Original Article

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Immunization of BALB/c mice with *Brucella abortus* 2308Δ*wbkA* confers protection against wild-type infection

Zhi-qiang Li[1,2,†], Dan Gui[1,†], Zhi-hua Sun[1], Jun-bo Zhang[3], Wen-zhi Zhang[1], Hui Zhang[1,4,*], Fei Guo[3,5], Chuang-fu Chen[1,4,*]

Colleges of [1]Animal Science and Technology, [5]Medicine, and [4]Co-Innovation Center for Zoonotic Infectious Diseases in the Western Region, Shihezi University, Shihezi 832000, China
[2]School of life sciences, Shangqiu normal university, Shangqiu 476000, China
[3]College of Biology, Agriculture and Forestry, Tongren University, Tongren 554300, China

Brucellosis is a zoonotic disease that causes animal and human diseases. Vaccination is a major measure for prevention of brucellosis, but it is currently not possible to distinguish vaccinated animals from those that have been naturally infected. Therefore, in this study, we constructed the *Brucella* (*B.*) *abortus* 2380 *wbkA* mutant (2308Δ*wbkA*) and evaluated its virulence. The survival of 2308Δ*wbkA* was attenuated in murine macrophage (RAW 264.7) and BALB/c mice, and it induced high protective immunity in mice. The *wbkA* mutant elicited an anti-*Brucella*-specific immunoglobulin G response and induced the secretion of gamma interferon. Antibodies to 2308Δ*wbkA* could be detected in sera from mice, implying the potential for use of this protein as a diagnostic antigen. The WbkA antigen would allow serological differentiation between infected and vaccinated animals. These results suggest that 2308Δ*wbkA* is a potential attenuated vaccine against 16M. This vaccine will be further evaluated in sheep.

Keywords: 2308Δ*wbkA*, *Brucella*, vaccine

Introduction

Brucellosis is a zoonotic disease caused by the Gram-negative genus *Brucella* [3]. Brucellosis has been widely disseminated throughout the world. In animals, brucellosis causes epididymitis in males and infertility or abortion in pregnant females. In humans, brucellosis causes inflammation and undulating fever [18]. However, there is no effective or approved *Brucella* vaccine for humans. Therefore, new low residual virulence marker vaccines that offer high levels of protection are required.

The live vaccine, *Brucella* (*B.*) *abortus* strain 19 (S19), is a spontaneously attenuated mutant [6] that deletes the erythritol catabolic gene. S19 had been used to prevent the infection of *B. abortus*. Some studies have shown that S19 provided protection against cattle abortion. S19 is also effective at protecting animals against *B. abortus* infections. S19 is an effective vaccine in animals that has been widely applied [15]. However, the vaccine is less effective, can induce abortion and milk excretion, and interferes with classical serological diagnostic tests [15]. Accordingly, one potential approach to these problems is development of a marker vaccine through deletion of virulence genes from these parental vaccine strains with good immunogenicity and vaccine efficacy. However, a great deal of research is needed to develop live vaccines against *B. abortus* that are superior to S19.

Lipopolysaccharides (LPS) are important virulence factors that have special pathogenicity. The LPS of *Brucella* has three structural regions: the O-antigen, the core oligosaccharide and lipid A. At present, *Brucella* LPS encodes 32 virulence factors [5,8,12]. *WbkA*, which is one of these virulence factors, encodes *N*-formyl-glutamine transferase, which is essential to biosynthesis of the *Brucella* LPS O antigen. The deletion or expression of the truncated form of *wbkA* may affect the antigenic structure of *Brucella*. Early studies have shown that *B. melitensis* 16MΔ*wbkA* is a rough mutant, and deletion of the *wbkA* flanking transposase improves the stability of *B. melitensis* Rev 1 vaccine. In this report, we describe the

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*Corresponding authors: Tel: +86-993-2058002; Fax: +86-993-2058512; E-mails: allanzhh@sohu.com (H Zhang), chuangfu_chen@163.com (C Chen)
[†]The first two authors contributed equally to this work.
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construction of a *B. abortus* 2308Δ*wbkA* mutant (2308Δ*wbkA*) and evaluate its role in intracellular multiplication in RAW 264.7 macrophages and protection of mice with the goal of producing a candidate vaccine against *B. abortus* 2308.

