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ORIGINAL ARTICLE

Construction and Evaluation of the *Brucella* Double Gene Knock-out Vaccine Strain MB6 Δbp26ΔwboA (RM6)

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Abstract

Objective: Brucellosis is a serious zoonotic infection worldwide. To date, vaccination is the most effective measure against brucellosis. This study was aimed at obtaining a vaccine strain that has high protective efficacy and low toxicity, and allows vaccination to be differentiated from infection.

Methods: Using homologous recombination, we constructed a double gene-deletion *Brucella* strain MB6 Δbp26ΔwboA (RM6) and evaluated its characteristics, safety and efficacy.

Results: The RM6 strain had good proliferative ability and stable biological characteristics in vivo and in vitro. Moreover, it had a favorable safety profile and elicited specific immune responses in mice and sheep.

Conclusion: The RM6 strain may have substantial practical application value.

Key words: brucellosis, *Brucella*$_2$, vaccine$_3$, bp26$_4$, wboA$_5$, homologous recombination$_6$

INTRODUCTION

Brucellosis is a serious zoonosis worldwide, which is caused by gram-negative, facultative intracellular bacteria of the *Brucella* genus [1]. *Brucella* can cause abortion and infertility in its natural animal hosts, thus resulting in major economic losses; it can also infect humans, causing excessive fever, endocarditis, arthritis and osteomyelitis [2-4]. To date, vaccination is the most effective prevention measure against brucellosis.

Live attenuated vaccines, compared with other vaccination types, such as cell extracts or DNA vaccines, are the most effective in preventing brucellosis in animals [5,6]. Many attenuated vaccine strains against brucellosis are available for domestic animals [7], such as *Brucella melitensis* M5 and *B. suis* S2. However, these vaccines have many drawbacks, including interference with classical serological diagnostic tests, the ability to cause many diseases in humans and the risk of virulence recurrence [8,9]. The *Brucella abortus* RB51 vaccine is a live vaccine with high safety and immunogenicity [10]. The *B. abortus* RB51 vaccine contains an intervening sequence (IS711) causing disruption or deletion of the wboA gene, thus enabling vaccinated animals to be differentiated from naturally infected animals. Although many vaccine studies have shown that gene-deleted marker vaccines elicit favorable immunogenicity

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and protective immunity, their biosecurity and genetic stability require further investigation [11-14]. Restoration of expression of these deleted genes might result in full or partial recovery of virulence, thus posing a potential threat to vaccinated animals. Furthermore, no vaccine has been approved to prevent human infections by any *Brucella* species [15]. Therefore, new vaccines, particularly marker vaccines, are needed to improve protection and biosecurity against brucellosis.

In this study, we generated the double gene-deletion *Brucella* strain MB6 Δbp26ΔwboA (RM6) by homologous recombination. The virulence recurrence risk was decreased to the greatest extent with bp26 and wboA double knockout. The wboA gene encodes an essential glycosyltransferase associated with production of the O polysaccharide (O antigen) for lipopolysaccharide biosynthesis, and it is involved in colony morphology variations [16-19]. Therefore, wboA was considered a candidate gene whose deletion might allow immunity and natural infection to be differentiated. In addition, some studies have indicated that mutation or knockout of the bp26 gene decreases the bacterium's virulence. The sera of animals inoculated with the bp26 deletion strain do not contain bp26 specific antibodies [20-23]. Therefore, we considered bp26 an ideal marker for a *Brucella* gene recombinant vaccine.

We analyzed the safety, immunogenicity and protective efficacy of our vaccine strain in protecting against *Brucella* infection in mice and sheep. MB6 Δbp26ΔwboA (RM6) was found to have highly favorable safety and efficacy in target animals, nontarget animals, susceptible populations and the environment. Thus, the *Brucella* RM6 deleted strain may have practical application value and important implications for effective control of brucellosis.

METHODS

Construction of *Brucella* MB6 Δbp26ΔwboA (RM6)

Resuscitation and genomic DNA extraction of the MB6 strain

Freeze-dried *Brucella* strain MB6 was dissolved in phosphate buffered saline, then spread plated on a tryptone soy agar (TSA) plate at 37°C. Single colonies were picked after 72 hours and inoculated in 5 mL tryptone soy broth (TSB) medium at 37°C at 200 r/min for 24 hours. Bacteria were centrifuged and collected according to the manufacturer's instructions (Sinopharm, and details of critical reagents are listed in S3 Table). The MB6 strain genomic DNA was extracted and stored at −20°C.

Primer design

The corresponding specific primers bp26-N-F/bp26-N-R and bp26-C-F/bp26-C-R were designed on the basis of the nucleotide sequences of the upstream and downstream homologous arms of the bp26 gene (S1 Table). The 5'-end of the forward primer bp26-N-F and the reverse primer bp26-C-R added SacI, pstI and corresponding protective sites.

Upstream and downstream homologous arm amplification of the bp26 gene

The upstream homologous arm sequence of the bp26 gene was amplified with the bp26-N-F/bp26-N-R primers, and the downstream homologous arm sequence (bp26-C) of the bp26 gene was amplified with the bp26-C-F/bp26-C-R primers. The amplified products were recovered and purified with an agarose gel recovery kit.

The PCR reaction system comprised 10 × Ex Taq buffer, 5 μL; dNTPs (10 μM), 4 μL; upstream primer (10 μM), 2 μL; downstream primer (10 μM), 2 μL; Ex Taq polymerase, 0.5 μL; template DNA, 1 μL; and pure water, 35.5 μL.

