Journal of Animal and Veterinary Advances 13 (5): 306-312, 2014
ISSN: 1680-5593
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Development and Evaluation of Vaccines in the Mice Using *Brucella suis* (*B. suis*) Deletion Mutant of the *vjbR* Gene

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Abstract: Brucellosis is an infectious disease that brings great economic burdens for developing countries. The vaccine S2 which is an attenuated *Brucella suis* (*B. suis*) strain has been used on a large scale in China. However, the immunity induced by S2 declined relative to those vaccinated with Rev-1 and S19 vaccines. Moreover, the vaccine S2 cannot differentiate natural from vaccinated infection. Therefore, a safer and more potent *B. suis* vaccine is needed. In this study, a vjbR mutant of *B. suis* (*B. suis*ΔvjbR) was constructed overcome these drawbacks. The *B. suis*ΔvjbR strain showed reduced survival capability in RAW264.7 macrophage and mice and induced high protective immunity in mice. In addition, *B. suis*ΔvjbR induced an anti-Brucella-specific IgG (immunoglobulin G) response and stimulated the expression of gamma interferon (INFγ). Further, the vjbR antigen allowed serological differentiation between natural and vaccinated infection in mice. Therefore, *B. suis*ΔvjbR was suggested as a safe and efficacious live vaccine candidate against virulent *Brucella suis* (*B. suis*) infection.

Key words: vjbR, vaccines, mice, *Brucella suis*, (*B. suis*), gamma interferan

INTRODUCTION

Brucellosis is one of the most important bacterial zoonosis, being transmitted to humans mainly from ruminants. *Brucella* sp. cause acute, transmissible infections in animal reservoirs and abortion in pregnant animals (Ficht, 2003). It is listed as a class II disease in both the law on prevention and control of infectious diseases of the People's Republic of China and in the implement detailed rules of the by-laws on disease prevention and control of livestock and poultry. The primary hosts for *Brucella melitensis* are goats and sheep in which the organism causes abortions during the third trimester of pregnancy. The pathogen goes undetected in infected flocks until lambing season, inducing abortions when colonized in pregnant animals. Following an abortion, the infected female excretes copious amounts of the bacteria in the uterine exudate and milk. This results in the spread of the organism throughout the flock and to human caretakers (Blasco, 1997).

B. suis infects a broad range of animals including swine (*Sus* sp.), reindeer (*Rangifer tarandus*), caribou (*Rangifer tarandus*), hares (*Lepus* sp.), various murine species (*Mus* sp.) and occasionally cattle and dogs (*Canis* sp.) and is the most diverse in its genome sequence. Although, each species has a relative host preference, cross-species infections occur frequently.

B. canis, a species mostly limited to infecting dogs is also infectious in cattle (Li, 1988) as *B. melitensis* is in swine (Borts *et al*., 1946). *B. suis* Strain 2 (S2) which is an attenuated strain *Brucella suis* (*B. suis*) has been used on a large scale in China. S2 is a laboratory adapted strain isolated in 1953 from an aborted sow which was attenuated by serial passage (Bosseray and Plommet, 1990). Although, S2 possesses a smooth phenotype, its virulence is significantly less than wt *B. suis* (Xin, 1986). However, the immunity induced by S2 declined relative to those vaccinated with Rev-1 and S19 vaccines (Bosseray and Plommet, 1990). Moreover, serological diagnosis cannot distinguish natural infection and vaccination. Therefore, numerous efforts have been made to develop new vaccines.

Quorum Sensing (QS) Systems are used by many bacterial species to sense their environment and drive a population-wide transcriptional response. Thus, the use of this regulatory system by the intracellular pathogen, *Brucella melitensis*, presents a new adaptation of a conserved signaling system. One of the two *QS* genes has been proved to be involved in Brucella virulence, the HTH type quorum sensing dependent transcriptional regulator, *vjbR* gene.