Materials and Methods

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

Bacterial strains, plasmids, cells and mice

B. abortus strain 2308 (S2308) and the vaccine strain S19 were obtained from the Center of Chinese Disease Prevention and Control (Beijing, China). *Brucella* was cultured in tryptone soy agar (TSA) or tryptone soy broth (TSB; Oxoid, UK). *E. coli* strain DH5α was grown on Luria–Bertani (LB) medium. Plasmid pGEM-7Zf^{+} was purchased from Promega (USA). The RAW 264.7 murine macrophage was purchased from Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China). Six-week-old BALB/c female mice were obtained from the Experimental Animal Center of Academy Military Medical Science (Beijing, China). All experimental procedures and animal care was performed in compliance with institutional animal care regulations.

Construction of the 2308Δ*wbkA*

The deletion mutant 2308Δ*wbkA* was constructed with pGEM-7Zf^{+} as follows. Two pairs of primers with restriction sites at the 5' ends were designed for amplification of the upstream (1100 bp) and downstream (1086 bp) arms of the S2308 *wbkA*, in which the *Xba* I, *Kpn* I, *Kpn* I, and *Sac* I (italicized) sites were integrated into both PCR fragment ends. The designs of the primers were based on *B. abortus* 2308. The primer sequences were as follows: *wbkA*-N-F, *TCT AGA* TTA CAG ATG AGC AAT GGA ACC; *wbkA*-N-R, *GGT ACC* TCC TTC TAT GAA GCT AAT TGT TTG; and *wbkA*-C-F, *GGT ACC* ATT ACT TGC GAA TAT CGG TCG C; *wbkA*-C-R, *GAG CTC* GCG TCA TTG GTC TCT TTG CAC. One pair of primers with restriction sites at the 5' ends was designed for amplification of the *SacB* DNA fragment (1477 bp), which is a selectable marker gene from *Bacillus subtilis*. The primer sequences were as follows: *SacB*-F, *GAG CTC* GGG CTG GAA GAA GCA GAC CGC TA, *SacB*-R, *GAG CTC* GCT TAT TGT TAA CTG TTA ATT GTC C. The two homologous arms and the *SacB* DNA fragment were sequentially cloned into the multi-cloning sites of pGEM-7Zf^{+} (*wbkA*-N cloned at the *Xba* I/*Kpn* I sites, *wbkA*-C at the *Kpn* I/*Sac* I sites and *SacB* at the *Sac* I site) to generate the suicide plasmid, pGEM-*wbkA*-SacB (pWS). Competent S2308 were electroporated with pWS and potential *wbkA* deletion mutant 2308Δ*wbkA* was isolated in the presence of 100 μg/mL ampicillin for the first screening and 5% sucrose for the second screening. The mutant was further confirmed by PCR amplification with primers *wbkA*-I-F (GAA AAC TAC CTC GCA GCC GT) and *wbkA*-I-R (AGC GGA TGA AAC GGT TGT AG), located in the upstream and downstream portion of the homologous arm of *wbkA*, respectively. PCR products were sequenced for confirmation. The deletion mutant was further confirmed by PCR amplification and RT-PCR sequencing, as previously described [20].

Complementation of 2308Δ*wbkA*

To restore the *wbkA* activity, PCR amplification fragments primers *wbkA*-N-F and *wbkA*-N-R, and the products were ligated with pMD19-T simple vector (Takara Bio, Japan) to generate pMD-*wbkA*, which was then transformed into *E. coli* DH5α. Plasmid pMD-*wbkA* was isolated from these transformants and analyzed by agarose gel electrophoresis following restriction digestion, after which it was electroporated into competent 2308Δ*wbkA*. The transformants were then selected on TSA containing 100 μg/mL ampicillin. The transcription restoration of *wbkA* in the complementary strain (2308-*wbkA*) was further confirmed by PCR and RT-PCR.

