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An Alternative Test Procedure for the Protective Efficacy of *Brucella* Vaccines

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Abstract

Administration of live *Brucella* vaccine is required to prevent the spreadof ruminant brucellosisin affected livestock herds. This study optimized the mouse model to test the protective efficacy of live *Brucella* vaccine. To optimize the protective efficacy test procedure, the absolute infective doses were determined in mice as 10 Colony Forming Units (CFU)/mouse for *B. melitensis* 16M and 50 CFU/mouse for *B. abortus* 2308, which were then used as the challenge doses for the respective strains. The optimal vaccination doses of vaccine *Brucellasuis* S2 in mice were $10^{2.25}$ CFU and $10^{3.5}$ CFU/mouse, which could confer ≥ 80% protection in mice against challenge by *B. melitensis* 16M or *B. abortus* 2308, respectively. In addition, challenge with *B. melitensis* 16M or *B. abortus* 2308 should occur just at 3.63 weeks and 4.75 weeks post-inoculation, respectively. The protective efficacy test not only was more accurate and took less time but also was consistent with the evaluation index in host animals. Our study indicated that the mouse model could be used to test the protective efficacy of live *Brucella* vaccines during their production and development.

Keywords: *Brucella* vaccine; Mouse model; Protective efficacy; Test procedure

Introduction

Brucellosis is an important zoonotic disease with great socio-economic impact in many countries. Infected animals and the resulting contaminated animal products are the main source of human infection [1]. Although animal brucellosis may be controlled by means of adequate testing and slaughtering programs in areas with a high prevalence of the disease and low socioeconomic conditions, vaccination has become the only practical way to control the disease in ruminants [2]. The vaccines used extensively in epidemic areas include *B. abortus* vaccine S19 for cattle and *B. melitensis* vaccine Rev.1 and *B. suis* vaccine S2 for cattle, sheep, and goats [3]. However, low degree of protective immunity and serious adverse effects have been reported for both *B. abortus* vaccine S19 and *B. melitensis* vaccine Rev.1 vaccines in some vaccination trials. Such variability might be due, at least in part, to the heterogeneity of the vaccines used mainly because of unsuitable quality control during the manufacturing process [4]. So, it is very important to conduct quality tests of vaccine products during the manufacturing of brucellosis vaccines.

Until recently, testing procedures for brucellosis vaccines were performed according to the procedures in the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* [3], in which mice were used in the protective efficacy test. Given that experiments in ruminants, humans, and primates have economical and ethical concerns, a standardized mouse model has been preferred in brucellosis research areas including pathogenesis, vaccine development, and therapies [5]. For immunogenicity tests, mice have been vaccinated subcutaneously with a dose of 10^5 CFU of tested vaccine and reference vaccine S19 and then were challenged with a dose of 2×10^5 CFU of *B. abortus*2308 after 30 days [3]. With this procedure, the vaccine strains often survive long beyond the time of the challenge, which interferes with the isolation and bacterial count of the challenge strain. To solve this problem, duplicate plates are required to differentiate the challenge strains from the vaccine strains, but spontaneous mutation and interference between vaccine and challenge strains may lead to an underestimation of the protective efficacy of *Brucella* vaccines. Therefore, a more convenient and effective test is needed for *Brucella* vaccine products. The present study aimed to optimize the mouse model for protective efficacy test of *Brucella* vaccines with S2 as a representative by optimizing the immunizing dose, the challenge dose, and the time interval between inoculation and challenge.

Material and Methods

Ethics statement

All animal research was approved by the Beijing Association for Science and Technology. The approval ID is SYXK (Beijing) 2007-0001, and the animal research complied with the guidelines of Beijing laboratory animal welfare and ethics of Beijing Administration Committee of Laboratory Animals.

