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A *Brucella melitensis* M5-90 *wboA* deletion strain is attenuated and enhances vaccine efficacy

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ABSTRACT

Brucella spp. are Gram-negative intracellular pathogens of both humans and animals that cause great economic burdens in developing countries. Live attenuated vaccines are the most efficient means for the prevention and control of animal Brucellosis. However, *Brucella* vaccines (strain M5-90 and others) have several drawbacks and do not allow serological differentiation between vaccinated and infected animals. A *wboA* mutant was derived from *Brucella melitensis* (*B. melitensis*) vaccine strain M5-90 and tested for virulence and protective efficiency. T-cell responses ($CD4^+$, $CD8^+$), levels of immunoglobulin G (IgG), and cytokine production were observed. WboA was also assessed as a diagnostic marker for Brucellosis. *B. melitensis* strain M5-90Δ*wboA* exhibited reduced survival in murine macrophages (RAW 264.7) and BALB/c mice and induced protective immunity in mice comparable to that from the parental strain M5-90. In mice, the *wboA* mutant elicited an anti-*Brucella*-specific IgG response and induced the secretion of gamma interferon (IFN-γ) and interleukin-2 (IL-2). In sheep, M5-90Δ*wboA* immunization induced the secretion of IFN-γ, and serum samples from sheep inoculated with M5-90Δ*wboA* were negative by Bengal Plate Test (RBPT) and Standard Tube Agglutination Test (STAT). In mice, probes against WboA antigen allowed for serological differentiation between natural infection and vaccination. The M5-90Δ*wboA* mutant is a potential attenuated live vaccine candidate against virulent *B. melitensis* 16M infection. It will be further evaluated in livestock.

1. Introduction

Brucella spp. are pathogens of various domestic and wild mammals (Boschiroli et al., 2001). Within hosts, *Brucella* spp. can replicate within professional and non-professional phagocytes, resulting in heavy economic losses and human suffering (Lacerda et al., 2010). It is important to strengthen the prevention and control *Brucellosis*. At present, live attenuated vaccines are the most effective and are used worldwide for prevention of animal *Brucellosis* (Schurig et al., 2002). However, such vaccines have some inconveniences: they retain some virulence in pregnant animals manifested in abortions and milk excretion of the vaccine; they are virulent in humans; and they cause interference in the classical serological diagnostic tests (Moriyon et al., 2004). Therefore, new vaccines, low in residual virulence, highly protective, and with clear markers are required to overcome these drawbacks.

Vaccination of animals is considered the most efficient way to control *Brucellosis*. An attenuated *B. melitensis* vaccine strain M5-90, similar to another live attenuated *B. melitensis* vaccine strain Rev.1, is mostly used for vaccination of sheep and goats in China (Zhang et al., 2010). M5-90 was derived from a virulent *B. melitensis* strain M28 isolated from a sheep and serially passaged for 90 generations in chicken embryo fibroblasts (Research Group of Brucellosis, 1991). Vaccination with *B. melitensis* M5-90 is one of the important strategies that decreased the incidence of *Brucellosis* in animals from the 1970s to 1990s in China (Deqiu et al., 2002). However, the antibody responses raised by M5-90 vaccine are difficult to distinguish from those of animals naturally infected with *Brucella* using conventional serological tests. In addition, those attenuated vaccines retain residual virulence in humans and may result in abortion in sheep (Ficht et al., 2009). A potential approach to tackling these problems is to develop a marker vaccine by deletion of the virulence and/or antigenic genes from these effective parental

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vaccine strains. To develop live vaccines against *B. melitensis* that are superior to M5-90, much research is required into the deletion of virulence genes.

Brucella spp. are divided into smooth and rough phenotypes; the virulence of smooth strains is greater than that of rough strains (Reeves et al., 1996). Lipopolysaccharide (LPS) is the major antigen used in *Brucella* diagnosis (Cardoso et al., 2006). Animals infected with smooth *Brucella* can produce anti-LPS O-chain antibodies. The *wboA* gene encodes a glycosyltransferase that is essential for the biosynthesis of the *Brucella* O antigen (McQuiston et al., 1999). The deletion or mutation of *wboA* may affect the *Brucella* phenotype. *B. melitensis* 16MΔ*wboA* exhibited extreme attenuation in BALB/c mice and human monocytes (hMDMs) (Nikolich et al., 2010). Another study found that a *Brucella wboA* mutant could enhance activation of the lectin pathway of complement (Fernandez-Prada et al., 2001). Therefore, *wboA* is an important virulence factor of *Brucella*. In this report, we described the construction of a *B. melitensis* M5-90Δ*wboA* deletion strain and evaluated the roles of *wboA* in the virulence of M5-90 in RAW 264.7 macrophages and mice, and then, we also detected the immune affections of M5-90Δ*wboA* mutant in sheep, with the aim of producing a candidate vaccine against wild-type *Brucella*.

2. Materials and methods

2.1. Ethics statement

The study was approved by the Institutional Committee of Post-Graduate Studies and Research at Shihezi University, China. All efforts were made to minimize animal suffering.