The PCR amplification conditions comprised initial denaturation at 94°C for 5 min, denaturation at 94°C for 45 s, annealing at 58°C for 45 s and extension at 72°C for 1 min, for 30 cycles.

Upstream and downstream homologous arm junction of bp26

The bp26-N-F/bp26-C-R primers were used to introduce the bp26-N and bp26-C fragments by overlapping splice PCR. The product was recovered and purified with an agarose gel recovery kit.

The PCR reaction system comprised 10×Ex Taq buffer, 5 μL; dNTPs (10 μM), 6 μL; upstream primer (10 μM), 4 μL; downstream primer (10 μM), 4 μL; Ex Taq polymerase, 1 μL; template (bp26-N) (200 ng/μL), 1 μL; template (bp26-C) (200 ng/μL), 1 μL; and pure water, 28 μL.

The PCR amplification conditions comprised 35 cycles of initial denaturation at 94°C for 5 min, denaturation at 94°C for 50 s, annealing at 48°C for 50 s and extension at 72°C for 2 min.

Construction of the suicide vector PUC19-SacB-bp26N/C

The deletion mutant cassettes bp26-NC and suicide vector PUC19-SacB were digested with SacI and pstI restriction endonucleases, respectively, and the products were recovered and purified with an agarose gel recovery kit. The recovered deletion mutant cassette bp26-NC was ligated with the vector PUC19-SacB with T4 ligase. The ligated product was transformed into DH5a competent cells through thermal shock. The plasmid was extracted with a plasmid extraction kit and examined via PCR amplification and restriction enzyme digestion. The products were sent to Sangon Biosciences (Shanghai, China) for sequence validation, and the plasmid was named PUC19-SacB-bp26N/C.

The reaction system comprised 10× buffer, 5 μL; SacI, 1 μL; pstI, 1 μL; DNA template, 25 μL; and pure water, 18 μL, and was incubated at 37°C overnight.

The ligation system comprised 10× T4 ligase buffer, 1 μL; fragment, 3 μL; carrier, 1 μL; T4 DNA ligase, 1 μL; and pure water to 10 μL, and was incubated at 16°C overnight.

Construction and screening of the Brucella *MB6Δbp26 strain*

The MB6 strain was transferred to 100 mL TSB and cultured at 200 rmp at 37°C. When the OD_{600} reached 0.4–0.6, the

bacterial precipitate was collected, washed and resuspended in 10% glycerol solution. Products were stored at −80°C.

A total of 3 μg of PUC19-SacB-bp26N/C plasmid DNA was added to 100 μL of competent cells and incubated on ice for 30 min. Then the mix was added to the electroporation cup for electroporation. After shocking, preheated TSB medium was immediately added to resuscitate cells at 37°C at 140 r/min for 24 hours. After resuscitation, all products were inoculated on a TSA plate containing 50 μg/mL ampicillin and cultured at 37°C for 5–7 days.

Afterward, single colonies were picked and cultured in TSB medium without antibody overnight. The products were then diluted and inoculated onto TSA plates containing 5% sucrose and cultured at 37°C for 5–7 days. The single colony was randomly selected and identified with the PCR primers bp26J-F/bp26-J-R. Finally, the strain selected for further studies was named *Brucella* MB6Δbp26.

Construction and screening of the Brucella *MB6Δbp26ΔwboA strain*

The wboA suicide vector PUC19-SacB-WboAN/C and *Brucella* MB6Δbp26 competent cells were prepared as described above.

A total of 3 μg PUC19-SacB-wboAN/C plasmid DNA was added to 100 μL *Brucella* MB6Δbp26 competent cells for 30 min in an ice bath. Then the mix was added to the electroporation cup for electroporation. After shocking, the preheated TSB medium was immediately added to resuscitate cells at 37°C at 140 r/min for 24 hours. After resuscitation, all products were inoculated on a TSA plate containing 50 μg/mL ampicillin and cultured at 37°C for 5–7 days.

The culture and identification methods were as described above. Finally, the strain selected for further studies was named the *Brucella* MB6Δbp26ΔwboA strain (RM6).

Gene identification

The acquired PCR products in each step were used as templates for another round of PCR with the indicated specific primers (S1 Table). The PCR products were identified by 1% agarose gel electrophoresis.

The reaction system comprised 10×PCR buffer, 2.5 μL; dNTP, 2 μL; Ex Taq DNA polymerase (2.5 U/μL), 0.3 μL; primer (10 μM), 1 μL; template, 1 μL; and purified water to 25 μL.

The reaction conditions comprised initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 1 min, for a total of 30 cycles.

Biological identification of the *Brucella* gene deletion strain

Bacterial morphology

The RM6 strain was inoculated into TSA medium and cultured at 37°C. The morphological characteristics were observed at 24 h, 48 h, 72 h and 96 h. The bacterial cells were gram-stained and visualized under a light microscope. Experiments were repeated three times, in triplicate each time.

Determination of proliferative ability

Single colonies were picked and inoculated into 2 mL TSB for 36 hours at 37°C and 200 rpm, and this was followed by 20 subcultures under the same conditions. T1, T5, T10, T15 and T20 generations were cultured in 100 mL of TSB at 37°C at 200 rpm. The growth curve of the bacteria was constructed from the measured OD_{600} at 12 h, 24 h, 36 h, 48 h, 60 h and 72 h. Experiments were repeated three times, in triplicate each time.

Phenotypic identification and serum agglutination tests

The hydrogen sulfide, bacteriostasis and agglutination tests, and crystal violet staining were performed with conventional methods. Experiments were repeated three times, in triplicate each time.

