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Immunity Efficacy of a DNA Vaccine Encoding VirB8 of Brucella in BALB/c Mice

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Abstract: Brucella is a intracellular bacterial pathogen. Due to current available attenuated vaccine against it has certain defects. The development of an effective DNA vaccine against brucellosis is an area of intense research. This study was conducted to evaluate the immunogenicity and protective efficacy of a DNA vaccine encoding Brucella VirB8 which is a essential components of type IV Secretion Systems (T4SSs) and bacterial virulence factor. The BALB/c mice inoculated Intramuscularly with pCDNA-VirB8 plasmid developed both humoral and cellular immune responses. They produced VirB8-specific antibodies with a dominance of IgG2a over IgG1 and a T-cell-proliferative response. Recombinant VirB8 also induced the production of gamma Interferon but not Interleukin-4 (IL-4) after stimulating lymphocytes with it *in vitro*. This suggested the induction of a typical T-helper-1-dominated immune response in mice. The protective ability of the DNA vaccine was evaluated by systemic bacterial clearance after challenging the mice with S2. The pCDNA-VirB8 induced a significant level of protection in BALB/c mice. Altogether, these data suggest that VirB8 is a good candidate for studies of DNA vaccination against brucellosis in future.

Key words: Brucella, VirB8, DNA vaccine, immunity efficacy, *in vitro*

INTRODUCTION

Brucellosis can induce abortion and infertility after affecting humans and several other animal species whose pathogen, the brucella is a facultative intracellular Gram-negative bacterium.

Until now, a few of attenuated vaccines such as *B. melitensis* Rev.1 and *B. abortus* S19 and RB51 have been used to control brucellosis in domestic animals. However, they are considered unsuitable to humans because of rather virulence (Perkins *et al*., 2010). For researching safe and efficacious vaccines such strategies as subunit vaccines, utilization of bacterial vectors and DNA vaccines have been developed (Jain-Gupta *et al*., 2012; Al-Mariri *et al*., 2012; Hu *et al*., 2010; Commander *et al*., 2010). Considering the capability of inducing strong Cell Mediated Immune response (CMI) which is vital in the protection against the intracellular pathogen, DNA vaccines can be very effectiv (Xu *et al*., 2008; Ge *et al*., 2012). At present, some DNA vaccine can already protect against many viral, fungal and parasitic diseases in different animal models (Roodbari *et al*., 2012; Rittner *et al*., 2012; Siqueira *et al*., 2012; De Amorim *et al*., 2013; Chen *et al*., 2013; Wang *et al*., 2012). On the brucellosis, DNA vaccines such as the Brucella ribosomal *L7/L12* gene (Luo *et al*., 2006a, b; Yu *et al*., 2007a, b; Kurar and Splitter, 1997), Cu-Zn Superoxide Dismutase (SOD) (Yu *et al*., 2007a), Outer-membrane protein 31 (Omp31) (Cassataro *et al*., 2007) or lumazine synthase gene (Velikovsky *et al*., 2002) have been demonstrated to induce significant levels of protection in the mice. Because some of them are inside the cell, this indicated that the gene of intracellular protein also can be used for DNA vaccine. Moreover, intracellular proteins are not as changeable as outer proteins of the cell so that they are rather suitable for the DNA vaccines.

Bacterial secretion system which have six types is closely related to bacterial survival in the host and pathogenicity to the host. Type IV Secretion Systems (T4SSs) which span outer and inner membranes are secretion machineries in Gram-negative bacteria (Terradot *et al*., 2005). They play an important role in the infection of human, animals and the propagation of antibiotic resistance by mediating the translocation of effector molecules from bacterial pathogens into eukaryotic cells (Backert and Meyer, 2006).