Survival and replication inside host cells by *Brucella* sp. requires a Type IV Secretion System (T4SS) which is encoded by the virB locus. The *vjbR* gene is a

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LuxR family regulator and is known to directly regulate virB operon by binding a fragment of the virB promoter containing an 18 bp palindromic motif (virB promoter box). It has been shown that for both Type III Secretion System (T3SS) and *T4SS* genes encoding the secretion apparatus are often coregulated with the secreted substrates. For example, the same regulators control expression of genes encoding the structural components of *Salmonella enterica* serotype Typhimurium T3SS-1 and T3SS-2 and their effectors (Thijs *et al*., 2007; Worley *et al*., 2000). Furthermore in *Legionella pneumophila* the two-component system regulators PmrA and CpxR were found to regulate genes of the Dot/Icm T4SS as well as several Dot/Icm effector proteins (Altman and Segal, 2008; Zusman *et al*., 2007). It has been reported that vjbR co-ordinates expression of the T4SS and at least two of its secreted substrates.

Published reports have showed that an encapsulated *B. melitensis* vjbR mutant could generate higher level of protection against wild type challenge in BALB/c mice than non-encapsulate does (Arenas-Gamboa *et al*., 2008). S19ΔvjbR is another vaccine candidate constructed by the same laboratory. The mutant was safer than S19, induced protection in mice and should be considered as a vaccine candidate when administered in a sustained-release manner (Arenas-Gamboa *et al*., 2009).

In this study, researchers constructed a vjbR deletion mutant of *B. suis* (*B. suis*ΔvjbR), *B. suis* 5ΔvjbR was attenuated in RAW264.7 macrophage and mice and induced good protective immunity in mice. These results indicated for the first time that *B. suis*ΔvjbR may be useful as an attenuated vaccine with low virulence and high-protective effect.

MATERIALS AND METHODS

Bacterial strains and cell culture: *Brucella suis* (*B. suis*) and the vaccine strain S2 were obtained from the center of Chinese Disease Prevention and Control. *B. suis* deletion mutant of the *vjbR* gene was constructed in this study. Brucella was cultured in Tryptic Soybroth (TSB) or Tryptic Soy Agar (TSA). Murine macrophage RAW264.7 cells were routinely cultured at 37°C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS).

Mice: The 8 weeks old female BALB/c mice were obtained from the Experimental Animal Center of Academy of Military Medical Science (Beijing, China). All experimental procedures and animal care were performed in compliance with institutional animal care regulations.

Construction of vjbR deletion mutant: A pair of primers was designed for vjbR DNA fragment amplification. The primers designations were based on *B. suis*. Primer sequences were the following: H-F, 5' TAAGCGATTG ACGGCCTCT3' and P-R, 5' ATTTCTATCCCGGCACAC TG 3'. This pair of primers was designed for amplifying vjbR and 1,211 bp PCR product was cloned into pMD18-T simple vector for sequencing.

For construction of the vjbR deletion mutant, two pairs of primers with restrictionsites at 5' ends were designed for amplification of the upstream (1043 bp) and downstream (1002 bp) arms of the *B. suis* ORF in which EcoRI, KpnI, KpnI and BamHI (underlined) sites were integrated into both of the PCR fragment ends. Primer sequences were the following: up-F, GAATCCACAA GAAAGGCGAGCTTCGT; up-R, GGTACCTGGAAATA TCCTTGGTGATG and dn-F, GGTACC TCAACATG GTCGCGCGG; dn-R, GGATCCGGCAGGAGGTGAAGGAT GAAT. The two homologous arms were sequentially cloned into pGEM-7Zf$^+$ (Promega, Madison, WI) to generate suicide plasmid pGEM-7Zf$^+$-vjbR. Then a pair of primers was designed for SacB DNA fragment amplification: S-F, GGATCCGGGCTGGAA-GAAGCAGA CCGCTA (containing a BamHI site); S-R, GGATCCGCT TATTGTTAACTGTTAATTGTCC (containing a BamHI site) a 1500 bp fragment was amplified by PCR from Bacillus subtilis. The BamHI-BamHI insert of this plasmid containing the PCR amplified DNA was subcloned in plasmid pGEM-7Zf$^+$-vjbR which generated plasmid pGEM-7Zf$^+$-vjbR-SacB. Competent *B. suis* was electroporated with pGEM-7Zf$^+$-vjbR-SacB and Brucella transconjugants were selected in the presence of 100 μg mL^{-1} ampicillin for the first screening and 5% sucrose for the second screening. The deletion mutant was further confirmed by PCR amplification and sequencing and absence of vjbR expression confirmed by SDS-PAGE and Western blotting. One isolate was retained and named *B. suis*ΔvjbR.

