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Research Article

An Novel Strategy for Brucella Differential Vaccine Combined with Colloidal Gold Immunochromatographic Strips Based on Mutants of *Brucella melitensis* M5-90

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Abstract

There is a requirement to develop an novel vaccine to distinguish between vaccinated and infected animals after vaccination. Two mutants of *Brucella melitensis* M5-90Δ*bp*261 and M5-90Δ*bp*262, knockout of the fragments amino acids 55-152 and 22-185 respectively, were primarily generated. Then the two mutants were inoculated 7 weeks old BALB/c mice with 3.0×10^6 CFU/0.2 mL parent or mutant strains, Serum samples were evaluated by Rose Bengal plate tests, serum agglutination tests, colloidal gold immunochromatographic strips (ICS) coated with VirB5 or BP26 and indirect enzyme-linked immunosorbent assays. Spleen tissue samples were used to determine *B. melitensis* abundance at 10, 20, 30, and 40 days post-immunization (dpi). The serological results showed that the parent and mutant strains elicited a weak immunological reaction at 10–30 dpi. Fewer colony forming units (CFUs) were recovered from spleens in mutant strains group than the parent strain group during 20-40 post-immunization (dpi). The ICS were able to distinguish between the sera from mice immunized with either a parent or mutant strain of *B. melitensis*. These results indicate that the two mutants strain of *B. melitensis* have potentially perspectives in distinguishing infected animals from vaccination combined with ICS in this study.

Keywords: *Brucella melitensis, Distinguish, Vaccine, Colony forming units, ICS*

Brucella melitensis M5-90 Mutantları Temelli Kolloidal Altın İmmunokromatografik Şerit İle Kombine Brucella Ayırıcı Aşı İçin Yeni Bir Strateji

Özet

Aşı sonrası aşılı ve enfekte hayvanları ayırt edebilecek yeni bir aşının geliştirilmesine ihtiyaç vardır. Öncelikli olarak *Brucella melitensis*'in iki mutantı, sırasıyla 55 ile 152 arası ve 22 ile 185 arası amino asitleri çıkarılmış olan M5-90Δbp261 ve M5-90Δbp262, üretildi. Sonrasında, her iki mutant da 7 haftalık BALB/c farelere 3.0×10^6 CFU/0.2 mL miktarında inokule edildi. Serum örnekleri Rose Bengal lam testi, serum aglutünasyon testi, VirB5 veya BP26 ile kaplı kolloidal altın immunokromatografik şerit (ICS) ve indirek enzim bağlı immunsorbent assay ile değerlendirildi. Dalak doku örnekleri immunizasyon sonrası 10, 20, 30 ve 40. günlerde *B. melitensis* varlığını belirlemek amacıyla kullanıldı. Serolojik sonuçlar doğal ve mutant suşların inokulasyon sonrası 10-30. günler arasında zayıf immünolojik reaksiyon oluşturduğunu gösterdi. İnokulasyon sonrası 20-40 günler arasında dalaktan mutant suşların gruplarından doğal suş grubuna oranla daha az koloni oluşturan birim (CFU) belirlendi. ICS *B. melitensis*'in doğal ve mutant suçlar ile immunize edilmiş farelerin serumlarını ayırt edebildi. Elde edilen sonuçlar, her iki *B. melitensis* mutant suşun enfekte hayvanlar ile aşılanmış hayvanların ayırt edilmesinde ICS ile kullanıldığında başarılı olabileceğini göstermiştir.

Anahtar sözcükler: *Brucella melitensis, Ayırt etme, Aşı, Koloni oluşturan birim, ICS*

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INTRODUCTION

Brucellosis is a major bacterial zoonosis caused by members of the *Brucella* genus [1]. This infectious disease occurs worldwide, has resulted in significant economic losses, and also is a public health concern to humans, particularly in developing countries [2]. In 2012, the Chinese Center for Disease Control and Prevention reported 39,515 new cases of human brucellosis, with this number increasing by 10% each year. Approximately 85% of cases were caused by *Brucella melitensis via* direct contact with infected sheep or goats, or their products [3]. Vaccination is considered the most effective strategy for preventing brucellosis in animals. In China, the *B. melitensis* M5-90 strain has been widely used as a live attenuated vaccine for sheep and goats.