Evaluation of 2308Δ*wbkA* attenuation intracellular survival in RAW264.7 murine macrophages

RAW264.7 murine macrophages were seeded in 6 well plates and infected with 2308Δ*wbkA*, S19, 2308-*wbkA* or parental strain S2308 at a multiplicity of infection of 150, as previously described [13]. Culture plates were centrifuged at 350 g for 5 min at room temperature, then placed in a 37^{o}C, 5% CO_2 incubator. At 45 min post-incubation, the cells were washed three times with phosphate buffered saline (PBS) and then incubated with 50 μg/mL of gentamicin (Invitrogen, USA) for 1 h to kill extracellular bacteria. Subsequently, the culture was replaced with Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, USA) containing 25 μg/mL of gentamicin (time zero). At different time points post-infection, the supernatant was discarded, cells were lysed by PBS containing 0.1% (v/v) TritonX-100, and the live bacteria were enumerated by plating on TSA plates. All assays were performed in triplicate wells, and the results represent the means from at least three separate experiments.

Evaluation of 2308Δ*wbkA* attenuation virulence in mice

Six-week-old female BALB/c mice were inoculated intraperitoneally (i.p.) with 1×10^{6} CFU of 2308Δ*wbkA*, S19, 2308-*wbkA* or S2308. Infected mice were held in microisolator cages in biosafety level 3 facilities. Bacterial counts in the spleen were calculated for five mice of each group at 3, 7, 14, 21 and 28 days post-inoculation. Mice were euthanized by CO_2 asphyxiation and their spleens were removed aseptically. The spleens were then homogenized in 1 mL PBS containing 0.1%

(v/v) TritonX-100, 10-fold serially diluted, and then plated on TSA. The plates were incubated at 37°C and the number of CFU counted after three days. All assays were performed three times with similar results [22].

Protection efficiency evaluation induced by 2308Δ*wbkA* in mice

Groups of six-week-old female BALB/c mice (n = 10 per group) were injected i.p. with 1×10^6 CFU (200 μL) of 2308Δ*wbkA* (experimental vaccine group), S19 (reference vaccine control group) or 200 μL PBS (unvaccinated control group). The mice were challenged i.p. with 1×10^6 CFU per mouse (200 μL) of virulent strain S2308. Mice (n = 5/time point per group) were euthanized at 2 and 4 weeks post challenge, and bacterial CFUs in the spleens were determined as described above. The mean value for each spleen count was obtained after logarithmic conversion. Log units of protection were obtained by subtracting the mean log CFU for the experimental group from the mean log CFU for the control group, as previously described [1,11]. The experiments were repeated three times.

Evaluation of antibody

Six-week-old female BALB/c mice were randomly divided into three groups. Group 1 and 2 were inoculated i.p. with 1×10^6 CFU of 2308Δ*wbkA* or S19, respectively, and group 3 was inoculated i.p. with PBS as a negative control. Serum samples were obtained from immunized mice at 3, 7, 14, 21 and 28 days post-immunization, and IgG levels were determined by enzyme-linked immunosorbent assay (ELISA) as previously described [9]. Serum samples were diluted with sample diluent buffer (1∶5). The levels of IgG in the supernatants were measured using an ELISA Quantikine Mouse Kit (R&D Systems, USA) according to the manufacturer's instructions. All assays were performed in triplicate and repeated at least three times.

Cytokine production assay

IFN-γ detection was carried out as previously described [19]. Specifically, IFN-γ was determined using an ELISA Quantikine Mouse Kit (R&D Systems) according to the manufacturer's instructions. All assays were performed in triplicate and repeated at least three times.

Cloning, expression, and purification of recombinant protein

The WbkA open reading frame was amplified by PCR of DNA from S2308, after which the amplified DNA fragment was cloned into the pET-32a vector (Novagen, USA) and expressed in *E. coli* BL21 as N-terminally His-tagged recombinant protein. The expression of the recombinant protein was analyzed by SDS-PAGE (12%). The recombinant protein, WbkA, was then purified by affinity chromatography with Ni_2-conjugated Sepharose (GE Healthcare Life Sciences, USA).