Evaluation of *B. suis*ΔvjbR attenuation in murine macrophages RAW264.7: Murine macrophages RAW264.7 were used to assess survival capability of *B. suis*ΔvjbR. Monolayers of macrophages of 10^5 cells/well were cultured in 24 well plates for 16 h at 37°C, infected with Brucella at an MOI of 50. At 45 min post-infection, the cells were washed twice with PBS and then incubated with 50 μg mL^{-1} of gentamicin. At 4, 12, 24 and 48 h post-infection, the cells were lysed and the live bacteria were enumerated by plating on TSA plates. All assays were performed in triplicate and repeated at least three times.

Evaluation of *B. suis*ΔvjbR attenuation in mice: Female BALB/c mice were used to evaluate the survival of the *B. suis*ΔvjbR. Briefly, 8 weeks old mice (n = 10 per group) were intraperitoneally (i.p.) inoculated with a total of 1×10^6 CFU of *B. suis*ΔvjbR or S2. The survival or persistence of the bacteria in mice was evaluated by bacteria enumeration in the spleens at different time points after the infection. At 1, 2, 4 and 6 weeks after the inoculation, the mice were euthanised and their spleens were removed aseptically. The spleens were collected and homogenised in 1 mL of PBS. The suspensions were diluted in sterile saline and plated on TSA. The plates were incubated at 37°C and the number of CFU was counted after three days. The experiments were repeated three with similar results.

Immuno-protection induced by *B. suis*ΔvjbR in BALB/c mice: Protective activity was evaluated by comparing the abilities of mice receiving *B. suis*ΔvjbR (experimental group); S2 (vaccine control group) and PBS (unvaccinated control group) to detect spleen infection after the virulent challenge with *B. suis* (a standardised virulent). The 8 weeks old female BALB/c mice (n = 10 per group) were vaccinated i.p. with 1×10^6 CFU of *B. suis*ΔvjbR or S2. 10 unvaccinated mice was injected i.p. with 200 μL of PBS as controls. The mice were challenged i.p. with 1×10^6 CFU (200 μL) per mouse of virulent strain *B. suis* at 12 weeks after vaccination. All mice were euthanized by cervical dislocation at 2 and 6 weeks post challenge and the bacterial CFUs in the spleen were determined. A mean value for each spleen count was obtained after logarithmic conversion. Log_{10} units of protection were obtained by subtracting the mean log_{10} CFU of the experimental group from the mean log_{10} CFU of the control group as described previously (Adone *et al*., 2005). The experiment was repeated twice.

Evaluation of antibody production: Serum samples were obtained from immunized mice at 2, 4 and 6 weeks after the immunization and IgG titers were determined by ELISA as indices of antibody production in inoculated mice. Briefly, heat-killed and sonicated *B. suis* whole-cell antigen was used to coat 96 well plates at a concentration of 25 μg protein/well. After overnight incubation at 4°C, the plates were washed once with 100 mL PBST buffer (PBS containing 0.05% Tween-20) and blocked with 200 mL blocking buffer (PBS with10% heat-inactivated FBS, pH 7.4) for 2 h at 37°C. Serial dilutions of serum were added to wells and incubated for 2 h at 37°C. After incubation, horseradish peroxidase-conjugated goat anti-mouse IgG antibody (SBA) was added and then the plates were incubated for 1 h at 37°C. After two washes with PBS, 100 mL per well of TMB substrate solution was added and incubated in darkness for 15 min at 37°C. The reaction was terminated by adding 50 μL of H_2SO_4; absorbance was measured at 450 nm. All assays were performed in triplicate and repeated at least three times.

Cellular response: The 11 weeks after the immunisation, BALB/c mice (n = 10 per group) were sacrificed and their spleens were removed under aseptic conditions. Single cell suspensions from the spleens were obtained by homogenization. The cells were suspended in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% (v/v) FBS. Erythrocytes were eliminated with ACK lysis solution (150 mM NH_4Cl, 1 mM Na_2-EDTA, pH 7.3). Splenocytes were cultured in 96 well plate with 4×10^5 cells/well, the culture was stimulated by adding 40 μg of S2 or heat-killed *B. suis* lysate/well, 1 μg of ConA (concanavalinA) (as positive control) or medium alone (as negative control). The cells were then incubated with 5% CO_2 for 72 h at 37°C. IFN-γ (Interferon-γ) in the supernatants were measured using an ELISA Quantikine Mouse kit (R&D Systems).

