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Brucella suis strain 2 vaccine is safe and protective against heterologous *Brucella* spp. infections

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ABSTRACT

Brucellosis is a wide spread zoonotic disease that causes abortion and infertility in mammals and leads to debilitating, febrile illness in humans. *Brucella abortus*, *Brucella melitensis* and *Brucella suis* are the major pathogenic species to humans. Vaccination with live attenuated *B. suis* strain 2 (S2) vaccine is an essential and critical component in the control of brucellosis in China. The S2 vaccine is very effective in preventing brucellosis in goats, sheep, cattle and swine. However, there are still debates outside of China whether the S2 vaccine is able to provide protection against heterologous virulent *Brucella* species. We investigated the residual virulence, immunogenicity and protective efficacy of the S2 vaccine in BALB/c mice by determining bacteria persistence in spleen, serum antibody response, cellular immune response and protection against a heterologous virulent challenge. The S2 vaccine was of low virulence as there were no bacteria recovered in spleen four weeks post vaccination. The vaccinated mice developed *Brucella*-specific IgG in 2–3 weeks, and a burst production of IFN-γ at one week as well as a two-fold increase in TNF-α production. The S2 vaccine protected mice from a virulent challenge by *B. melitensis* M28, *B. abortus* 2308 and *B. suis* S1330, and the S2 vaccinated mice did not develop any clinical signs or tissue damage. Our study demonstrated that the S2 vaccine is of low virulence, stimulates good humoral and cellular immunity and protects animals against infection by heterologous, virulent *Brucella* species.

1. Introduction

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Brucellosis is a chronic infectious zoonotic disease worldwide, which is characterized by infectious abortion and sterility in various domestic animal species and potentially debilitating infection in humans [1,2]. Brucellosis is caused by the facultative intracellular Gram-negative bacteria of the genus of *Brucella* [1]. There are ten species of *Brucella* based on preferential host specificity, with three species, *Brucella melitensis* (mainly infecting goats and sheep), *Brucella abortus* (cattle) and *Brucella suis* (swine) highly pathogenic to humans [3,4]. Although some developed countries, such as those in Northern Europe, Australia, USA, and Canada, have eradicated brucellosis from livestock, the disease remains endemic in many regions of the world including Latin America, Middle East, Africa, Asia, and the Mediterranean basin [2]. Brucellosis can result in great economic loss, particularly in the food animal production sector [5]. Human brucellosis occurs when humans come in direct contact with fluid discharges from an infected animal or through the consumption of unpasteurized dairy products [2,6]. Thus brucellosis is of a significant public health issue and economic concern.

Up to date, quarantine, slaughter and vaccination are mainstay arsenals for the control of brucellosis. Since there is no licensed vaccine for human brucellosis, much research has been conducted in an attempt to develop safer and more effective *Brucella* vaccines for animals, to prevent animal brucellosis and ultimately human infection of *Brucella* [7]. Among several vaccines, only live attenuated *B. melitensis* Rev. 1, *B. abortus* S19, and *B. abortus* RB51 vaccines are recommended by the WHO and OIE and widely used to control animal brucellosis in most countries [7,8]. Three different live attenuated *B. suis* S2, *B. melitensis* M5 and *B. abortus* A19 vaccines are licensed and used for the control of animal brucellosis in China [9,10].

Abbreviations: S2, *Brucella suis* strain 2; TSA, trypticase-soy agar; CVCC, China Veterinary Culture Collection Center; RBT, Rose Bengal test; SAT, serum agglutination test.

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B. suis strain 2 (S2) vaccine was developed from a *B. suis* naturally attenuated variant, which was isolated from the embryo of an aborted sow in 1952 by the researchers of China Institute of Veterinary Drug Control. The S2 vaccine has been used for vaccination of pigs and other domestic animals in China since 1971. Over several decades of vaccination in goats, sheep, cattle and swine, the S2 vaccine was proved to be safe [11]. Moreover, after wide applications of S2, the positive rate of brucellosis decreased from 20 to 30% in the 1960s to less than 0.1% in the 1990s in livestock, and from 10 per million in the 1970s to 0.15 per million in the 1990s in humans [12,13]. The S2 vaccine is the most widely used brucellosis vaccine in China and comprises over 95% of the whole usage, compared to the other two vaccines [14]. However, the S2 vaccine has been rarely used in areas out of China except Libya [15].

It is recommended by OIE and European Union that *Brucella* vaccine efficacy could be evaluated in a mouse model [3,16]. The susceptibility to *Brucella* infection differs in different mouse strains; for example C57BL shows relative resistance to *Brucella* than other strains including BALB/c [17–21]. It was reported that S19 was more protective than RB51 in CD-1 and Swiss mice, but S19 and RB51 had similar protective efficacy in BALB/c mice [22]. Usually, different mouse strains are used for the testing of different *Brucella* vaccines, for example, CD-1 mice of 5–7 weeks old are used for the evaluation of S19 and Rev.1, and BLAB/c mice for RB51. Till now, there are only few reports about the efficacy of the S2 vaccine in laboratory animals, and among these no study was carried out in BALB/c mice, which is a common experimental mouse strain [23–25].

