 1993).

Bacterial ghosts (BGs) are non-living Gram-negative bacterial cell envelopes lacking cytoplasmic contents while maintaining their cellular morphology and natural surface antigenic structures (Jalava et al., 2002). They are constructed through controlled expression of bacteriophage PhiX174 lysis E gene which forms transmembrane lanes, draining the content of bacteria. The technology of BGs as vaccine candidates represents a relatively new approach in developing safe and potent vaccines against Gram-negative pathogens (Langemann et al., 2010; Szostak et al., 1996; Mayr et al., 2005; Eko et al., 2003; Wang and Lu, 2009), such as *Escherichia coli* O157, *Salmonella*, *Edwardsiella tarda*, *Vibrio cholerae*, *Pseudomonas aeruginosa*. As to *Brucella*, it was reported that *Brucella* S2 ghosts induce better IgG antibody and T cell responses than inactivated bacteria in mice (Liu et al., 2015). These recombinant *Brucella* ghost strains were generated through the transformation of suicide plasmid carrying lysis fragment, which have some drawbacks such as loss of plasmid, horizontal transfer of antibiotic resistance gene to wild strains and the costly use of antibiotics in large scale production. Thus, there is great demand in generating a safe and genetically stable *Brucella* ghost strain to meet the requirements of clinical use and commercial production.

Additionally, as dogs are closely related to human beings, the vacuum of canine *Brucella* vaccine will ultimately pose great danger to human health. These facts necessitate a safe and efficient vaccine against *B. canis*. In this study, we combined the advantage of HR and BG technologies for the first time, integrated the lysis *E* gene compartment into the genome of *B. canis* RM6/66. After kanamycin positive screen and *SacB* gene counter-selection, the nonantibiotic *B. canis* ghost strain was selected. Its growth kinetic, lysis kinetic, genetic stability were subsequently measured, and optimal lysis condition were determined. The BGs derived from this ghost strain showed satisfying safety, immunogenicity and cross-protection against both homologous and heterologous virulent strains. BGs immunization can also be distinguished from natural infection by specific protein ELISA. In general, we developed a safe, efficient, molecularly distinguishable ghost vaccine candidate against *B. canis*, which provide a new strategy for the prevention of animal brucellosis.

2. Materials and methods

2.1. Animals

BALB/c mice (female, 4–6 weeks old) were purchased from the Changchun Institute of Biological Products Co., Ltd, China. All animal experiments were carried out according to the experimental practices and standards approved by the Animal Welfare and Research Ethics Committee at Jilin University (Approval ID: 2015077410-1). The challenged mice were kept in animal biosafety level 3 (ABSL-3) facility.

2.2. Bacterial strains and plasmids

Brucella strains *B. canis* RM6/66, *B. suis* S2 and *B. melitensis* 16 M (Lab stock) were grown on tryptic soy broth (TSB, Sigma, St. Louis, MO, USA) agar at 37 °C, and the *B. canis* ghost strain was grown at 28 °C. All live *Brucella. spp* manipulations were performed in BSL-3 facility. Plasmid pBKSacB carrying the *SacB* gene was constructed in our previous work (Wang et al., 2014c).

2.3. Construction of suicide plasmid and selection of B. canis ghost strain

The upstream and downstream regions of *BCAN_B0419* gene (GenBank: ABX63601) were amplified from the genome of *B. canis* RM6/66 strain (ATCC 23365, Accession: NC_010104) using the following primers: *B0419f*-F, 5′-GGATCCGTGATTATCTCCGACGTTTCATC-3′ and *B0419f*-R, 5′-CTCGAGGGTTTGCAGCCTTGCCTGCGCGGT-3′; *B0419r*-F, 5′-CTCGAGAGGCCAGCCCAGACGACCGCGCT-3′ and *B0419r*-R, 5′-TCTAGACTATGCCTGTTCTTCCATCGG-3′ containing *BamH* I, *Xho* I and *Xba* I restriction sites (underlined) at 5′ ends. The PCR conditions were: 95 °C 5 mins; 94 °C 30 s, 58 °C 30 s, 72 °C 1 min, 30 cycles; 72 °C 10 mins. These DNA products were amplified and ligated into the plasmid pBKSacB to construct pBKSacB-*ΔB0419*. Lysis *E* gene from *Bacteriophage* ΦX174 RF I DNA was inserted in plasmid pBV220 containing temperature sensitive fragment *cI857-λpR/pL* to construct pBV220-E, as previously described (Liu et al., 2012). PCR amplification of temperature-sensitive lysis components (TLC) was performed using the primer TLC-F-XhoI (5′-CTAGTCTAGACCATTCTT-CATAATTCAATCC-3′) and TLC-R-XhoI (5′-CCGCTCGAGAAATAAACA-AAGAGTTTGTAGAAAC-3′). The amplified DNA product was cloned into pBKSacB-*ΔB0419* through *Xho* I restriction site to construct suicide plasmid pBKSacB-*ΔB0419*-TLC.

Electroporation of a suicide plasmid pBKSacB-*ΔB0419*-TLC bearing *B0419* gene homologous arms and TLC fragment into *B. canis* RM6/66 competent cells was performed at 1500 kV and 6 ms (1 mm bottom; BTX, Holliston, MA, USA). Transformants experienced kanamycin positive selection and glucose counter selection. Briefly, they were inoculated to TSB agar plates (100 μg/ml kanamycin) for 6 days at 28 °C. The survived clones were subsequently inoculated to glucose TSB agar plates (20 mM). The resultant strains were then passaged for 30 generations and determined by PCR.

2.4. Growth and lysis characterization of B. canis ghost strain and production of B. canis ghosts

Wild-type *B. canis* and ghost strains were inoculated into 100 ml of TSB medium at 28 °C, the growth curve was measure by OD_{600} value and colony forming units (CFU). When the culture reached OD_{600} of 0.6, the culture temperature of *B. canis* ghost strain was converted to 42 °C to induce lysis. The growth curves under shifted temperature of both ghost strain and parental strain RM6/66 were measured. The lysis was monitored by examining the OD_{600} value and viable cell counts through CFU every 6 h. The resulting cell ghosts were pelleted by centrifugation at 8000 × *g* for 30 min at 4 °C, then washed three times with sterile phosphate-buffered saline (PBS, pH 7.2) and stored at −70 °C prior to use. BGs were fixed for ultracryotomy and were subsequently visualized on a Hitachi H-7650 transmission electron microscope (TEM) operating at 80 kV.

2.5. Animals immunization and safety evaluation

Mice were randomly divided into four groups (n = 6 for each group) and immunizated twice with PBS (100 μl), *B. canis* ghosts (BGs, 1.0×10^8 CFU/mouse in 100 μl), formalin-killed *B. canis* RM6/66 strain (FKB, 1.0×10^8 CFU/mouse in 100 μl) and *B. suis* S2 strain (S2, 1.0×10^7 CFU/mouse in 100 μl). The initial injection was set as day 0 and the boost was at day 7, through intraperitoneal (i.p.) route. To evaluate the safety of these antigens, mice were euthanized by cervical dislocation to assess the spleen index (spleen weight/body weight, mg/g) and the number of CFU/spleen (CFU/g) two weeks after immunization.

2.6. Antibody response assessment

The specific IgG antibody titer was estimated by indirect ELISA. Briefly, the 96-well plates (Costar, Corning, NY, USA) were coated with

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