Gene identification

The genomes of RM6 generations and the wild MB6 strain were extracted as templates with a bacterial genome extraction kit. Specific primers are shown in Supplementary Table 1. The reaction conditions and reaction system were as in **1.8**. Experiments were repeated three times, in triplicate each time.

Virulence tests

According to the World Organization for Animal Health method for determination of virulence with the S19/Rev.1 strain, we determined the virulence of the RM6 deletion strain to mice by using a live brucellosis vaccine (S2 strain) as a control. Male and female Bal B/C mice weighing 18–22 g were selected, and 50 m of suspension was used to infect each mouse with an inoculum of 2.5×10^8 CFU MB6Δbp26ΔwboA strain or S2 vaccine. All injections were administered intraperitoneally. Each measurement was repeated at least three times.

Genetic stability tests

A group of mice was inoculated with RM6 strain under the above conditions. These mice were euthanized 14 days after inoculation, and the spleens were collected aseptically and mixed with physiological saline at 1:2 (w/v) to obtain the subculture inoculation, which was denoted the second generation. A new group of mice was inoculated with the second generation, and each generation was repeatedly sub-cultured in the same manner. After inoculation, the mice were followed to assess clinical manifestations, and the genotype and phenotype features of the strain from each generation were compared. Each measurement was repeated at least three times.

A group of sheep was inoculated subcutaneously 1×10^{10} CFU at the base of the neck and euthanized 30 days after inoculation. Lymph nodes (anterior shoulder LN, groin LN, bronchus LN, mammary gland LN and submandibular gland LN) were collected aseptically. The other methods and conditions were as described above.

Safety tests

Vaccine preparation

The RM6 strain and *Brucella* vaccine S2 were diluted appropriately. The inoculation dose of each experimental group is shown in S2 Table.

Animal immunity

Healthy sheep (1–2 years of age) and pregnant sheep (day 75–90 of pregnancy) were selected and inoculated subcutaneously at the base of the neck.

Clinical characterization

The animals were observed for 60 days after inoculation, including pregnancy, mental state, diet and behavioral activities. Their body temperatures were measured continuously for 14 days after inoculation.

Pathological changes

At the end of the observation period, the sheep were euthanized. Representative sections of tissues were collected, including the heart, liver, spleen, lungs, kidneys, and lymph nodes, and H & E staining was used to evaluate pathological changes in these tissues.

Proliferation and distribution of RM6 in sheep after inoculation

Sheep were euthanized on the 30^{th}, 60^{th}, 90^{th}, 120^{th}, 150^{th} and 180^{th} days after inoculation. Heart, liver, spleen, lung, kidney, lymph node (anterior shoulder L, groin L, bronchus L, breast L and submandibular gland L), breast and uterus tissues were collected aseptically and mixed with physiological saline at 1:5 (w/v). Grinding conditions were set according to the instructions of the grinding instrument. After homogenization, 100 μL of homogenate was inoculated on a *Brucella* selective medium plate at 37°C for 10 days. Bacterial count measurements were performed with the plate-count method.

Excretion of RM6 in sheep after inoculation

Urine and feces were continuously collected to detect the excretion of bacteria within 30 days. Fecal samples (per 100 mg) were added to 1 mL of phosphate buffered saline and allowed to stand for 30 min; urine samples were diluted appropriately. All samples were inoculated on a *Brucella* selective medium plate at 37°C for 10 days. A single colony was selected and inoculated into TSB medium.

The genomic DNA of bacteria was extracted to detect the vaccine strains in excreta by PCR detection. Placenta, colostrum, spleen, liver, lymph node, blood and milk samples of pregnant sheep were collected to detect the vaccine strains with the method described above.

Immunogenicity

Sera from the sheep were collected on the 7^{th}, 14^{th}, 30^{th}, 60^{th}, 90^{th}, 120^{th} and 150^{th} days after inoculation. The antibody positive conversion rate was determined with the standard tube agglutination test, and the antibody titer of positive serum was detected with enzyme linked immunosorbent assay performed according to standard procedures.

Protective immunity

Immune protection of RM6 in mice

According to the World Organization for Animal Health methods, the protective immunity of RM6 strain in mice was evaluated by protection unit determination, and the live brucellosis vaccine (S2 strain) was used as the control.

Immune protection of RM6 in sheep

Individual sheep and pregnant sheep were observed daily for clinical signs until 60 days after immunization. Then the *Brucella* M28 WT strain was subcutaneously inoculated at 1×10^8 CFU per sheep. At 30 days after the challenge, sheep were euthanized, and the liver, spleen, left and right submandibular glands, anterior shoulder, inguinal lymph nodes, mesentery, vaginal secretions and placenta were collected aseptically from aborted sheep and mixed with physiological saline at 1:5 (w/v). All samples were homogenized completely with tissue grinders.

The homogenates (100 μL) were inoculated on TSA medium at 37°C for 3–5 days. When one or more M28 strains were isolated from any of the above tissue samples, the sheep were considered infected or unprotected. The protection rate was determined as follows: protection rate (%) = 100 (total number of animals-infected animals)/total number of animals.

Ethics

Experimental procedures were approved by the Animal Ethics Committee of PLA Military Science and complied with the Chinese Code of Practice for the Care and Use of Animals for Scientific Purposes (IACUC-DWZX-2015-030).

Statistical analysis

Statistical analyses were performed in SPSS 22 software. Comparisons between two groups were performed with two-tailed Student's t-test. One-way analysis of variance was used to compare three or more groups. $p < 0.05$ was considered significant.