Bacterial strains and media

Virulent *B. abortus* 2308, *B. melitensis* 16M, and vaccine strain *B. suis* S2 were all kindly donated by Dr. Qianni He (Institute of Veterinary Research, Xinjiang Academy of Animal Sciences, Urumqi, China). These strains were originally collected and preserved in the Chinese Veterinary Culture Collection Center (Beijing, China). Freeze-dried rehydrated bacteria were routinely grown in Tryptic Soy Broth (TSB) or Tryptic Soy Agar (TSA) at 37. To prepare organisms for animal infection, the *Brucella* strains were harvested from the

surface of the plates after 3 days of incubation using Phosphate Buffered Solution (PBS), adjusted to the required concentration, and injected into mice as indicated for each experiment. Exact doses expressed in CFU were verified retrospectively by serial dilution, plating, and counting. All work with live *Brucella* virulent strains was performed in Biosafety Level 3 facilities at China Agricultural University.

Mice

Female BALB/c mice (6 to 8 weeks old) were purchased from Weitong Lihua Laboratory Animal Services Centre (Beijing, China), raised in individually ventilated cage rack systems, and subsequently transferred to the Biosafety Level 3 facilities at the beginning of the infection and challenge experiments. All experiments involving animals followed the regulations enacted by the Beijing Administration Office of Laboratory Animals.

Determining the absolute infective dose (ID100) of B. melitensis16M and B. abortus2308 in mice

To confirm the challenge doses for *B. melitensis* 16M and *B. abortus*2308 in mice, the ID_{100} of each of these two wild-type strains was determined. Six groups of four mice each were inoculated intraperitoneally with *B. melitensis* 16M at a dose of 1,000, 500, 100, 50, 10, and 5 CFU respectively, and another six groups were inoculated with *B. abortus* 2308 at the same doses gradient. Two weeks post-infection, all mice were euthanized via carbon dioxide asphyxiation. Spleens in all the mice were collected aseptically for bacteriological examination as described [6]. The bacteria recovered from the spleens were counted. If no bacteria grew, the spleen was assumed to contain fewer than five bacteria, below the limit of detection of 5 CFU/spleen. The minimum dose of each strain that infected all four mice was determined to be the ID_{100}.

Determining the virulence of vaccine strain S2 in mice at different doses

Four groups of 25 mice each were inoculated intraperitoneally with 100, 1,000, 10,000, and 100,000 CFU/mouse, respectively, in 0.1 mL PBS for vaccine strain S2. Five mice from each group were randomly selected and euthanized via carbon dioxide asphyxiation at 2, 3, 4, 5, and 6 weeks post-inoculation. At each time point, spleens were collected aseptically for bacteriological examination. The bacteria recovered from the spleens were counted to evaluate the survival in mice.

Evaluating the protective efficacy of vaccine strain S2 against challenge with wild-type strains

To evaluate the protective efficacy of vaccine strain S2 at different inoculation doses, four groups of 10 mice each were inoculated intraperitoneally with 100, 1,000, 10,000, and 100,000 CFU/mouse, respectively, in 0.1 mL PBS. Another 10 mice received 0.1 mL PBS per mouse as a control. Six weeks post-immunization, two groups of five mice from each group were challenged with *B. melitensis*16M or *B. abortus* 2308, respectively, using the ID_{100}. Two weeks later, the challenged mice were euthanized, and their spleens were collected for isolation of bacteria as described above. Mice were scored for infection or protection based on the presence or absence of the challenge *Brucella* strain in their spleens [4]. The protective efficacy was evaluated by the protection ratio in accord with the efficacy index in ruminants [7]. The Protection Ratio (PR) was calculated as PR = (AB)/A 100, where A is the number of mice in test and B is the number of mice that infected. Mice were considered infectedbased upon the presence of 1 CFU of challenge *Brucella* strain in spleen [6].

Statistical analysis

A Student's t-test was performed to analyze the data from the mouse virulence and protection experiments, and a P-value of < 0.05 was considered significant. To determine the optimal vaccination dose, and optimal time interval between vaccinate and challenge, curve fitting with the Solver function of Excel 2007 (Microsoft) was performed.