Application of the *B. melitensis* M5-90 vaccine strain is considered to be an important factor that led to a rapid decline in the incidence of brucellosis in animals and humans in China from the 1970s to the 1990s [4]. The vaccine M5-90 strain has been shown to confer protective effects against brucellosis [5]. However, it is difficult to distinguish between vaccinated and infected animals.

Clinical tests have revealed that the whole *Brucella* antigen is associated with false-positive results, due to serological cross-reactivity with other Gram-negative bacteria [6]. Various *Brucella*-dominant antigens have been used to increase the specificity of brucellosis diagnoses. As an example, the BP26 protein of *Brucella* species is a periplasmic protein that is highly immunogenic [7]. Seco-Mediavilla and colleagues characterized the antigenicity of BP26 using a panel of 18 BP26-specific monoclonal antibodies and recombinant fragments of the protein itself [8]. They found that amino acids 55-152 and 1-191 of BP26 were the immunodominant epitopes of this protein.

Previously, we generated *B. melitensis* strain M5-90r*bp26*, in which BP26 was deleted. This *B. melitensis* mutant elicited a weak serological reaction in mice and sheep [9]. To develop better candidate vaccines, and to differentiate infected and vaccinated animals, here we generated mutants of *B. melitensis* M5-90 based on BP26 partial fragments, the amino acids 55-152 and 22-185 respectively, and evaluated the immune responses of the BP261 and BP262 mutants in mice. Additionally, we sought to develop immunochromatographic strips (ICS) to discriminate between vaccinated and infected animals.

MATERIAL and METHODS

Bacterial Strains and Culture Conditions

We used *B. melitensis* strain M5-90 (Xinjiang Tiankang Animal Biotechnology Co. Ltd., China) as the parental vaccine strain, while *Bacillus subtilis* strain BAA12545 was used for *sacB* engineering and to screen for mutant strains by counter-selection. Bacteria were grown on *Brucella* agar or *Brucella* broth (BD Co., Sparks, MD, USA) at 37°C. To generate the *B. melitensis* M5-90 mutants, the medium was supplemented with ampicillin (100 mg/mL). Sucrose medium was used for *sacB* counter-selection as previously described [10]. *Escherichia coli* was used in the generation of M5-90Δ*bp261* and M5-90Δ*bp262* mutants, with cultures grown on Luria-Bertani (Difco/Becton Dickinson) plates or broth overnight at 37°C, with or without ampicillin (100 mg/L). *Brucella* cultures were harvested from plates using phosphate-buffered saline (PBS; pH 7.2) after 3 days. Based on optical density (OD) readings from a Klett meter and a standard curve, bacteria were centrifuged (8000 rpm) and resuspended to a final concentration (OD_{260nm}=0.18). The viability of bacteria was confirmed by serial dilution, plating, and enumeration.

Generation of BP26 Truncation Mutants

Genomic DNA from *B. melitensis* M5-90 was used as a template for the polymerase chain reaction (PCR) amplification of *bp26* fragments. The specific oligonucleotide primers which we used are summarized in *Table 1*. Flanking sequences were amplified separately, and overlap extension PCR [11] was used to combine sequences and generate fragments *bp261* (2445 bp including the DNA fragment encoding amino acids 55-152 of BP26) and *bp262* (2250 bp including the DNA fragment encoding amino acids 22-185 of BP26). Each fragment was cloned into the pMD18-T vector (TIAGEN, Beijing, China). Recombinant plasmids were digested with *Sph*I and *Sac*I, and the resulting fragments were introduced into pGEM-7zf (+) (Promega, Madison, USA). The *sacB* gene of *B. subtilis* was amplified by PCR and then inserted into pGEM-7zf (+) containing either fragment to yield pGB261 and pGB262.