Western blotting

The recombinant protein WbkA cell lysates were analyzed by Western blotting as previously described [21]. The membrane with recombinant protein WbkA cell lysates was then incubated with a primary *Brucella*-vaccinated sera and sheep anti-mouse IgG (horseradish peroxidase conjugated; EarthOx Life Sciences, USA) secondary Ab. The membrane was developed using an enhanced HRP-DAB substrate color kit (TIANGEN Biotech, China).

WbkA indirect ELISA (iELISA)

Serum samples were obtained from *Brucella*-infected mice. Antibody responses to the purified recombinant WbkA protein were estimated via WbkA-based iELISA, as previously described [14].

Statistical analysis

Data were analyzed by one-way ANOVA for each triplicate or quintuplicate set using SPSS (ver 17.0; SPSS, USA). Values for all parameters are expressed as the mean ± the standard deviation (SD). P values < 0.05 were considered statistically significant.

Results

Construction and complementation of the *B. abortus* 2308Δ*wbkA* mutant

The *wbkA* gene deletion mutant of *B. abortus* strain 2308 (2308Δ*wbkA*) was successfully obtained. A 433 bp DNA fragment was amplified from 2308Δ*wbkA*, but a 1131 bp DNA fragment was amplified from S2308 with primers *wbkA*-I-F and *wbkA*-I-R, indicating that *wbkA* was correctly knocked out. These findings were subsequently confirmed by sequencing (data not shown). The complementary strain was constructed by integration of *wbkA* at the disrupted locus of 2308Δ*wbkA*. RT-PCR showed that *wbkA* was not transcribed in 2308Δ*wbkA*, but was restored in 2308-*wbkA* (data not shown). Taken together, these findings indicate that both 2308Δ*wbkA* mutant and complementary strain 2308-*wbkA* were correctly constructed.

2308Δ*wbkA* mutant is attenuated relative to *B. abortus* 2308 based on survival in RAW 264.7 murine macrophages

RAW 264.7 murine macrophages were infected with 2308Δ*wbkA*, S19, 2308-*wbkA* and S2308, after which their survival capability in macrophages was determined. Macrophages were infected with the three strains at 1∶150, and the surviving bacteria were calculated. At 4 h post-infection, no differences in the amount of bacteria were observed among strains (Fig. 1). By 8 h post-infection, there was a 1.08 log ($p < 0.05$), 0.87 log ($p < 0.05$) and 0.27 log ($p > 0.05$) decrease in the number of 2308Δ*wbkA* bacteria compared to that of S2308 and 2308-*wbkA* or S19. At 24 h post-infection, there was a 3.19

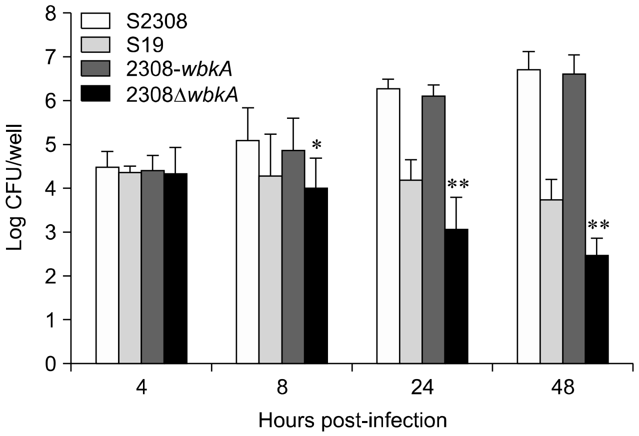

Fig. 1. Intracellular replication of *Brucella abortus* 2380 *wbkA* mutant (2308Δ*wbkA*) within RAW264.7 macrophages. RAW264.7 Macrophages were infected with 2308Δ*wbkA*, S19, 2308-*wbkA* and S2308 at a multiplicity of infection of 150. The level of initial infection was the same for these strains. At 4, 8, 24 and 48 h post-infection, infected macrophages were lysed and bacteria were calculated by plating serial dilutions on tryptone soy agar plates. The results demonstrate that the 2308Δ*wbkA* was unable to achieve the level of colonization reached by the conventional vaccine strain S19, parental strain S2308 and complementary strain 2308-*wbkA*. Significant differences between the mutant and S2308 or 2308-*wbkA* are indicated by $^*p < 0.05$ and $^{**}p < 0.01$.