RESULTS

Construction and identification of the *Brucella* gene deletion strain MB6Δbp26ΔwboA (RM6)

Our group successfully constructed the rough type *Brucella* MB6Δbp26ΔwboA gene deletion strain. The bp26 gene sequences at 58–582 bp and 1–897 bp were deleted (S4 Table) by insertion of the suicide vectors PUC19-SacB-bp26N/C and PUC19-SacB-wboAN/C into MB6 (Fig 1A, B).

First, the *Brucella* MB6Δbp26 strain was constructed and identified. The upstream homologous arm bp26-N of 504

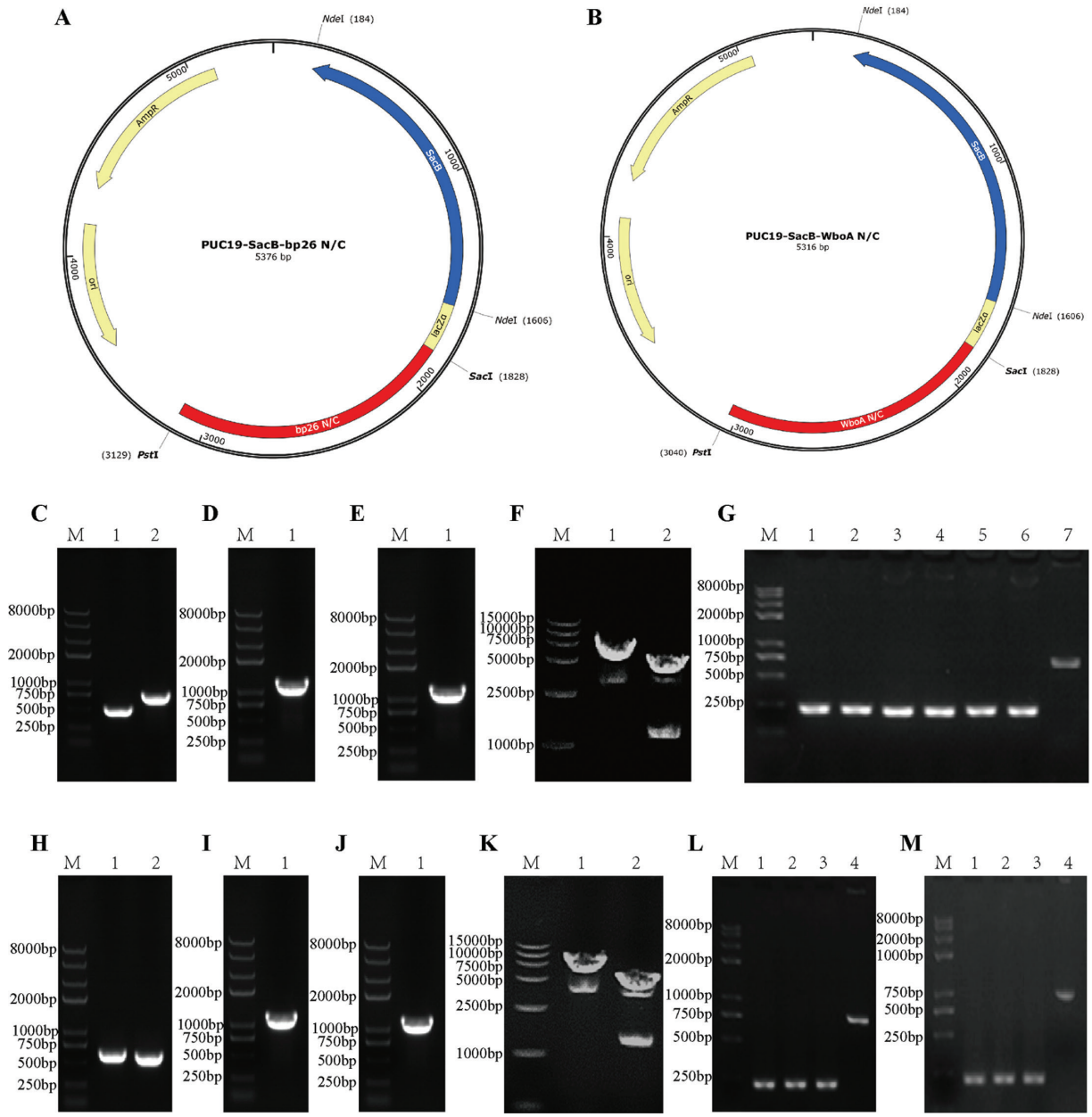

FIGURE 1 | Construction of the *Brucella* deletion mutant RM6Δbp26ΔwboA.
A. Schematic representation of the construction of the recombinant vector PUC19-SacB-bp26N/C. B. Schematic representation of the construction of the recombinant vector PUC19-SacB-wboAN/C. C. Up- and downstream homologous arms of the gene bp26, respectively. 1, upstream homologous arm of bp26 (bp26-N); 2, downstream homologous arm of bp26 (bp26-C). D. Overlapping regions of bp26 amplified by PCR (bp26-NC). E. PCR identification of the recombinant plasmid PUC19-SacB-bp26N/C (1266 bp). F. Identification of recombinant PUC19-SacB-bp26N/C plasmid double and single enzyme digestion. 1: SacI single enzyme digestion; 2: SacI and PstI double enzyme digestion. G. Identification of the recombinant strain *Brucella* RM6Δbp26 by PCR. 1–6: PCR results for RM6Δbp26 with primer bp26-J-F/bp26-J-R (228 bp); 7: PCR results for the MB6 wild strain with primer bp26-J-F/bp26-J-R (753 bp). H. Up- and downstream homologous arms of the gene wboA, respectively. 1, the upstream homologous arm of wboA (wboA-N); 2, the downstream homologous arm of wboA (wboA-C). I. Overlapping regions of bp26 amplified by PCR (wboA -NC). J. PCR identification of recombinant plasmid PUC19-SacB-wboAN/C (1209 bp). K. Identification of recombinant PUC19-SacB-wboAN/C plasmid double and single enzyme digestion. 1: SacI single enzyme digestion; 2: SacI and PstI double enzyme digestion. L. Identification of the recombinant strains *Brucella* RM6Δbp26ΔwboA by PCR. 1–3: PCR results of the RM6Δbp26ΔwboA with primer bp26-J-F/bp26-J-R (228 bp); 4: PCR results of the MB6 wild strain with primer bp26-J-F/bp26-J-R (753 bp). M. Identification of the recombinant strains *Brucella* RM6Δbp26ΔwboA by PCR. 1–3: PCR results of the RM6Δbp26ΔwboA with primer wboA-J-F/wboA-J-R (225 bp); 4: PCR results of the MB6 wild strain with primer wboA-J-F/wboA-J-R (1122 bp).