The pGB261 and pGB262 vectors were introduced separately into *B. melitensis* M5-90 by electroporation as previously described, with ampicillin-resistant and sucrose-sensitive integrants selected for [12]. Two identified mutants were designated M5-90Δ*bp261* and M5-90Δ*bp262*, subcultured for 15 generations, and then subjected to western blotting analysis and various microbiological tests [12].

Bacteriological and Typing Methods

Conventional bacteriological and typing methods were carried out as described previously by Alton et al.[13]. Briefly these includes lysis by Tb, Wb, Iz, and R/C phages; urease test; CO_2 requirement; H_2S production; growth on dyes (thionin, basic fuchsin, safranin O) and oxidative metabolism tests. Then the characteristics of the two mutants strain were assessed by above methods.

Expression and Purification of BP261 and BP262

The nucleotide sequences encoding the BP261 and BP262 proteins were cloned into the pET-32a expression vector to generate pET32a-BP261 and pET32a-BP262,

Table 1. *PCR primers used for the construction and identification of the M5-90Δbp261 and M5-90Δbp262 mutants*

Primer	Primer Sequences (5'-3')
bp261-left flanking-forward	*GCATGC*TTTCTAAGCGCAGACCTTCGGG (*Sph* I)
bp261-left flanking-reverse	GTTCAAATCACCGCCCTGATTCATATCGGGCGAGGCCGTCAT
bp261-right flanking- forward	AATCAGGGCGGTGATTTGAACCT
bp261-left flanking-reverse	*GAGCTC*TACTGGTGGCATCCCCTTGTTTC (*Sac* I)
bp262-left flanking-forward	*GCATGC*TTTCTAAGCGCAGACCTTCGGG (*Sph* I)
bp262-left flanking-reverse	AGCGTCGGCAAGCGTCTTAGCGCCGACGAGCATGATT
bp262- left flanking-forward	AAGACGCTTGCCGACGCT
bp262-left flanking-reverse	*GAGCTC*TACTGGTGGCATCCCCTTGTTTC (*Sac* I)
sacB-forward	*GAGCTC*GGGCTGGAAGAAGCAGACCGCTA (*Sac* I)
sacB-reverse	*GAGCTC*GCTTATTGTTAACTGTTAATTGTCC (*Sac* I)
detecting rbp261/rbp262 -forward	TCCACAATCATGCTCGTCG
detecting rbp261/rbp262 -reverse	GCGTTTTGTATCAGGTGGC
expressing BP261-forword	*GAATTC*GCCATTCTCAATCTCTCGGTGC (*EcoR* I)
expressing BP261-reverse	*GTCGAC*AACACCGAGCGTGACGGATTC (*Sal* I)
expressing BP262-forword	*GAATTC*TTCAGCCTGCCCGCTTTCG (*EcoR* I)
expressing BP262-reverse	*GTCGAC*CGCTTGGCAATGGCATTG (*Sal* I)

respectively. Plasmids were introduced into *E. coli* strain BL21, and expression induced with isopropyl β-D-1-thiogalactopyranoside. BP261 and BP262 contained a 6×His tag, therefore Ni-NTA column chromatography was used to purify these proteins from their lysates.

Vaccination of Mice

Female BALB/c mice (7 weeks old) were obtained from the Xinjiang Center for Disease Prevention and Control and allowed to acclimate for 3 days prior to vaccination. Three groups of mice (n = 20 per group) were inoculated intraperitoneally with 3.0×10^6 CFU/0.2 mL of either *B. melitensis* M5-90, M5-90Δ*bp261*, or M5-90Δ*bp262* in 200 µL of PBS. The fourth group was injected with 200 µL of PBS as negative control.