Statistical analysis: The data were analyzed using Student's t-test and were expressed as mean value±standard error of the mean (SEM). The $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Construction of the *B. suis*ΔvjbR: *B. suis*ΔvjbR was successfully obtained. The vjbR deletion was confirmed by PCR and Western blot. Bacteriological analysis and typing of the mutant showed that deletion of the *vjbR* gene was stable after passage either in culture media or mice. It had no detectable effects on conventional species, biovar phenotypic properties or on differential bacteriological tests characteristic of strain S2.

***B. suis*ΔvjbR is attenuated for survival in macrophages:** To further assess attenuation of *B. suis*ΔvjbR, RAW264.7 macrophages were infected with *B. suis*ΔvjbR and S2. The surviving bacteria were enumerated and by 4 h post-infection there were no differences in the number of surviving bacteria in RAW264.7 cells (Fig. 1; $p>0.05$). This indicates the deletion of vjbR does not affect invasion of Brucella into macrophage. However, at 12 h post-infection there was a 1.01-log decrease ($p<0.01$) in the number of *B. suis*ΔvjbR compared to S2 (Fig. 1). At 48 h post-infection this decrease for *B. suis*ΔvjbR was 1.52-log compared to S2 (Fig. 1; $p<0.01$). These results show that the *B. suis*ΔvjbR mutant had a decreased replication capability in RAW264.7 macrophages.

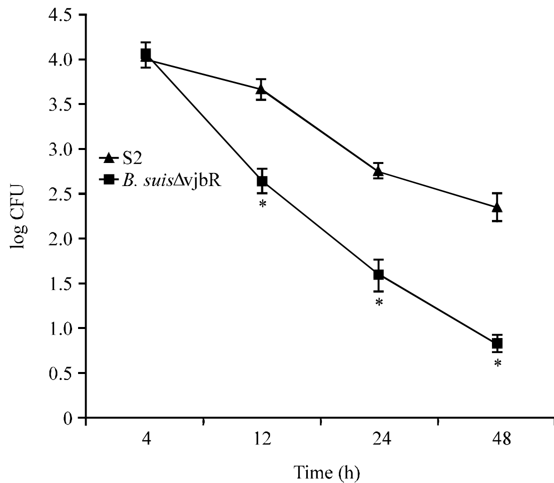

Fig. 1: Survival of bacterial strain *B. suis*ΔvjbR and S2 in RAW 264.7 macrophages. Monolayers of macrophages were infected with Brucella. At 4, 12, 24 and 48 h postinfection, infected macrophages were lysed and supernatants were diluted for CFU enumeration. Significant differences between the mutant and other *B. melitensis* strains are indicated as follows: *p<0.01

***B. suis*ΔvjbR mutant is attenuated in BALB/c mice:** To narrow the study to the mutant strains that had an attenuated phenotype in an animal model, researchers infected wild-type BALB/c mice 1×10^6 CFU of *B. suis*ΔvjbR and S2. The number of Brucella CFU was evaluated at 1, 2, 4 and 6 weeks postinfection in the spleen of each animal. The *B. suis*ΔvjbR mutant was significantly attenuated (p<0.001) at 2, 4 and 6 weeks when compared to S2 (Fig. 2). In addition, the *B. suis*ΔvjbR mutant was cleared by week 6. These results showed that the *B. suis*ΔvjbR strain was greatly attenuated in mice.

***B. suis*ΔvjbR immunization induced humoral immune and cytokine responses:** Serum from mice inoculated i.p. with 1×10^6 CFU of *B. suis*ΔvjbR, S2 or PBS were collected to monitor total IgG levels by ELISA. The *B. suis*ΔvjbR and S2 vaccinated mice expressed significantly higher IgG levels than that in PBS-infected mice (p<0.001; Fig. 3).