bp was amplified with the primers bp26-N-F/bp26-N-R, and the downstream homologous arm bp26-C of 762 bp was amplified with the primers bp26-C-F/bp26-C-R (Fig 1C). The upstream and downstream homologous arms of the bp26 gene were used as templates, and the 1266 bp fragments were amplified by overlapping PCR with the

primers bp26-N-F/bp26-C-R (Fig 1D). The recombinant plasmid PUC19SacB-bp26N/C was used as a template, and the fragment of 1266 bp was amplified with the primers bp26-N-F/bp26-C-R (Fig 1E). The recombinant plasmid PUC19-SacB-bp26N/C was digested with SacI and pstI to obtain two fragments of 4108 bp and 1266 bp (Fig 1F). The specificity of the gene deletion strain MB6Δbp26 of *Brucella* was identified, and a target band of approximately 228 bp was amplified (Fig 1G), in agreement with the expected results.

Second, the recombinant plasmid PUC19-SacB-wboA N/C was constructed and identified with the same method described above (Fig 1H-K).

Finally, the *Brucella* MB6Δbp26ΔwboA gene deletion strain was constructed and identified, and the target bands of approximately 228 bp and 225 bp were amplified (Fig 1L, M). The results were consistent with the expected results.

Biological characteristics of the *Brucella* RM6 gene deletion strain

The RM6 gene deletion strain was inoculated on TSA at 37°C. At 72 hours after inoculation, small translucent colonies were visible. At 96 hours after inoculation, the colonies were mostly circular, smooth and translucent. Gram staining revealed that the bacteria were gram-negative, ball-shaped, and monodisperse, with no flagella, spores or capsules (Fig 2A). The RM6 strain grew well in medium containing thionine (20 μg/ml) and alkaline fuchsin (20 μg/ml), and the H_2S test was negative (Table 1). Agglutination assays yielded positive results; agglutination occurred in a reaction with 0.2% acridine yellow; and crystal violet staining was observed. The RM6 deletion strain agglutinated with R serum but not with S, A or M single factor serum.

The RM6 deletion strain was subcultured for 20 generations in TSB liquid medium. The generations F1, F5, F10, F15 and F20 were inoculated into TSB medium in the same proportion and under the same culture conditions.

All generations demonstrated comparable growth rates. The stable growth period was 36 h, and the bacterial counts exceeded 10^9CFU/ml. The RM6 strain showed good proliferative ability, and the gene deletion did not affect linear growth and reproduction (Fig 2B). Each generation strain was inoculated on TSA medium at 37℃. At 24 h after inoculation, only traces of bacteria were observed. At 48 h after inoculation, needle-tip-sized, translucent colonies were observed. At 72 h after inoculation, clear colonies (1 mm) and smooth, slightly yellowish-white colonies were observed, in agreement with the morphology of the primary bacterial strain. The PCR genomic amplification products of each generation strain were the same, and the target bands of 228 bp and 225 bp were successfully amplified (Fig 2C, D), in agreement with the morphology of the primary bacteria strain.

In addition, the *Brucella* RM6 strain was passaged in mice five times, and no restoration of virulence was observed. With increasing inoculation time, the number of spleen bacteria gradually decreased. At week 8, the bacteria were completely cleared. In sheep, the RM6 strain was detected after five blind passages but was undetectable after four passages. No differences were apparent among the generations.

In summary, the *Brucella* RM6 deletion strain presented stable biological characteristics.

Safety evaluation

After RM6 inoculation, the mice were in good condition and showed no adverse reactions during the experiment. However, mice inoculated with the S2 vaccine strain were in poor condition, and one died 2 days after inoculation. On the fourth day after inoculation, the hair luster was gradually restored. In addition, the RT_{50} of the RM6 strain was 2.83 weeks, whereas that of the S2 strain was 4.16 weeks (Fig 2E). Thus, the results indicated that the RM6 strain, compared with the S2 strain, had lower toxicity and was easily eradicated from the body by the immune system. In the normal dose group, the sheep and pregnant sheep experienced no adverse effects. The mean body temperature was 38.5±0.5°C. No difference was observed between a single dose and multiple doses (Fig 2F). The sheep and pregnant sheep inoculated with a overdose of the RM6 strain had no clinical symptoms of shortness of breath, loss of appetite, retardation or lameness, except for a transient elevation of temperature in the RM6 and S2 groups (Fig 2G, H). Compared with the control group, the inoculated group showed no clear pathological changes in the heart, liver, spleen, lungs, kidneys or lymph nodes (Fig 2I). After overdose inoculation of pregnant sheep, only one sheep had an abortion on the 7th day, and the rest delivered normally. Moreover, the lambs had good vitality, normal physical signs and stable weight gain. The other pregnancy ewes in this group remained well, and no clinical symptoms, such as death, lethargy, shortness of breath, or slow movement were found; moreover, no redness, swelling or ulceration were found at the injection sites. However, all pregnant sheep inoculated with the overdose S2 vaccine strain aborted, showing symptoms such as dyspnea, retardation, myoplegia, redness, swelling and ulceration. All samples of vaginal secretions, placenta and stomach contents of aborted sheep showed positive bacterial isolation results, and only *Brucella* S2 was identified by PCR, thus demonstrating no cross infection among sheep. In summary, the safety of the RM6 strain was better than that of the S2 vaccine strain, and overdose inoculation of sheep and pregnant sheep was found to be safe.