Detection of Immunoglobin G (IgG)

Peripheral blood samples were collected by tail incision from five mice which selected randomly in each group at 0, 10, 20, 30, and 40 days post-inoculation (dpi). Serum samples were tested for *Brucella* spp. antibodies by Rose Bengal plate test (RBPT; Qingdao Yebio Bioengineering Co., Ltd, China) as described previously [14], and using a serum agglutination test, which has also been described previously [15]. Levels of serum IgG titers with specificity to *Brucella melitensis* M5-90, M5-90Δ*bp261* and M5-90Δ*bp262* were determined by indirect enzyme-linked immunosorbent assay (iELISA) according to manufacturer's instructions (RD, Minneapolis, Minnesota, USA). Subsequently the absorbance (450 nm) was recorded using a microplate reader (Molecular devices, Sunnyvale,CA). Then the measurement value was obtained through standard curve which is used to determine the amount in an unknown sample and the standard curve is generated by plotting the average OD (450 nm) obtained for each of the 6 standard concentrations on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis. The actual value of the sample is 5-fold to measurement value. The sensitivity by this assay is 1.0 µg/mL. The cutoff value for the assay was calculated as the mean optical density at a 1:5 dilution. All of these data was analyzed using SPSS version 17.0 software and the concentration of IgG titers in serum samples were determined. Meanwhile the serum samples were tested in duplicates.

Assessment of Brucella Abundance

Five mice from each group in randomly were euthanized using diethyl ether at 10, 20, 30, and 40 dpi. Spleens were removed aseptically. Then suspended in 1 mL of sterile PBS and were homogenized using tissue grinder [16]. Ten-fold serial dilutions of the tissue homogenates were prepared in saline, and 100 µL of each dilution was plated on *Brucella* agar medium. After 3-5 days of incubation at 37°C, the *Brucella* CFUs were counted and the bacterial burden per spleen was calculated.

Preparation and Evaluation of Colloidal Gold ICS

Colloidal gold ICS were coated with either VirB5 or BP26, as outlined in our patent [No: 201410405061.6]. For each test, the ICS was immersed in diluted serum samples obtained from *B. melitensis*-infected sheep, and from mice that immunized with *B. melitensis* M5-90, M5-90Δ*bp261*, or M5-90Δ*bp262* respectively. In addition, all serum samples were evaluated by RBPT and serum agglutination tests.

Ethics

All animals used in our experiment were treated humanely and in accordance with institutional animal care guidelines. Our study was approved by the Animal Care and Use Committee of Shihezi University.

RESULTS

Generation of B. melitensis M5-90Δbp261 and M5-90Δbp262

The *B. melitensis* mutants which we generated *(Table 1)* were identified using PCR and the relevant inserted nucleotide sequences were confirmed by sequencing analysis (data not shown). The serum samples collected from mice which immunized with *B. melitensis* M5-90 were analyzed by Western blotting. We found that they reacted strongly with BP261 and BP262, while serum from mice immunized with either M5-90Δ*bp261* or M5-90Δ*bp262* failed to react with BP261 or BP262 *(Fig.1)*.