Results

ID100 of *B. melitensis* 16M and *B. abortus* 2308

The protection units of a vaccine strain are typically determined 2 weeks after challenge [3] and bacterial survival curves indicate that the number of wild-type *Brucella* bacteria peak in mice 2 weeks post-inoculation [6]. Thus, to optimize the challenge doses of wild-type *Brucella* strains, the ID_{100} values of *B. melitensis*16M and *B. abortus* 2308 were determined 2 weeks post-infection. All of the mice were infected at 10 CFU for *B. melitensis* 16M (Figure 1A) and at 50 CFU for *B. abortus*2308 (Figure 1B). Thus, the ID_{100}values for *B. melitensis* 16M and *B. abortus* 2308 in mice were determined as 10 CFU and 50 CFU respectively.

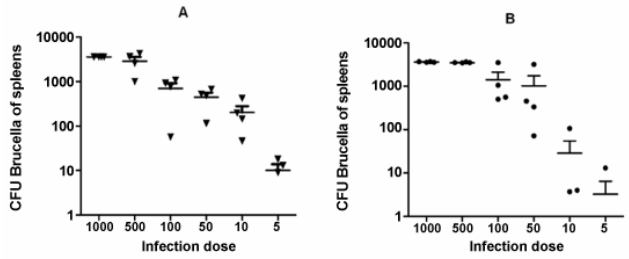

Figure 1: Infection with the different doses of *B. melitensis* 16M (A) and *B. abortus* 2308 (B) in mice. Six groups of four mice each were inoculated intraperitoneally with *B. melitensis* 16M or B. *abortus* 2308 at a dose of 1,000, 500, 100, 50, 10, or 5 CFU. Two weeks post-inoculation, all mice were euthanized, and the bacteria recovered from the spleens were counted. Data are expressed as the number of CFU in each spleen.

Persistence time in mice for different doses of vaccine strain S2

To measure the persistence time in mice for different vaccine strain S2 doses, we inoculated BALB/c mice with four doses of vaccine S2 and determined the bacterial loads in the infected mice at different time points post-inoculation. The number of viable bacteria recovered from the spleens of mice in each group decreased continuously. The vaccine strain S2 had disappeared from the mice in the 100 CFU vaccination group by 3 weeks post-inoculation, whereas it persisted until 5 weeks post-inoculation in the 1,000 CFU and 10,000 CFU vaccination groups and until 6 weeks in the 100,000 CFU group

(Figure 2). These results indicated that the persistence time of vaccine strain S2 in mice was positively correlated with the inoculation dose ($P<0.05$).

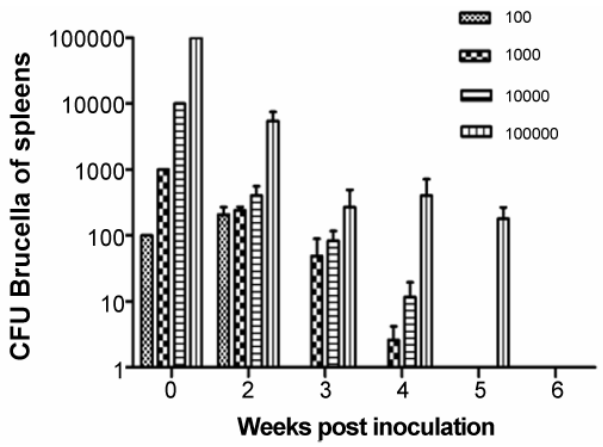

Figure 2: Persistence of different vaccination doses of vaccine strain S2 in mice. Twenty-five mice were inoculated with each strain at a dose of 100, 1,000, 10,000, or 100,000 CFU/mouse. Five mice per group were euthanized at 2, 3, 4, 5, and 6 weeks post-inoculation, and the persistence time of vaccine strain S2 was determined based on the number of CFU recovered from each spleen, which is expressed as the mean ± SD.

Protective efficacy of vaccine strain S2 against a challenge by wild-type strains

Because the vaccine strain S2 was not detected in spleens 6 weeks after inoculation, all vaccinated mice were challenged at 6 weeks post-inoculation with *B. melitensis* 16M or *B. abortus* 2308 at the respective ID_{100}. The challenge strains caused 100% infection in the control groups (Figure 3 and Table 1). The protection ratio was somewhat dose dependent in the vaccination groups. The protection ratio against challenge with either strain was 100% after inoculation with 100,000 CFU of vaccination strain S2. However, at the lowest dose (100 CFU/mouse), the protection ratios were 80% against *B. melitensis* 16M and 60% against *B. abortus* 2308.