In order to examine the T-cell responses to *B. suis*ΔvjbR, levels of IFN-γ in splenocytes of *B. suis*ΔvjbR and S2 vaccinated mice were assessed at 8 weeks post-immunization mice inoculated with homologous immunogen ConA and heat-inactivated *B. suis* were used as control. Splenocytes of *B. suis*ΔvjbR and S2 vaccinated mice expressed significantly higher levels of IFN-γ than that of PBS-injected mice

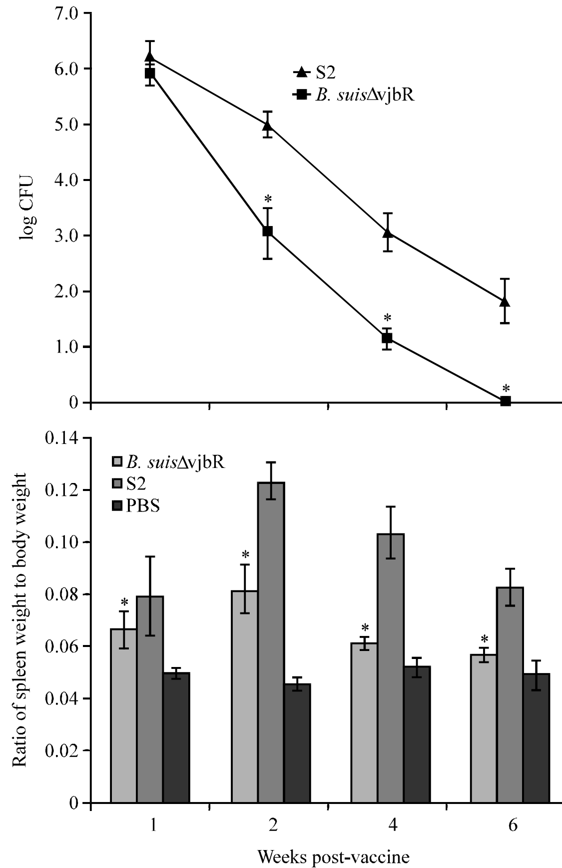

Fig. 2: Clearance of *B. suis*ΔvjbR after infection. BALB/c mice were infected with 1×10^6 CFU/mouse of *B. suis*ΔvjbR and S2. At 1, 2, 4 and 6 weeks postinfection, the spleens were harvested at different time points and individual spleens were assessed for colonisation. Values are the means of individual mice±SD and differences in splenic weight and colonisation were determined via unpaired test between *B. suis*ΔvjbR and S2 (*p<0.001)

(p<0.001; Fig. 4) and the splenocytes of *B. suis*ΔvjbR vaccinated mice expressed higher levels of IFN-γ than that of S2 vaccinated mice.

***B. suis*ΔvjbR-vaccination provided good immune protection against *B. suis* infection:** Mice were vaccinated i.p. with 1×10^6 CFU of *B. suis*ΔvjbR, S2 or PBS to determine whether *B. suis*ΔvjbR vaccination can provide protection against wild-type *B. suis* infection. The 14th week after vaccination, mice were challenged with 1×10^6 CFU of *B. suis*. Mice immunized with the *B. suis*ΔvjbR exhibited significantly fewer splenic Brucella than the non-immunized ones at 2 (1.70 log unit) and

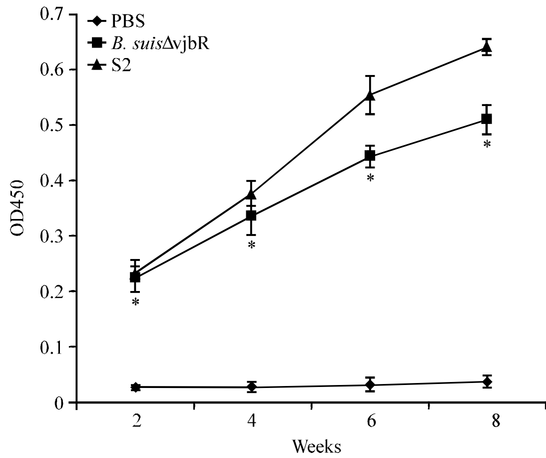

Fig. 3: Immunization with *B. suis*ΔvjbR induces antibody responses. BALB/c mice were immunized with 1×10^6 CFU of either *B. suis*ΔvjbR, S2 or PBS. At 2, 4 and 6 weeks post-immunisation, serum samples were collected and analyzed for IgG levels by ELISA. Results are means±SD (n = 10) of the absorbance at 450 nm (OD450). Significant differences between *B. suis*ΔvjbR and PBS are indicated as follows: *p<0.001