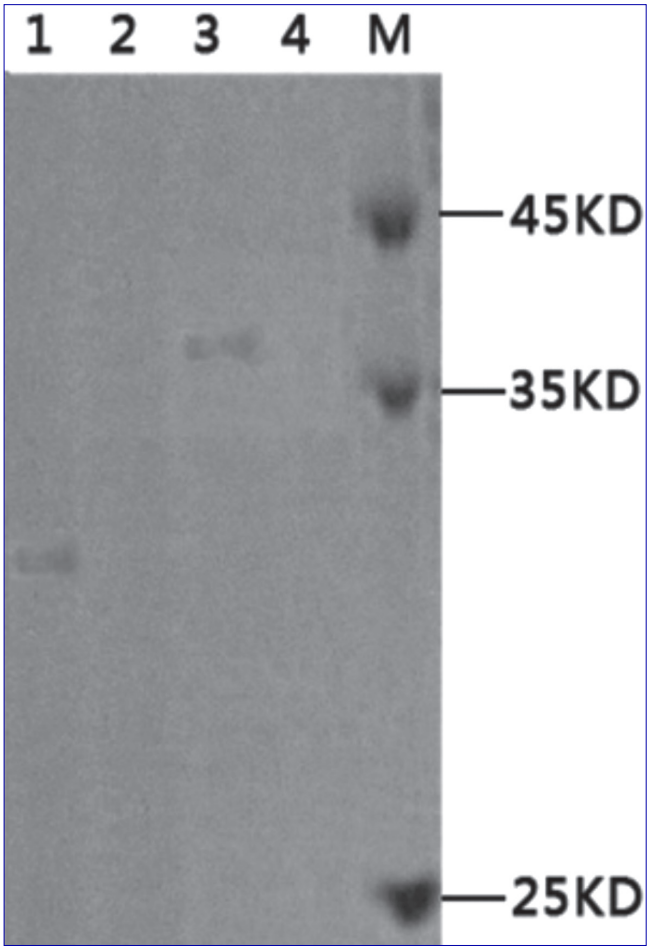

Fig 1. The result of Western blot of sera from animals which were infected with the parent or mutant strains that reacted with BP261 and BP262 (Lane 1 and Lane 3: The results from sera induced by the *M5-90* vaccine strain that reacted with BP261 and BP262 proteins; Lane 2 and Lane 4: The results from sera induced by the *Brucella* M5-90Δ*bp261* and M5-90Δ*bp262* mutants that reacted with BP261 and BP262 proteins. Lane M: Protein Molecular Weight Marker)

Phenotypic Characterization of Mutants

Microbiological testing indicated that mutants M5-90Δ*bp261* and M5-90Δ*bp262* were *B. melitensis* biotype 1. Growth rates and staining profiles for the mutants were similar to those of the parental strain.

Serum IgG Profiles

According to our iELISA results, the level of serum specific IgG titers in immunized mice ranged from 11.49 to 27.32 μg/mL. However, mice immunized with M5-90Δ*bp261* or M5-90Δ*bp262*, IgG titers were not significantly lower than those in mice immunized with M5-90 during 10 - 40 dpi [17] *(Fig. 2)*.

Residual Virulence of M5-90Δbp261 and M5-90Δbp262 Mutants

We compared the levels of viable bacteria recovered from the spleens of mice immunized with the parent or mutant strains of *B. melitensis*. Levels of bacteria in spleen tissues were slightly lower in mice immunized with the mutant strains of *B. melitensis* (M5-90Δ*bp261* and M5-90Δ*bp262*) than mice immunized with the parent strain (M5-90) *(Fig. 3)*. Bacteria recovered from spleen tissues proliferated at low levels at 0-10 dpi, peaking at 10-20 dpi for all three strains we investigated. Levels of bacteria decreased rapidly between 20 and 40 dpi, approaching 3.93-5.66 log units *(Fig. 3)*.

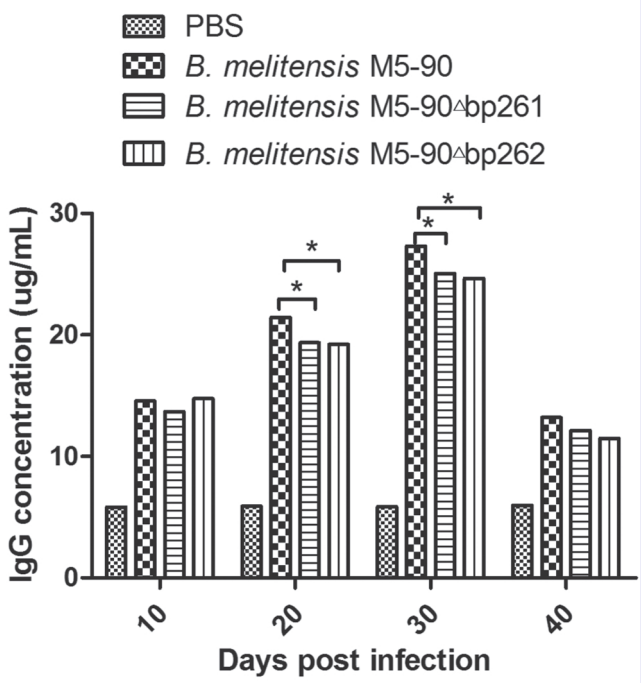

Fig 2. IgG antibody serum profiles at different time spots after immunization of BALB/c mice with *B. melitensis* M5-90 mutants