Challenge strain	S2 vaccination dose	No. of mice in test	No. of mice infected	Protection ratio (%)
B. melitensis 16M	100	5	1	80
	1,000	5	1	80
	10,000	5	0	100
	100,000	5	0	100
	PBS	5	5	0
B. abortus 2308	100	5	2	60
	1,000	5	1	80
	10,000	5	1	80
	100,000	5	0	100
	PBS	5	5	0

Table 1: Protective efficacy of vaccine strain S2 with different vaccination doses.

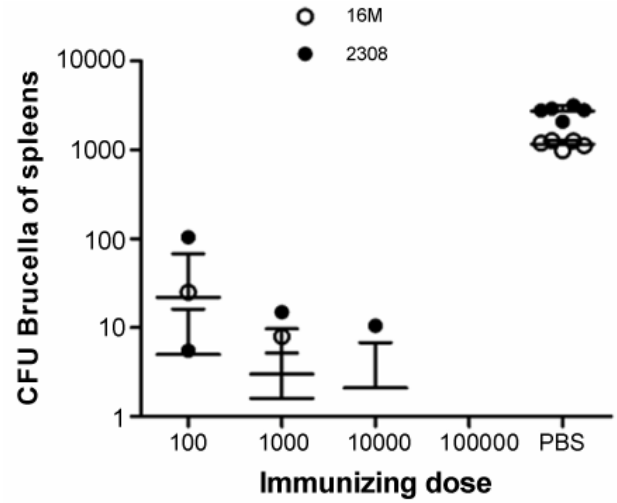

Figure 3: Wild-type strains recovered from spleens of vaccinated mice at 2 weeks post-challenge. Groups of five mice each were vaccinated with PBS (control) or 100, 1,000, 10,000, or 100,000 CFU of vaccine strain S2 at 6 weeks prior to the challenge with 10 CFU *B. melitensis* 16M or 50 CFU *B. abortus* 2308. Data are expressed as the number of CFU recovered from the spleens.

The optimal immunizing dose and optimal time interval between vaccination and challenge to determine the greatest protective efficacy test procedure of brucellosis vaccination that can occur over a short period of time, the immunizing dose and time interval between vaccination and challenge were optimized. As described above, we observed that the vaccine strain S2 survival time in mice was linear correlated with the vaccination dose, and the protective efficacy was also linear related to the vaccination dose in a range of doses. Thus, the optimal vaccination dose and the optimal time interval between vaccination and challenge was determined for vaccine strain S2 by linear curve fitting. When the vaccine strain S2 conferred at least 80% protection for the animals, the optimal vaccination dose against challenge with *B. melitensis*16M or *B. abortus*2308 was $10^{2.25}$ CFU or $10^{3.5}$ CFU per mouse, respectively (Figure 4A and 4B). The optimal time interval between vaccination and challenge was 3.625 weeks for challenge by *B. Melitensis* 16M and 4.75 weeks for challenge by B. *abortus* 2308 at the optimal vaccination dose of vaccine strain S2 (Figure 4C).

Discussion

Brucella infection in mice can persist throughout their lives and is accompanied by characteristic pathological signs [5]. Therefore, mice have been used to evaluate the in vivo character of *Brucella* vaccine and mutation strains [8-11]. For protective efficacy test of *Brucella* vaccine, different inoculation doses, time intervals between vaccination and challenge, and challenge doses have been used in in mice [12,13-15]. To unify and optimize these factors, we first

determined the ID_{100} values for *B. melitensis* 16M and *B. abortus* 2308, which were 10 CFU and 50 CFU in mice, respectively. While the ID_{100} values for *B. melitensis* 16M in our study was lower than that extrapolated by Teske et al. [16], which may be resulted from the differences in the mouse age, the different source and cultivation conditions of *Brucella* strains, and the bacteria-counting methods. We subsequently used the ID_{100} as the challenge doses. Although these challenge doses were also significantly lower than that (2×10^5 CFU/mouse) proposed by Office International des Epizooties and other studies [3,17] they successfully infected all mice.