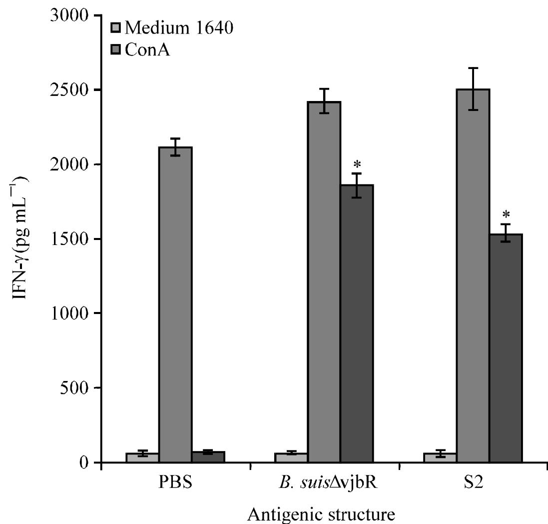

Fig. 4: Splenocyte production of IFN-γ induced by *B. suis*ΔvjbR. Spleens from BALB/c mice were inoculated intraperitoneally with 1×10^6 CFU of either *B. suis*ΔvjbR, S2 or PBS. Splenocyte culture supernatants were harvested after 72 h of culture. The analysis of IFN-γ secretion was measured by ELISA from the supernatant. Significantly different from the same stimulus in PBS-immunised mice are indicated as follows: *p<0.001

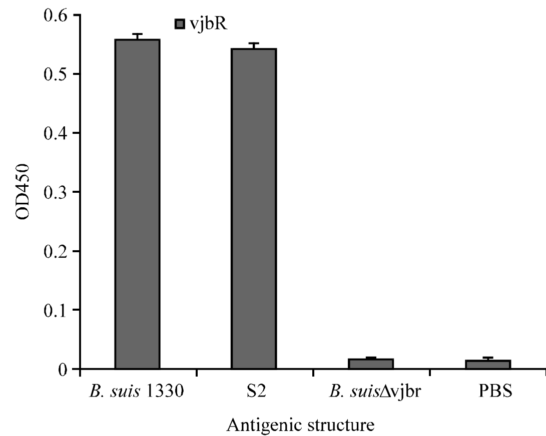

Fig. 5: Reaction of vjbR to *B. suis*ΔvjbR immunization sera. Antibodies to vjbR protein were not detected in the serum from *B. suis*ΔvjbR immunised mice by vjbR-iELISA but they were detected in the serum from *B. suis* immunised mice

6 (3.05 log unit) weeks after challenge (p<0.001, Table 1). As expected, S2 also induced significant protection at 2 (1.33 log unit) and 6 (2.17 log unit) weeks after challenge and the protective efficacy of *B. suis*ΔvjbR was higher than that in S2 vaccinated mice. The results indicated that the *B. suis*ΔvjbR-vaccination can provide stronger protection than S2-vaccination against the virulent *B. suis*.

Differentiation of *B. suis*ΔvjbR: Serum from mice vaccinated with *B. suis*ΔvjbR, S2, *B. suis* or PBS were collected to determine whether the protein vjbR can be used as a diagnostic antigen. Antibodies against vjbR protein could be detected in serum of *B. suis* or S2 vaccinated mice and could not be detected in serum of *B. suis*ΔvjbR or PBS-vaccinated mice, indicating that *B. suis*ΔvjbR-vaccination did not induce antibodies against vjbR (Fig. 5). Further, serum from *B. suis*-vaccinated mice showed positive reaction and serum from those *B. suis*ΔvjbR-vaccinated showed negative reaction by vjbR-iELISA using HIS-vjbR as solid-phase antigen. These results indicate that the vjbR protein can be used to differentiate between natural and vaccinated infections by vjbR-iELISA after the confirmation of Brucellosis infection using LPS-based serological tests.