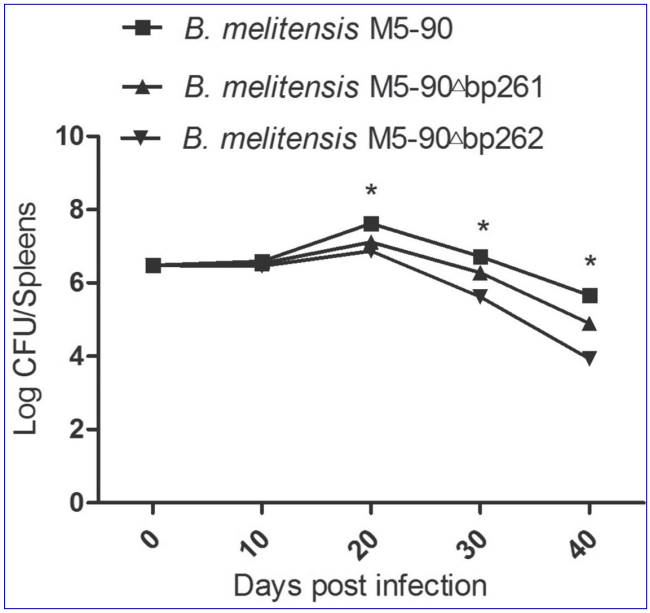

Fig 3. Replication profiles of *B. melitensis* M5-90 mutants in mice spleens

Screening Serum Samples with ICS

The colloidal gold ICS coated with VirB5 reacted with the sera from mice immunized with *B. melitensis* M5-90, M5-90Δ*bp261*, and M5-90Δ*bp262* at 10 and 30 dpi. However, ICS coated with BP26 failed to react with the sera from mice immunized with either the parent or mutant strains *(Fig. 4)*.