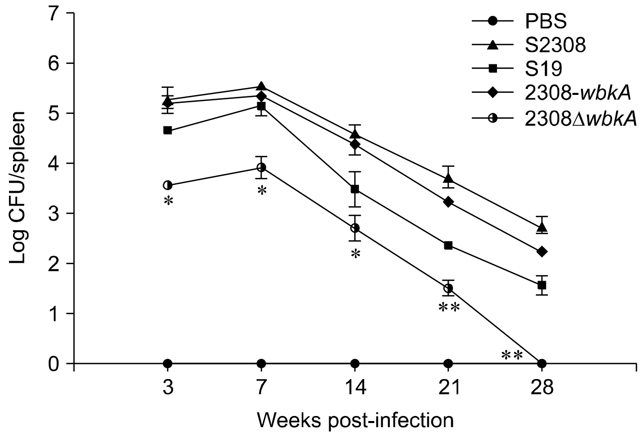

Fig. 2. Clearance of 2308Δ*wbkA* after infection. BALB/c mice were inoculated i.p. with 1×10^6 CFU/mouse of 2308Δ*wbkA*, S19 and S2308. Control groups were inoculated i.p. with phosphate buffered saline (PBS). Infected mice were sacrificed by CO_2 asphyxiation at 3, 7, 14, 21 and 28 days post-infection, after which spleens were removed aseptically and assessed individually for colonization. Values are the means ± SD (n = 5 per time point). Differences in splenic colonization were determined by one-way ANOVA. $^*p < 0.05$, $^{**}p < 0.01$.

log ($p < 0.01$), 3.05 log ($p < 0.05$) and 1.11 log ($p < 0.05$) decrease in the number of 2308Δ*wbkA* bacteria compared to that of S2308, 2308-*wbkA* or S19 bacteria, respectively, indicating that the numbers of 2308Δ*wbkA* were significantly lower than in these strains. At 48 h post-infection, decreases of 4.23 log, 4.13 log and 1.27 log were observed inside the macrophages ($p < 0.01$) (Fig. 1). These results showed that 2308Δ*wbkA* mutant had a decreased survival capability in RAW 264.7 murine macrophages relative to S2308, 2308-*wbkA* and S19, indicating that 2308Δ*wbkA* was attenuated compared with *B. abortus* 2308 based on survival in RAW 264.7 murine macrophages.

2308Δ*wbkA* mutant is attenuated compared with *B. abortus* 2308 based on survival in BALB/c mice

To determine the survival capability in BALB/c mice, animals were inoculated i.p. with 1×10^6 CFU of 2308Δ*wbkA*, S19, 2308-*wbkA* and S2308. When compared with S19, 2308-*wbkA* and S2308, splenic CFU in 2308Δ*wbkA* infected mice were significantly reduced ($p < 0.01$) at days 3, 7, 14, 21, and 28. At 28 days post-inoculation, 2308Δ*wbkA* had been cleared from the spleens of mice (Fig. 2). Taken together, these results showed that 2308Δ*wbkA* mutant was greatly attenuated in BALB/c mice.

2308Δ*wbkA* induces immune protection against challenge with *B. abortus* 2308

To determine the protection efficiency of 2308Δ*wbkA*, mice were vaccinated i.p. with 1×10^6 CFU of 2308Δ*wbkA*, S19 or PBS. At 4 weeks post-vaccination, mice were challenged with 1×10^6 CFU (200 μL) of virulent strain S2308. The mice immunized with 2308Δ*wbkA* exhibited significantly fewer splenic *Brucella* than non-immunized mice at 2 (2.22 log units) and 4 (1.76 log units) weeks post-challenge ($p < 0.05$) (Table 1). As expected, S19 also induced significant protection at 2 (1.83 log units) and 4 (1.34 log units) weeks after challenge, and the protective efficacy of S19 was lower than that in the 2308Δ*wbkA*-vaccinated mice. The 2308Δ*wbkA* showed similar protective efficacy to that of S19 (Table 1). Taken together, these results indicate that 2308Δ*wbkA* can provide similar protection efficacy against challenge as the S19 vaccine.