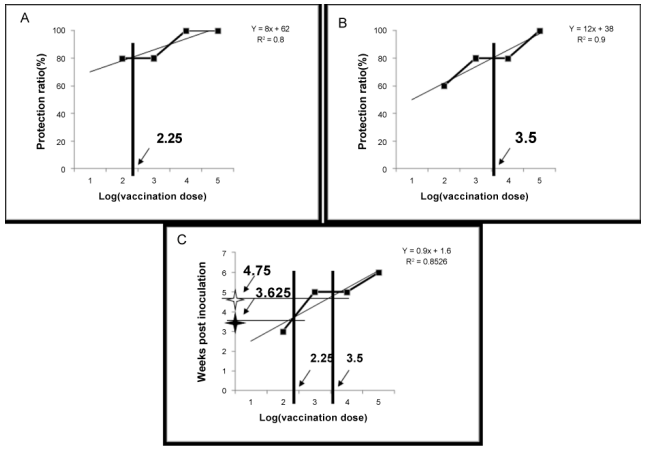

Figure 4: The optimal vaccination doses for vaccine strain S2 against challenge by *B. melitensis* 16M (A) and *B. abortus* 2308 (B); (C) The optimal time interval between vaccinate and challenge for the optimal vaccination dose of vaccine strain S2 against *B. melitensis* 16M ⊕ and *B. abortus* 2308 ⊕ challenges.

The live attenuated smooth *B. suis* strain S2 was developed in China by *in vitro* serial passages of a field strain, which was isolated in 1953 from an aborted sow. The vaccine has been widely utilized against *B. melitensis* and *B. abortus* infection in cattle, goat, and sheep herds in China since 1958 [18,19]. The vaccine is administered through drinking water; a dose of 10^{10} bacteria gives 1–3years of protection for cattle.In the middle of 1980s, *B. suis* S2 vaccine was introduced to the other countries, e.g. Spain, Turkey, Libya, Britain, France, Germany, and Zambia, and was recorded in "The *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*" [18]. In the present study, we selected vaccine S2 to optimize the vaccination dose and the corresponding time interval between vaccination and challenge. At lower vaccination doses, vaccine strain S2 died more quickly in mice. At 6 weeks post-inoculation, we observed that vaccine S2 was absent from all the mice of the different dose groups. In the protective efficacy test, we used the protection ratio as the evaluation index, which was similar to that used in the host animals [7,20], instead of comparing the relative protection units of the given vaccine strains to the reference vaccine or PBS control group. We observed that the protection ratio of vaccine strain S2 was 60% or higher even at the low vaccination dose. To optimize the results, when the protection ratio expected to be 80% the optimal vaccination dose of vaccine S2 was determined by linear curve fitting to be no more than $10^{3.5}$ CFU/mouse, which is much lower than 10^5 CFU, as proposed by others [3]. Corresponding to the optimal vaccination doses, the optimal time interval between vaccination and challenge was determined to be < 5 weeks, which was not required to consider the interference of the vaccine strains for the number of challenge strain. Therefore, determination of the optimal vaccination dose and survival time would be much practicable for testing the protective efficacy of *Brucella* vaccines in the course of production or development.

This study indicated that vaccination of *B. suis* vaccine S2 with $10^{2.25}$ CFU/mouse and $10^{3.5}$ CFU/mouse conferred 80% protection in mice against *B. melitensis*16M and *B. abortus* 2308, respectively. Furthermore, challenge with 10 CFU of *B. melitensis* 16M and 50 CFU of *B. abortus* 2308 should occur at 3.63 weeks and 4.75 weeks post-inoculation, respectively. These results from miceshould be the essentialdata for the specific *Brucella* vaccine strain S2, which has the intrinsic relations with the data from host animals vaccinated by this vaccine. Therefore, we suggested that the mouse model was used for evaluating the protective immunity of live *Brucella* vaccine, which would provide a reliable and fast method for quality control of the vaccines in production.

Acknowledgments

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