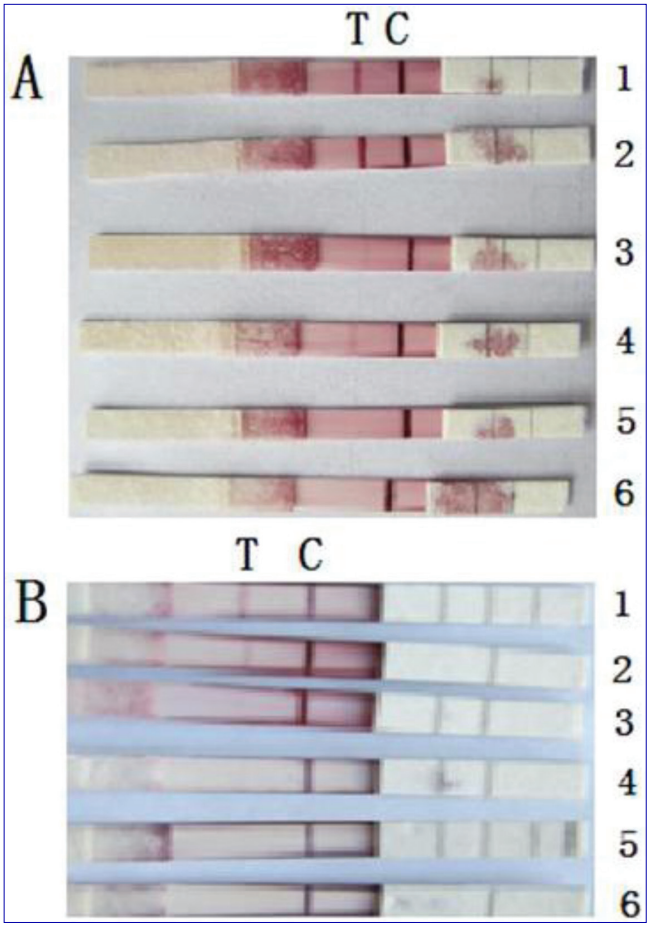

Fig 4. The result of using immunochromatographic test strips coated with VirB5 (A) or BP26 (B) protein. A. Lane 1: positive control (serum from sheep infected with *B. melitensis*). Lane 2,3,4: positive results (serum from mice immunized with *B. melitensis* M5-90, *B. melitensis* M5-90Δ*bp261* and *B. melitensis* M5-90Δ*bp262* respectively pi. 30d). Lane 5,6: negative results (serum from mice injected with PBS and brucellosis-free sheep). B. Lane 1: positive control (serum from sheep infected with *B. melitensis*). Lane 2: positive result (serum from mice immunized with *B. melitensis* M5-90 pi. 30d). Lane 3,4,5,6: negative results (serum from mice immunized with *B. melitensis* M5-90Δ*bp261*, *B. melitensis* M5-90Δ*bp262*, PBS and brucellosis-free sheep respectively pi. 30d). T = test zone, C = control zone

DISCUSSION

In previous studies, researchers have used attenuation or differential diagnostic markers to interrupt or delete genes associated with virulence or antigenicity to investigate novel vaccine strains [18,19]. The *B. abortus* vaccine strain S19, where *bp26* and *bmp18* are deleted, is attenuated and it is possible to differentiate between vaccinated and naturally infected animals [12]. In a separate study, *bp26* and/or *omp31* were found to be deleted in *B. melitensis* vaccine strain *Rev.1*. These genes encode the BP26 and OMP31 antigens and allow for serological differentiation between infected and vaccinated animals [20]. However, due to a lack of appropriate differential diagnostic methods and intellectual property issues, a candidate vaccine for brucellosis utilizing a mutant strain has not been commercially produced and marketed.

The M5-90 vaccine strain of *B. melitensis* was derived by a process that was significantly different from that used to generate the *S19* or *Rev.1* vaccine strains. The M5-90 strain was attenuated through the use of acriflavine and by consecutive subculturing in chickens and chicken embryo fibroblasts. *B. melitensis* M5-90 has been commonly used to vaccinate sheep and goats in China for at least two decades. Previously, a series of *B. melitensis* M5-90 mutants were developed where *virB2*, *WboA*, and/or *pgm* were deleted [21, 22]. To identify suitable methods for differential diagnosis, VirB2, VirB5, BP26, and OMP31 proteins were expressed, purified and coated onto ICS. Clinical tests revealed that ICS coated with VirB5, BP26, or OMP31 were specific and sensitive [23]. Evaluation of the humoral immune response in sheep and mice infected with *B. melitensis* M5-90 mutants where entire genes had been deleted revealed that only weak *Brucella*-specific antibodies were identified. In the current study, we generated truncated forms of BP26, with two fragments corresponding to epitopes omitted from the expressed proteins. However, similar results identified with the parent M5-90 strain, a weak humoral immune response was elicited in BALB/c mice immunized with M5-90Δ*bp261* or M5-90Δ*bp262*.

In a previous study, VirB5 was reported to be a

conserved protein and a serological marker suitable for brucellosis diagnosis [24]. The use of colloidal gold ICS to diagnose brucellosis is rapid and convenient [25]. The convective mass transfer of the immunoreactant to the binding partner allows the assay to be performed with no reagent handling [26]. An ICS coated with VirB5 and/or BP26 can used to test serum samples, and allowed us to differentiate between naturally infected mice and those immunized with M5-90Δ*bp261* and M5-90Δ*bp262*. We found that the sensitivity of ICS was around two-to-four fold higher than that for RBPT. Our developed methodology requires further confirmatory studies in humans and other animals, but could potentially be used in resource-poor rural communities.

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