2308Δ*wbkA* induces both humoral and cytokine responses

Serum samples from mice inoculated with 2308Δ*wbkA*, S19 or PBS were obtained from immunized mice at selected intervals post-immunization to monitor total IgG levels by ELISA. For mice inoculated with 2308Δ*wbkA* and S19, the total IgG levels peaked at 21 days post-inoculation. The 2308Δ*wbkA*-vaccinated mice expressed slightly higher IgG levels than the S19-vaccinated mice ($p > 0.05$) and significantly higher IgG levels than the PBS-infected mice ($p < 0.05$) (Fig. 3).

To characterize the cellular immune response, the IFN-γ levels in the splenocytes of the 2308Δ*wbkA* and S19 vaccinated

Table 1. Protection conferred by 2308Δ*wbkA* and S19 against S2308

Vaccination	Protection criteria			
	Log CFU/spleen (mean ± SD)*		Units of protection†	
	2	4	2	4
2308Δ*wbkA*	5.06 ± 1.05‡	4.79 ± 0.04‡	2.22	1.76
S19	5.45 ± 0.49‡	5.21 ± 0.67‡	1.83	1.34
PBS	7.28 ± 0.93	6.55 ± 0.47	–	–

*Statistical significance. †Log units of protection = average of Log CFU in spleens of control unvaccinated mice minus the average of Log CFU in spleens of vaccinated mice. ‡$p < 0.05$ with respect to unvaccinated controls.

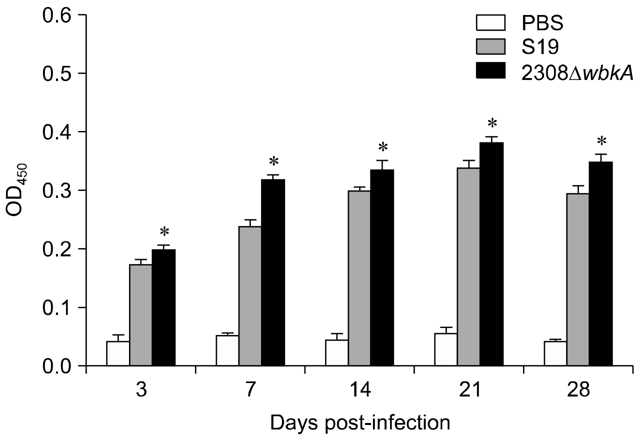

Fig. 3. Anti-*Brucella* in serum from mice immunized with 2308Δ*wbkA* or S19. Mice were inoculated i.p. with 1 × 10^6 CFU of 2308Δ*wbkA* or S19. Control groups were inoculated with PBS. Serum samples were collected at 3, 7, 14, 21 and 28 days post-vaccination (n = 5 per time point) and IgG antibodies were determined by enzyme-linked immunosorbent assay (ELISA). The results are the means ± SD of the absorbance at 450 nm (OD_{450}). Significant differences between the 2308Δ*wbkA* and PBS groups are indicated by $^*p < 0.05$.

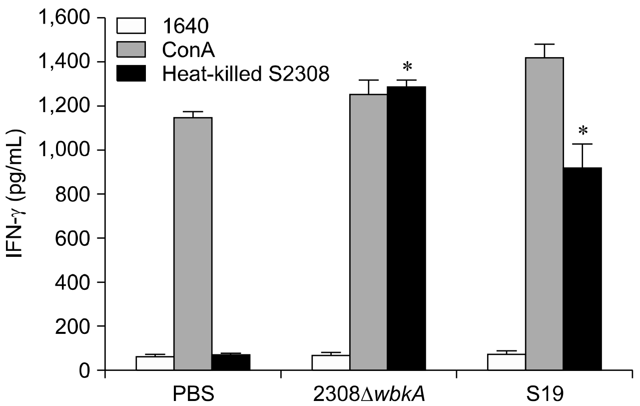

Fig. 4. IFN-γ production by spleen cells of BALB/c mice vaccinated with 2308Δ*wbkA* or S19. Spleens from mice were inoculated i.p. with 1 × 10^6 CFU of 2308Δ*wbkA* or S19. Control groups were inoculated with PBS. At 4 weeks post-vaccination, mice were euthanized and splenocytes were recovered and stimulated with either heat-killed S2308, concanavalin A (ConA) or RPMI 1640. Splenocyte culture supernatants were harvested and analysis of IFN-γ secretion (pg/mL) was assayed by ELISA. Significant differences from the same stimulus in PBS-injected mice are indicated by $^*p < 0.05$.