There are three classes of T4SSs: IVA subtype, IVB subtype and GI subtype (Nagai, 2010; Waksman and Fronzes, 2010; Wallden *et al*., 2010). T4SS of brucella classified into IVA subtype was known as VirB (Paredes-Cervantes *et al*., 2011). VirB8 is a essential components of VirB of brucella (Den Hartigh *et al*., 2008) which can interact with periplasmic domain of VirB6 and

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the Globular Domain of VirB5 (Villamil *et al*., 2012) and act as an assembly factor in secretion system assembly (Sivanesan *et al*., 2010) and which dimmer interface is required for T4SS function, the stabilization of many VirB proteins and targeting of VirB2 to the T-pilus assembly site.

In the present study, the immunogenicity and protective ability of the *VirB8* gene as the DNA vaccine was demonstrated.

MATERIALS AND METHODS

Bacterial strains and vector: *B. suis* Strain 2 (S2) and *B. abortus* Strain 19 (S19) were obtained from the Tecon Group, China. Its purity and biochemical characters were tested by standard biochemical tests before the experiment. The bacterial cells were grown under aerobic conditions in tryptose-soy broth (Qingdao Hope Bio-Technology Co., Ltd.) for 72 h at 37°C. For inoculation, the bacterial suspension was adjusted spectrophotometrically to 2×10^8 CFU. All experiments with live brucella were performed in bio-safety level 2 facilities.

E. coli strain DH5 and BL21(DE3) was conserved in the own laboratory, grown on LB medium and used for cloning preparations, producing the necessary plasmid constructs or expressing recombinant protein. The *E. coli* cultures were routinely grown at 37°C in LB broth or agar supplemented with 100 μg of ampicillin per milliliter.

The eukaryotic vector pCDNA3.0 and prokaryotic vector pET28a conserved in the laboratory was used to construct the DNA vaccine and recombinant expressing plasmid.

Animals: Specific pathogen-free female BALB/c mice were 6 weeks old and obtained from the Animal center at the Academy of Military Medical Sciences, China. The mice were fed with commercial mouse chow and water *ad libitum* in clean conditions at the laboratory animal center of Shenyang Agriculture University, China. They were randomly distributed into experimental groups.

Construction of VirB8 DNA vaccine: The primers for VirB8 were designed based on the corresponding genome sequence from GENEBANK. VIRB8L: 5'-AAAAGGATCCGCCACCATGGTTGGACGCAAACAATC-3'; VIRB8R: 5'-AAAACTCGAGGAATCTTTTCATTGCACCACTCC-3'. Restriction sites for EcoR I and Xho I were introduced, respectively to oligonucleotides to conveniently clone. Chromosomal DNA of *B. suis* strain 2 was used for the template in amplifying the *VirB8* gene by Polymerase Chain Reaction (PCR).

PCR was performed for 35 cycles at 94°C for 30 sec, 50°C for 30 sec and 72°C for 45 sec after pre-denaturation for 2 min. The PCR amplified product was digested by EcoR I and Xho I restriction enzyme and retrieved.

The vector pCDNA3.0 was used for construction of VirB8 DNA vaccine. The flanked *VirB8* gene was ligated into pCDNA3.0 downstream to the CMV promoter to form the resulting plasmid pCDNA-VirB8. Then, the pCDNA-VirB8 plasmid was verified by restriction enzymes digestion, PCR and sequencing after it was purified using the UNIQ-500 Column Enodotoxin-Free Plasmid Maxi-Preps kit (Sangon Biotech Co., Ltd. Shanghai, China).

Expression and purification of recombinant VirB8 (rVirB8): The expressing vector pET28a was used to construct express plasmid for express rVirB8. The above flanked *VirB8* gene was ligated into pET28a digested by EcoR I and Xho I restriction enzyme to construct the recombinant plasmid pET-VirB8. After verified by the same way like the pCDNA-VirB8, *E. coli* BL21 (DE3) harboring pET-VirB8 was induced in auto-induction media ZYP-5052 (Studier, 2005).

Polyhistidine tagged rVirB8 was purified by affinity chromatography with Histrap FF crude (GE Healthcare Bio-sciences AB, USA) and identified with anti-Histidine antibody by Western Blot analysis. Then, it was stored at -70°C until used for Enzyme-Linked Immunosorbent Assay (ELISA) or for *in vitro* stimulation of lymphocytes.