Various sheep tissue samples were collected under sterile conditions on the 30th day after inoculation. The results indicated that RM6 deletion strains were distributed mainly in the lymph nodes and uterine tissues (Fig 2J). The RM6 deletion strain was isolated from the lymph nodes of only one sheep 60 days after inoculation, and was not isolated 90 days after inoculation. Our findings suggested that the RM6 deletion strain had an excellent safety profile.

Within 30 days after inoculation, no bacteria were isolated from the feces and urine. After delivery, no bacteria were isolated from the placenta, colostrum, spleen, liver, lymph nodes, blood and milk samples from sheep, and PCR

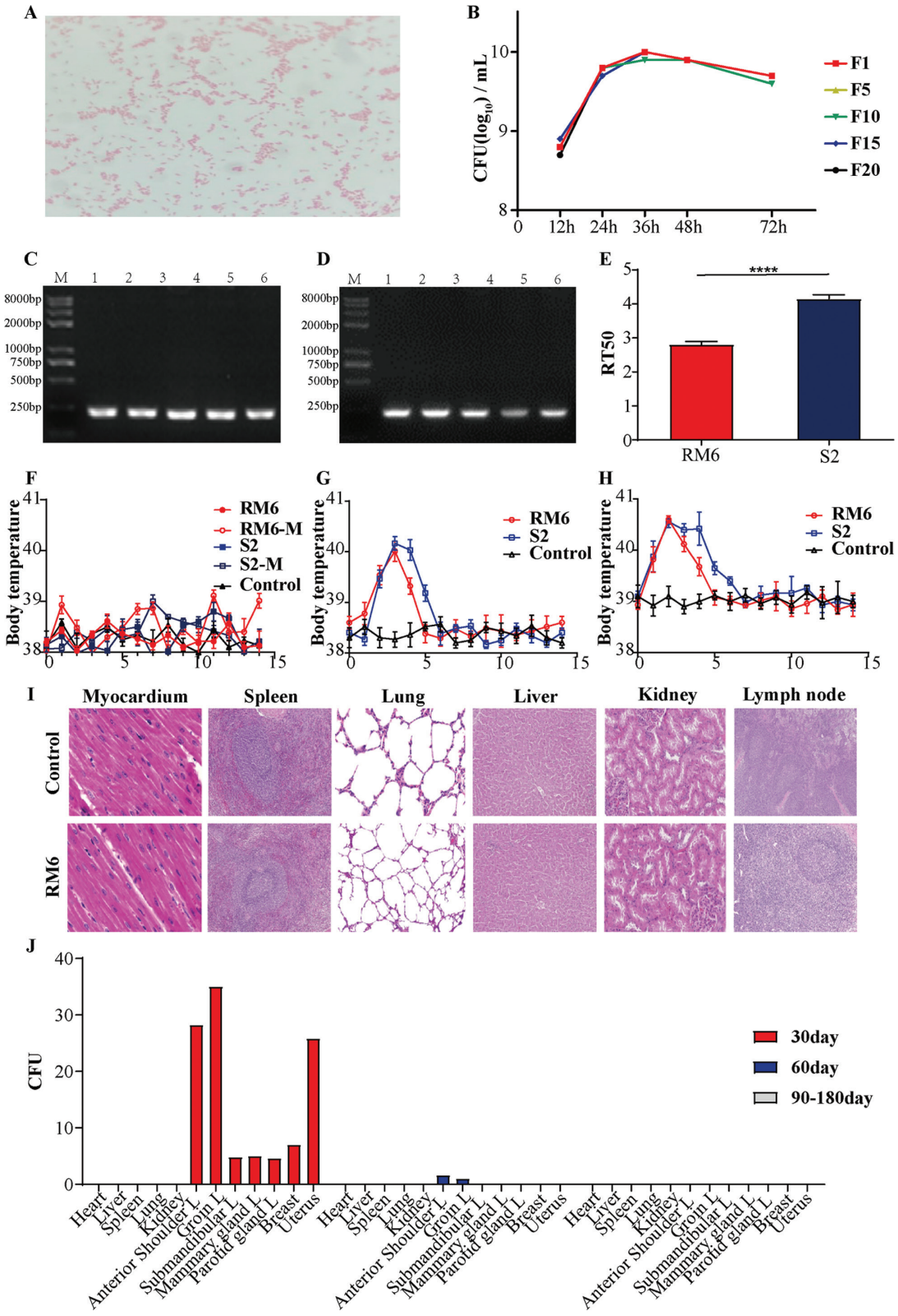

FIGURE 2 | Physiological characteristics of the *Brucella* deletion mutant RM6Δbp26ΔwboA.
A. Gram staining of RM6Δbp26Δwbo. B. Growth curves of various generations of RM6Δbp26Δwbo. C&D. Genotype identification of various generations of RM6Δbp26ΔwboA. 1–5: RM6Δbp26ΔwboA F1, F5, F10, F15 and F20. PCR with primer bp26-J-F/bp26-J-R (left); PCR with primer bp26-J-F/bp26-J-R (right). E. Virulence assays of RM6Δbp26ΔwboA on mice (RT50), expressed as mean ± SD. Significance values: ****$p < 0.0001$. F. Change in body temperature of susceptible sheep immunized with a regular dose, expressed as mean ± SEM. G. Change in body temperature of susceptible sheep immunized with a overdose, expressed as mean ± SEM. H. Change in body temperature of susceptible pregnant sheep immunized with a overdose, expressed as mean ± SEM. I. Pathological sections of the heart, spleen, lung, liver, kidneys and lymph nodes in sheep immunized with a overdose, expressed as mean ± SEM. G. In vivo distribution and bacterial burden in organs of immunized sheep.

TABLE 1 | Biochemical characterization and phenotype identification of *Brucella* RM6Δbp26ΔwboA

Inhibition test	Thionin (20 μg/ml)		Basic fuchsin (20 μg/ml)	
	+		+	
H2S test	–			
Thermal agglutination test	+			
Acriflavine agglutination test	+			
Crystal violet staining	+			
Serum specific	S	R	A	M
	–	+	–	–

"R" represents rough-type, "S" represents smooth-type; "+" represents positive agglutination reaction, "–" represents negative agglutination reaction.

detection of the deleted gene (bp26/wboA) was negative. Thus, the RM6 deletion strain appears to have good environmental safety.

Immunogenicity and immunity protection

Immunogenicity: In the RM6 group, *Brucella* agglutination antibodies in sheep sera became positive on the 7th day, and the antibody positive conversion rate was 75%. On the 14th day, the antibody positive conversion rate reached 100% and then decreased gradually. On the 120th day, all *Brucella* agglutination antibodies in immunized sheep became negative (Fig 3A). Moreover, the bp26 antibody was negative in the RM6 group and control group. The *Brucella*-specific antibody titer indicated little fluctuation (Fig 3B). Thus, the RM6 deletion strain appears to fully activate anti-infective immunity.