mice were evaluated at 28 days post-vaccination. The splenocytes of immunized mice were isolated and stimulated with heat-killed S2308, complete RPMI 1640 medium (Gibco BRL) (negative control) or concanavalin A (positive control). The 2308Δ*wbkA*-vaccinated mice expressed slightly higher IFN-γ levels in the splenocytes than the S19-vaccinated mice ($p > 0.05$) and significant higher levels than the PBS-injected mice ($p < 0.05$) (Fig. 4).

Differentiation of 2308Δ*wbkA*

Serum from mice inoculated with 2308Δ*wbkA*, S2308, S19 or PBS were collected to determine whether the WbkA protein can be used as a diagnostic antigen. Western blotting showed that antibodies against the WbkA protein could be detected in the serum of S2308- or S19-inoculated mice, and produced specific bands. However, the antibodies could not be detected in the serum of 2308Δ*wbkA*- or PBS-inoculated mice (Fig. 5). Furthermore, antibodies against the WbkA protein were detected by iELISA. Serum from S2308- or S19-inoculated mice showed positive reaction and serum from 2308Δ*wbkA*- or PBS-inoculated mice showed negative reaction according to iELISA using WbkA as antigen. The results of iELISA were similar to those obtained Western blotting (Fig. 6). Taken together, these results indicate that WbkA protein had good reactogenicity and could be used to differentiate vaccination from natural infection by WbkA-iELISA after confirmation of brucellosis infection using LPS-based serological tests.

Discussion

The development of an efficacious vaccine against brucellosis has long been a challenge to scientists. Most currently available

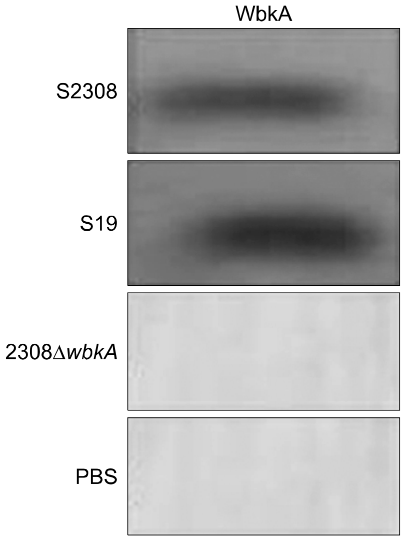

Fig. 5. Response of WbkA to 2308Δ*wbkA* immunization sera. Sera from mice immunized with 2308Δ*wbkA*, S2308, S19 and PBS were collected. Antibodies to WbkA were detected in these sera by Western blotting. Antibodies against the WbkA protein were not detected in sera from 2308Δ*wbkA* immunized mice.

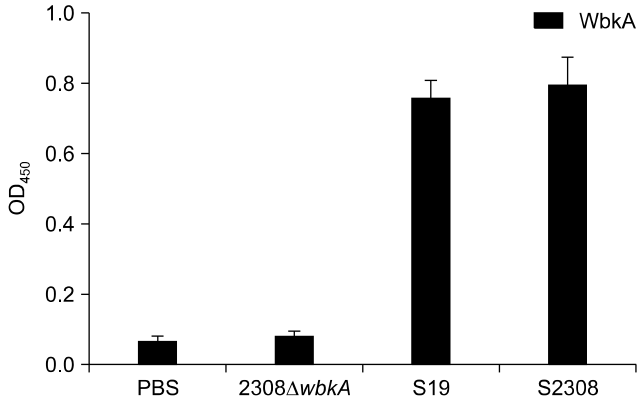

Fig. 6. Humoral immune responses to WbkA were assessed in serum by indirect ELISA. Sera from mice immunized with 2308Δ*wbkA*, S2308, S19 and PBS were collected. Humoral immune responses against the WbkA protein were not detected in serum from 2308Δ*wbkA* immunized mice.