Outside of China, there are still debates about whether S2 could confer good protection against the three highly pathogenic virulent strains of *Brucella menlitensis*, *B. abortus* and *B. suis*, which are pathogenic to goat and sheep, cattle, and swine, respectively [23–26].

To assess whether the S2 vaccine is able to elicit effective immunity against virulent *Brucella* strains of *B. menlitensis*, *B. abortus* and *B. suis*, we evaluated the protective efficacy of the S2 vaccine in BALB/c mice. In addition, we also investigated residual virulence, and humoral and cellular immune responses and analyzed the potential correlation between the protection against challenge and the associated immune responses.

2. Materials and methods

2.1. Brucella strains and animals

Brucella strains used in this study and their characteristics are shown in Table 1. The virulent strain *B. melitensis* M28, *B. abortus* 2308 and *B. suis* S1330 were used as the challenge strain [3,9,10,27]. BALB/c mice were used for the study based on their wide use in *Brucella* spp. studies. Animals were housed in an air-conditioned, air filtered, biosafety level III facility. The experimental protocol was approved by the institute Ethics Committee in Animal Experimentation.

2.2. Vaccine and immunization

The S2 vaccine was purchased from China Animal Husbandry Industry Co., Ltd. Each vaccine vial is specified for 80 doses with each dose containing approximately 8.5×10^9 CFU of live bacteria. Before use, the vaccine was checked for purity, viability and smoothness.

The S2 vaccine was dissolved, diluted in saline to the final concentration of about 5×10^5 CFU live bacteria per ml. Bacteria counts were confirmed by serial tenfold dilutions on trypticase-soy agar (TSA) (BD, USA) plates. Fifty-five female BALB/c mice (Vital River, China) at the age of 6 weeks, were selected for administration subcutaneously with 0.2 ml the above diluted S2 vaccine (about 1×10^5 CFU live bacteria per animals) [10,19,27]. Another fifteen mice were inoculated with saline as the unvaccinated control.

Blood samples were collected for serological studies from five vaccinated mice every week until the 8th week post vaccination. Sera were separated by centrifugation at 1700 × *g* for 10 min at 4 °C, and then stored at −80 °C until analysis. The same mice were then killed by cervical dislocation and their spleens were aseptically isolated. The collected spleens were defatted, weighed and divided to two equal parts for bacteriological examination (persistence assay described below) and the cytokine profile, respectively.

2.3. Bacteriological examination

Persistence of the bacteria in mice was measured by determining the bacterial CFU in spleen weekly 1–8 weeks post vaccination. The collected spleens (half-spleen) was homogenized aseptically in a glass grinder with 200 μl sterile saline. The obtained tissue suspension was ten-fold serially diluted by up to 10^4 fold. Selected dilutions 1×, 100× and 10^4× were plated onto two parallel TSA plates followed by incubation at 37 °C for 3–5 days. Bacterial colonies in culture plates with fewer than 300 CFU per plate were counted. Generally, the bacterial persistence in spleen is expressed as 50% survival time (recovery time RT50), which was calculated according to the Reed–Meunch method [28].

2.4. Evaluation of serum IgG

Serum IgG titers in the vaccinated mice were determined by the Rose Bengal test (RBT) according to OIE [3]. The collected serum was first diluted two fold serially up to 64 fold with PBS. The serially diluted test serum (30 μl) was pipetted to a white tile plate followed by adding the same volume of *Brucella* BRT reference antigen to the serum spot. After thorough mixing and gentle agitation for 4 min, the plate was visually examined. Any visible reaction was considered positive.

The serum IgG titers were also determined by the serum agglutination test (SAT) [3]. The collected serum was diluted by PBS to 5×, 10×, 20×, 40×, 80×, 160× and 320×. The serially diluted test serum (50 μl) was added to a 96-well micro agglutination plate. After 1:20 dilution by PBS, the *Brucella* SAT reference antigen was added to the 96-well plate, then it was incubated at 37 °C for 22 h and the results were recorded.

2.5. Detection of cytokine production

Cytokine production was evaluated in splenocytes 1–8 weeks post vaccination. The collected spleen (half-spleen) was incubated in DMEM containing 10% fetal bovine serum at 37 °C and a 5% CO_2 atmosphere with gentle agitation. When adherent monolayer cells appeared, splenocytes were separated from cell debris and blood cells. The splenocytes were stimulated by 0.5 mg/ml Brucellin (prepared according to the standard procedure) for 24–48 h at 37 °C and 5% CO_2 atmosphere [10,29,30]. The supernatant was collected for quantification of secreted IFN-γ, and the stimulated splenocytes for analysis of cytokine IFN-γ, TNF-α and IL-4 expression.

Cytokine expression in the stimulated splenocytes was determined by quantitative RT-PCR. The total RNA of the stimulated cells was extracted, purified, and quantified, and the cDNA was generated using 1–2 μg of RNA. For RT-PCR, the primers for amplifying IFN-γ, TNF-α, IL-4 and the housekeeping gene, actin were designed by the PerlPrimer software (Table 2). mRNA expression was assessed by RT-PCR on a 7500 Real Time PCR System (Ambion, USA). The analysis of relative change (relative to the non-stimulated control) in mRNA expression of the target gene was based on the $2^{-\Delta\Delta Ct}$ method [31].

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