Protective immunity: In the RM6 and S2 groups, the bacterial loads in the mouse spleens were significantly lower 14 days after challenge ($p<0.05$) than those in the control group (Fig 3C). Meanwhile, units of protection were induced at 2.65 log and 2.64 log ($p<0.05$) by the RM6 deletion strain and the vaccine S2, respectively. The same immunoprotective efficacy provided by RM6 and S2 was seen in mice. As shown in Fig 3D, all tissues from sheep were harvested 30 days after challenge and processed to detect infection conditions in each tissue. None of the tissues from sheep in the RM6 group had an infection, and one sheep in the S2 group had two infections in the lymph nodes. The protective rates were 100% (5/5) and 80% (4/5) in the RM6 group and S2 group, respectively. Thus, the immunoprotective efficacy of RM6 was slightly better than that of S2 in sheep. The immunoprotective efficacy of RM6 in pregnant sheep was further evaluated, and no significant adverse pregnancy outcomes associated with *Brucella* were observed after challenge in pregnant sheep, except for one case of abortion. The protective rates were 96% (29/30).

Finally, the long-term protective efficacy of RM6 was determined. Sheep were challenged on the 360th and 450th days after immunization. The protective rates were 90% (9/10) and 80% (8/10) on the 360th day and 450th day (Fig 3E, F), respectively. This finding indicated that the RM6 deletion strain confers lasting immunological memory and long-term immunoprotective efficacy.

In conclusion, the *Brucella* RM6 deletion strain has high application value and provides favorable immunoprotective efficacy in mice, sheep, and pregnant sheep.

DISCUSSION

Brucellosis is a disease caused by *Brucella*. A very serious zoonotic disease, brucellosis is widespread globally, and has caused large economic losses to the livestock industry, and severely threatened human health and public safety [2-4]. Vaccination is the most effective protection measure against brucellosis. However, owing to the severe intrinsic drawbacks of current live *Brucella* vaccines, vaccination is restricted in many countries prone to brucellosis [7,24]. Moreover, all vaccine-induced antibodies can affect the interpretation of serological test results [25-27], except those elicited by the RB51 vaccine. Furthermore, no vaccine has been approved to prevent human infections by any *Brucella* species. Therefore, new vaccines, particularly marker vaccines, are required to improve protection against brucellosis.

Through homologous recombination, we knocked down the genes bp26 (58–582 bp) and wboA (1–897 bp) in the *Brucella* RM6 strain. After multiple rounds of selection, a rough *Brucella* spp. strain was generated, denoted the *Brucella* MB6Δbp26ΔwboA gene-deletion strain (RM6). Bp26 is a strong *Brucella* immunogen considered to be a diagnostic biomarker for distinguishing immunization from natural infection. Some studies have indicated that mutation or knockout of the bp26 gene has no effect on biological characterization and protective immunity, and can decrease the bacterium's virulence [20-23]. In addition, the sera of animals inoculated with the bp26 deletion strain do not contain bp26 specific antibodies. Our results supported these conclusions. Overall, bp26 is an ideal marker for a *Brucella* gene recombinant vaccine. The gene wboA encodes an essential glycosyltransferase associated with production of the O polysaccharide (O antigen) for lipopolysaccharide biosynthesis, and it is involved in colony morphology variations [16-19]. Virulent rough strains attenuated by wboA