vaccines have several limitations, such as residual virulence, interference of serodiagnosis and splenomegaly [2,4,7,17]. One of the drawbacks to the rational development of new *Brucella* vaccines is limited virulence. Furthermore, serological interference has limited development of an effective vaccine. The ideal vaccine must be protective and should allow for differentiation of natural and vaccinated infection [15]. LPS are important virulence factors with unique pathogenicity. *Brucella* LPS encodes 32 virulence factors, including *WbkA*. To investigate whether 2308Δ*wbkA* maintains protective efficacy, we constructed *B. abortus* 2308Δ*wbkA* mutant and its virulence and protection efficacy were evaluated in cells and mice.

The 2308Δ*wbkA* was constructed to confirm that the reduced survival capability of the mutant was directly related to the deleted *wbkA* gene. As shown in this study, 2308Δ*wbkA* was defective for survival in RAW 264.7 cells and BALB/c mice, and it was cleared faster than S19, within 28 days. This finding showed that the lack of splenomegaly in inoculated mice increased the safety of 2308Δ*wbkA* as a vaccine.

An ideal *Brucella* live attenuated vaccine strain must be non-virulent towards the host and induce higher levels of protection. Therefore, we performed the protection experiment in BALB/c mice. We found that the 2308Δ*wbkA* mutant could provide slightly higher protection than S19.

The cytokine and humoral immune responses have been evaluated for the degree of protection conferred by 2308Δ*wbkA*. *Brucella* infects the host cells and mainly causes cellular immunity. Th1 immune responses characterized by production of IFN-γ are associated with protective immunity to *Brucella*. IFN-γ is a critical cytokine required for macrophage bactericidal activity that is produced by T lymphocytes and is a potent macrophage-activating factor [10,16]. IFN-γ plays an important role in killing intracellular *Brucella*. IFN-γ is antibacterial, and this study evaluated the antimicrobial capacity and cellular immunity of the host. Our results indicated that 2308Δ*wbkA* induced higher levels of IFN-γ than S19. In addition, one weak challenge, 2308Δ*wbkA*, induced anti-*Brucella* containing a high level of IgG. These results showed that immunization with 2308Δ*wbkA* elicits a Th1 response.

Brucella LPS was the most important antigen during immune response in brucellosis. Current serological diagnostic tests include the standard tube agglutination test, Rose Bengal plate test, and iELISA using LPS antigens of smooth *Brucella*. However, LPS-based serological tests cannot easily differentiate between the serum of vaccinated and infected animals. Therefore, we used WbkA protein as a diagnostic antigen and detected the antibody profiles in different sera. The results indicated that the antibodies against the WbkA protein could be detected in infected serum, but not in that from 2308Δ*wbkA*-immunized animals. These results showed that WbkA could be used as a diagnostic antigen to be differentiated from vaccination serum. Furthermore, 2308Δ*wbkA* vaccination was detected by iELISA. The results revealed that S2308- and S19-infected mice were positive, whereas 2308Δ*wbkA*-infected mice were negative. Therefore, 2308Δ*wbkA* allows for differentiation between infected and vaccinated animals.

In this study, 2308Δ*wbkA* mutant may be another ideal live vaccine candidate for S2308 because of its low virulence in macrophage RAW264.7 and BALB/c mice. Moreover, it provided protection similar to that provided by the S19 vaccine strain. The humoral responses indicated that 2308Δ*wbkA* could elicit an anti-*Brucella*-specific IgG response. Moreover, the

WbkA protein is an ideal diagnostic antigen for differentiation of immunization from infection by WbkA-iELISA. In future studies, comprehensive protection experiments must be conducted to determine whether the measurable immune responses in systemic compartments confer detectable protection against *Brucella* infection. In addition, further testing in livestock will determine whether 2308Δ*wbkA* will be a promising live vaccine candidate.

Acknowledgments

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Conflict of Interest

There is no conflict of interest.

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