Preparation and identification of hyperimmune sera: Two healthy adult rabbits (New Zealand White) were injected intramuscularly with purified rVirB8 (100 μg) for the preparation of anti-VirB8 hyperimmune serum. Two booster was given on the 14th and 28th days. The rabbits were bled for serum collection on 14th day after final booster. Specificity of the rabbit hyper-immune serum to the purified rVirB8 and the disintegrated cells of S2 was identified by western blot analysis.

Immunization: The immunological studies of the VirB8 DNA vaccine were performed in 2 groups of BALB/c mice. Each group consisted of thirty mice.

After being anesthetized with inhaled halothane, Different groups of experimental mice were injected separately in tibialis anterior muscle for immunization with 100 μg of pCDNA3.0 and pCDNA-VirB8 on days 0, 14 and 28.

At 0 day and inoculating days, six mice from pCDNA3.0 and pCDNA-VirB8 groups were bled through retro-orbital puncture with capillary tubes and blood was collected in sterile microfuge tubes for serum. The final preparation was kept at -70°C till further use.

Determination of antigen specific immunoglobulin and their isotypes by indirect ELISA (iELISA): Pooled serum from 6 mice of different groups bled individually at 0, 14, 28 and 42 days were used for detection of antibodies specific to purified rVirR8 by indirect ELISA.

The purified rVirB8 protein was diluted to 3 μg mL^{-1} in carbonate buffer (0.05 M, pH 9.6) and used to coat overnight the wells of a polystyrene plate at 4°C. Next day, plate was washed thrice with PBS-T and blocked with skimmed milk powder (3%) in PBS-T for 1 h at 37°C, then incubated with 100 times of dilution of the sera for 3 h at room temperature. Each serum sample was tested in triple. Anti-mouse IgG, IgG1 or IgG2a HRP conjugate (100 μL $well^{-1}$) was added to wells at an 1000 times of dilution and incubated at 37°C for 1 h after being washed 4 times. After 4 washing, 100 μL of substrate solution (200 mol of o-Phenylenediamine and 0.04% H_2O_2) was added to each well for 20 min of incubation in the dark at room temperature. Then, the enzyme reaction was stopped by addition of 100 μL of 0.5 M sulfuric acid/well and the absorbance was measured at 490 nm. The titer was expressed as Optical Density (OD) units.

Lymphocyte proliferation assay: On the days 0, 14, 28 and 42 after inoculation, six mice from pCDNA3.0 and pCDNA-VirB8 group, respectively were sacrificed to obtain their spleens under aseptic conditions. Splenocytes were collected by forceful flushing of spleen with chilled PBS. The flushed PBS containing splenocytes and RBCs was layered slowly on to equal volume of Mouse lymphocyte isolation liquid (Sangon Biotech Co., Ltd. Shanghai, China) and centrifuged at 1000 g for 40 min. The interface containing lymphocytes were collected and washed with chilled PBS followed by a final wash with RPMI-1640 containing 10% Newborn Calf Serum (NBCS), L-glutamine (2 mM), streptomycin (100 μg mL^{-1}) and penicillin (100 IU mL^{-1}). Splenocytes were cultured at 37°C with 5% CO_2 in a 96 well flat-bottom plate at a concentration of 4×10^5 viable cells/well in the presence of rVirB8 (1 μg mL^{-1}) for 72 h. Then, 10 μL of 5 mg mL^{-1} Thiazolyl blue (MTT) in RPMI 1640 was added to all the wells to further incubation at 37°C for 4 h. Thereafter, the plate was centrifuged at 1000 g for 10 min to settle down the formazen crystals. After aspirating supernatant, the crystals were dissolved with 150 μL $well^{-1}$ of Dimethyl Sulfoxide (DMSO, 100%) by vigorous pipetting. Finally, the absorbance was measured at 570 nm with a reference wavelength of 650 nm using ELISA Reader (Biorad). The Stimulation Indices (SI) were calculated as the ratio between the absorbance values of stimulated cells to the unstimulated cells.