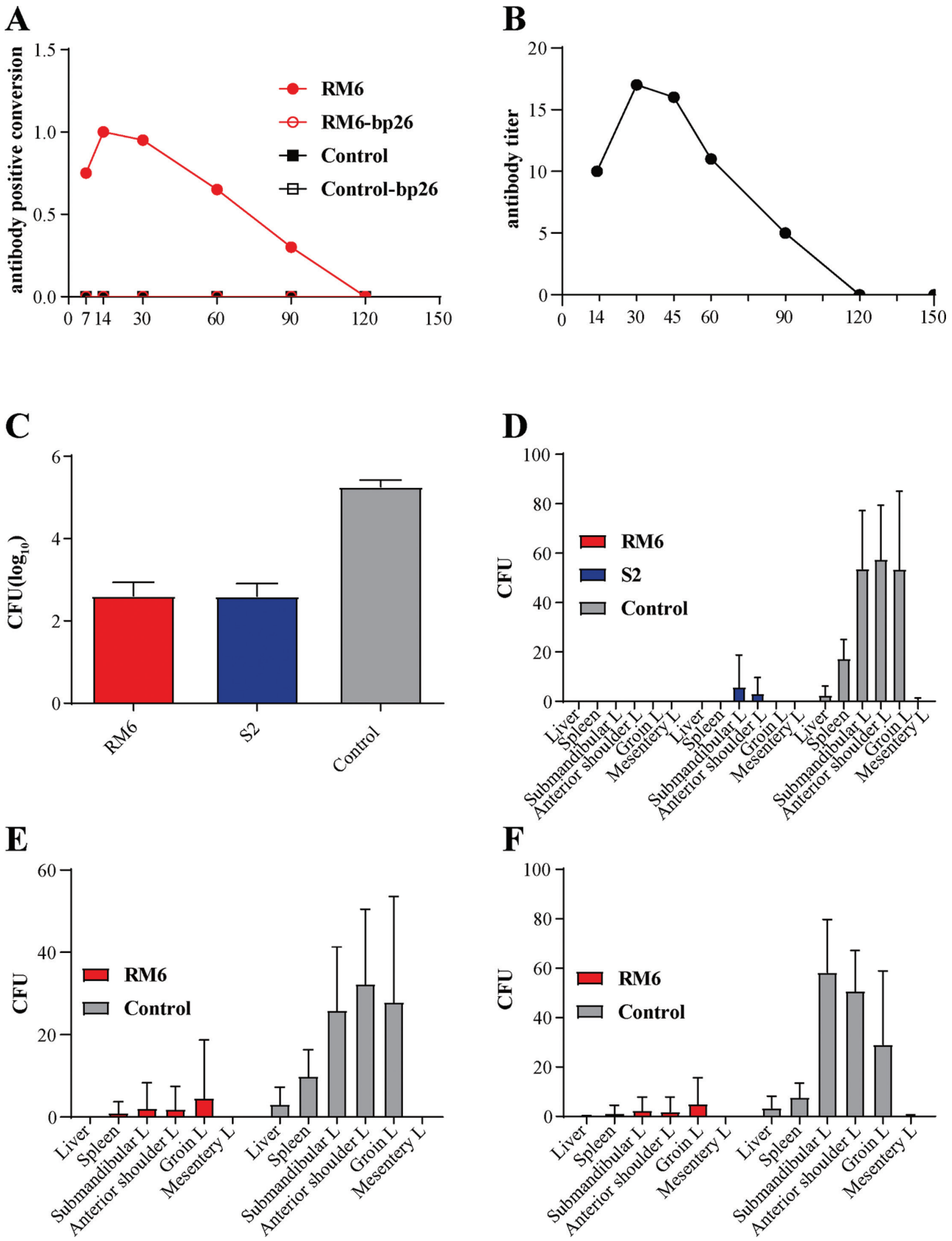

FIGURE 3 | Immunological evaluation of RM6Δbp26ΔwboA.
A. Antibody positive conversion in sheep. B. Antibody titers in sheep. C. Protective effects of RM6Δbp26ΔwboA and S2 immunization in mice, expressed as mean ± SD. D. Protective effects of RM6Δbp26ΔwboA and S2 immunization in sheep, expressed as mean ± SD. E. In vivo distribution and bacterial burden in organs of immunized sheep 360 days after inoculation, expressed as mean ± SD. F. In vivo distribution and bacterial burden in organs of immunized sheep 450 days after inoculation, expressed as mean ± SD.

mutation, knockout or silencing can induce cellular and humoral immune responses, but do not induce production of anti-lipopolysaccharide-O antibody. Therefore, wboA is a candidate gene for differentiating immunity from natural infection.

Our experiments demonstrated that the RM6 deletion strain had stable biological characteristics and strong immunogenicity, and induced long-lasting protective immunity, particularly in pregnant sheep. Compared with the S2 vaccine strain, RM6 knockout strains showed better protection against *Brucella* infection and showed faster clearance by the immune system.

Although gene-deleted marker vaccines have been shown to produce favorable immunogenicity and protective immunity in many vaccine studies [11-14], the biosecurity and genetic stability require further evaluation. Restored expression of these deleted genes might potentially result in full or partial recovery of virulence, thus posing a potential threat to vaccinated people. In our study, the virulence recurrence risk was decreased to the greatest extent with bp26 and wboA double knockout. Furthermore, no *Brucella* DNA was detected from a variety of secretions and excreta from immunized animals. Our findings suggested that RM6 does not have the possibility of horizontal propagation and vertical transmission, and has a highly favorable safety profile for target animals, nontarget animals, susceptible populations and the environment.

In conclusion, the *Brucella* RM6 deleted strain may have practical application value and important implications for effective control of brucellosis.

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CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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