2.2. Bacterial strains, plasmids and cell line

B. melitensis strain 16M and vaccine strain M5-90 were obtained from the Center of Chinese Disease Prevention and Control (Beijing, China). *Brucella* was cultured in tryptic soy agar (TSA) or tryptic soy broth (TSB) (Sigma, St. Louis, MO, USA). *Escherichia coli* (*E. coli*) strains DH5α and BL21 were purchased from Promega (Madison, WI, USA). Two strains were grown on Luria-Bertani (LB) medium. The culture media were supplemented with appropriate antibiotics (100 μg/mL ampicillin for M5-90 or 50 μg/mL kanamycin for *E. coli*) when necessary. Plasmid pGEM-7Zf$^+$ was purchased from Promega (Madison, WI, USA). The murine macrophage RAW264.7 line (obtained from Cell Resource Center, IBMS, CAMS/PUMC, Beijing, China) was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies, Rockville, MD, USA) at 37 °C with 5% CO_2 (vol./vol.).

2.3. Mice

Six-week-old BALB/c female mice were obtained from the Experimental Animal Center of the Academy of Military Medical Science (Beijing, China). Animals were maintained in barrier housing with filtered inflow air in a restricted-access room in pathogen-limited conditions. They were acclimatized for a minimum of 1 week before experimentation, and water and commercial food were provided *ad libitum*. All experimental procedures and animal care were performed in compliance with institutional animal care regulations.

2.4. Construction of the M5-90Δ*wboA* deletion mutant

The M5-90Δ*wboA* deletion mutant was constructed as described (Zhang et al., 2013) with some modifications. The sequence upstream (1000 bp) of *wboA* was amplified from *B. melitensis* M5-90 genome using the primer pair up-F and up-R (Table 1). The sequence downstream (1100 bp) of *wboA* was amplified from *B. melitensis* M5-90 genome using the primer pair dn-F and dn-R (Table 1). The two homologous arms of M5-90 *wboA* were cloned into pMD18-T Simple Vector (Takara, Japan) for sequencing and then subcloned into pGEM-7Zf$^+$ to generate the suicide plasmid pGEM-7Zf$^+$-*wboA*. SacB-F and SacB-R primers (Table 1) were designed for amplification of the *B. subtilis* SacB DNA fragment (1477 bp), which is a selectable marker gene. The PCR amplified DNA fragment (SacB DNA fragment) was subcloned into plasmid pGEM-7Zf$^+$-*wboA* to generate the plasmid pGEM-7Zf$^+$-*wboA*-SacB. Competent *B. melitensis* M5-90 was electroporated with pGEM-7Zf$^+$-*wboA*-SacB. Potential *wboA* deletion mutants M5-90Δ*wboA* were selected in the presence of 100 μg/mL ampicillin in the first screening and 5% (w/v) sucrose in the second screening. The deletion mutant was further confirmed by PCR amplification and RT-PCR sequencing, as described previously (Wang et al., 2009). W-F and W-R primers were designed for the detection of the deletion mutant.

Table 1
Primers used in this study.

Primer		5′–3′ sequence
up-F	Forward	TCTAGACAAGCAGCGTTGTTCCAT
up-R	Reverse	GGTACCATGTCTCGGAGCCATACTG
dn-F	Forward	GGTACCCTTGAACCAGGGTAGCGG
dn-R	Reverse	GAGCTCGCAGCGACAGCACATCAG
SacB-F	Forward	GAGCTCGGGCTGGAAGAAGCAGACCGCTA
SacB-R	Reverse	GAGCTCGCTTATTGTTAACTGTTAATTGTCC
W-F	Forward	GGACAAGCGTAAACCATCT
W-R	Reverse	CGTTTTCGTTTACTCGTCCGTC

2.5. Evaluation of M5-90Δ*wboA* attenuation in RAW 264.7 macrophages

Murine macrophages RAW264.7 were used to assess the survival capability of the M5-90Δ*wboA* mutant and the parental strain M5-90, as previously described (Hernández-Castro et al., 2008). Briefly, monolayers of macrophages of 1×10^6 cells/well were cultured in 12 well plates for 24 h at 37 °C under 5% CO_2, and then infected with *Brucella* at a multiplicity of infection (MOI) of 100. Culture plates were centrifuged at 350*g* for 5 min at room temperature. At 45 min post-infection, the cells were washed twice with medium without antibiotics and then incubated with 50 μg/mL of gentamicin (Invitrogen, Carlsbad, CA, USA) for 60 min to kill extracellular bacteria. Then, the culture was placed in fresh DMEM containing 25 μg/mL gentamicin (defined as time zero). At 0, 4, 8, 24 and 48 h post-infection, the supernatant was discarded and cells were lyzed by phosphate buffered saline (PBS) containing 0.1% (v/v) Triton X-100. Live bacteria were enumerated by plating on TSA plates. All assays were performed in triplicate and repeated at least three times.

2.6. Evaluation of M5-90Δ*wboA* attenuation in mice

Mouse survival assays were performed as previously described (Pugh et al., 1991). Briefly, female 6-week-old BALB/c mice ($n = 5$ per group) were inoculated intraperitoneally (i.p.) with a total of 1×10^6 colony-forming-units (CFU) (200 μL) of *B. melitensis* M5-90Δ*wboA*, M5-90 or PBS. Survival or persistence of the bacteria in mice was evaluated by enumerating the bacteria in the spleens at different time points post-infection. At 1, 3, 7, 14 and 28 days post-inoculation, mice were euthanized and spleens were removed aseptically. The spleens were collected, weighed, and homogenized in 1 mL PBS containing 0.1% (v/v) Triton X-100, serially diluted, and plated on TSA plates. Plates were incubated at 37 °C, and the