The development of an efficacious vaccine for brucellosis has been a challenge for scientists for many years. Most of the present license vaccines have several drawbacks such as residual virulence, splenomegaly and interference of serodiagnosis (Ashford *et al*., 2004; Strausbaugh and Berkelman, 2003; Yang *et al*., 2013). One

Table 1: Protection by *B. suis*ΔvjbR against *B. suist*

	$\log_{10}$ CFU Brucella per spleen (mean±SD) at week post-challenge		$\log_{10}$ units of potection[a, b] at week	
Vaccine	2	6	2	6
PBS	6.46±0.47	5.93±0.73	-	-
B. suisΔvjbR	4.76±0.08	2.88±0.35	1.70*	3.05*
S2	5.13±0.21	3.76±0.14	1.33*	2.17*

of the drawbacks for the development of new Brucella vaccines is the limited knowledge of virulence factors. The recent deciphering of the complete genome sequences of *B. melitensis* will help to identify genes involved in virulence and avirulent mutant candidates used as vaccines (Cosivi and Corbel, 1998; Paulsen *et al*., 2002). To investigate whether *Brucella melitensis B. suis*ΔvjbR maintains protective efficacy, *B. suis*ΔvjbR was constructed and its virulence and protection efficacy were assessed in mice.

The deletion mutant of vjbR was constructed and confirmed by PCR and transcription analysis. *B. suis*ΔvjbR was firstly evaluated in RAW 264.7 macrophages and BALB/c mice and showed that *B. suis*ΔvjbR was defective for survival in them and it was cleared faster than S2 and eventually cleared within 6 weeks mice. This indicated that *B. suis*ΔvjbR elicited a significantly reduced virulence. This is consistent with previous results and substantiated that vjbR is involved in virulence of Brucella. Then *B. suis*ΔvjbR was evaluated in mice and conferred higher protection against an experimental *B. suis* in mice compared to S2. The study showed that *B. suis*ΔvjbR mutant was safe and could elicit good protective efficacy against a subsequent challenge with the wild-type strain.

Current serological diagnostic tests such as the RBPT, SAT, CFT, iELISA or cELISA using hot saline extract and smooth LPS antigens. The LPS of smooth Brucella species is by far the strongest antigen when compared to other antigenic molecules (Wang *et al*., 2011). However, LPS-based serological tests are difficult to differentiate between serum of vaccinated animals and which of infected ones. Therefore, vjbR protein was purified by recombinant HIS-fusion protein and evaluated as diagnostic antigen. The results showed that mice infected with S2 were positive in the vjbR-iELISA based on a recombinant HIS-fusion protein as the solid-phase antigen while animals vaccinated with the *B. suis*ΔvjbR mutant were negative. Therefore, *B. suis*ΔvjbR provides choice for differentiation between infected and vaccinated animals.

The cytokine and the humoral immune response have been evaluated for detecting the protection conferred by the *B. suis*ΔvjbR. The IFN-γ that results from cytokines can gather to the infection sites to enhance bacterial killing. Th1 immune responses characterized by production of IFN-γ are associated with protective immunity to Brucella and these responses are best stimulated by live vaccines (Golding *et al*., 2001). Previous study showed that IFN-γ is a critical cytokine and is required for macrophage bactericidal activity (Sathiyaseelan *et al*., 2006). The results showed that *B. suis*ΔvjbR could induce high IFN-γ and IgG-LPS in mice. Therefore, it is essential for the vaccination *B. suis*ΔvjbR can induce cell-mediated immunity and humoral immunological responses.

CONCLUSION

This study indicates *B. suis*ΔvjbR gave good protection against challenge with virulent *B. suis* strain *B. suis*. In addition, *B. suis*ΔvjbR can elicit an anti-Brucella-specific IgG response and vjbR antigen could provide an ideal diagnostic for the differentiation of immunisation from infection by vjbR-iELISA. In future studies, it will be important to conduct comprehensive protection experiments and to determine if the measurable immune responses in systemic compartments via different routes confer detectable protection against Brucella infections in sheep and goats.

ACKNOWLEDGEMENTS

This research was supported by grants from the National Basic Research Program of China (973 Program) (2010CB530203) and National Twelfth Five-Year Plan for Scie-nce and Technology Support Program (2013BAI05B05) and the National Natural Science Foundation of China (31201863, 81360239).

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