Cytokine ELISAs: The presence of the cytokines in the culture supernatants of spleen cells were tested using Mouse IFN-γELISA Set and Mouse IL-4 ELISA Set (BD Biosciences, USA). All assays were performed in triplicate. The concentration of IFN-γ and IL-4 in the culture supernatants was calculated for each experimental group by using a linear-regression equation obtained from the absorbance values of the standards.

Protection experiments: In protection experiments a PBS control group and a positive control vaccinated intramuscularly with 10^8 CFU of *B. suis* S2 were added.

The protection experiments were performed using *B. abortus* S19. The 42 days after first vaccination, six mice from each group were challenged with 10^8 CFU of S19 by intramuscular injection. The 2 weeks later, the infected mice were sacrificed and their spleens were removed aseptically and triturated. A 10 fold serial dilution of spleen lysate was prepared in Tryptic Soy Broth (TSB) and 10 μL from each dilution was plated in triplicate to determine the number of Brucella CFU per spleen. All the plates were incubated at 37°C with 5% CO_2 for 3 days. Then, colonies were counted and expressed as $\log^{10}$ CFU per spleen. protection were obtained by subtracting the mean $\log^{10}$ CFU for the experimental group from the mean $\log^{10}$ CFU of the corresponding PBS control group.

Statistical analysis: The statistical analysis was evaluated using the SPSS 15.0 program for Windows (IBM Corp., New York, USA). The data for the antibodies, lymphocyte proliferation and cytokine were analyzed with paired-samples t-test. The data for the protection experiments were analyzed using Tukey's honestly significant difference procedure.

RESULTS AND DISCUSSION

Construction of VirB8 DNA vaccine: For rapidly constructing VirB8 DNA vaccine, researchers designed a pair of primers with restriction sites and redundant bases on their 5 terminal so that they can be digested straightly by restriction enzymes and inserted into the vectors after amplification. The constructed pCDNA-VirB8 plasmid was verified by restriction enzymes digestion, PCR and sequencing. Result showed it was a appropriate band (Fig. 1a) and right sequence of VirB8.

SDS-PAGE and immunoblot analysis of rVirB8: For obtaining recombinant protein, *VirB8* gene was inserted into pET28a expression vector system to construct a new pET-VirB8 recombinant plasmid. Identification result by restriction enzymes and PCR showed it contained *VirB8*

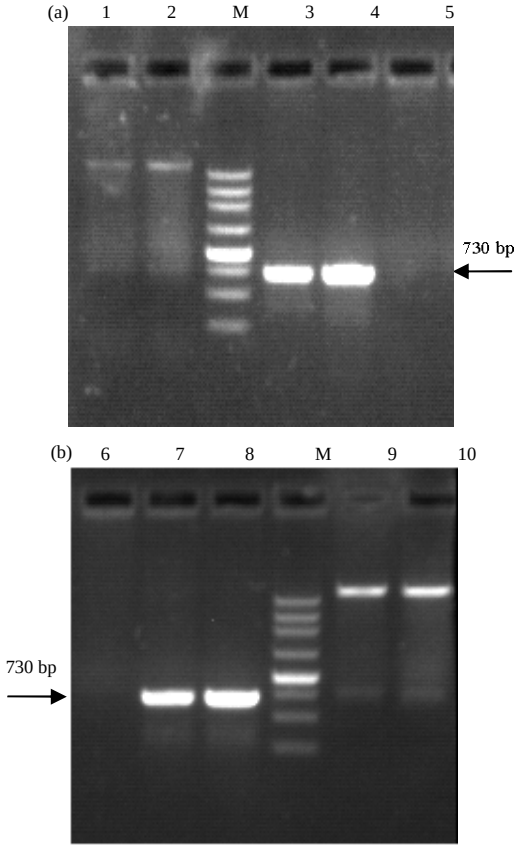

Fig. 1: Identification of recombinant plasmids by restriction enzymes digestion and PCR; a) identification of pCDNA-VirB8 plasmid; b) identification of pET-VirB8 plasmid 1, 2, 9, 10. The lanes of restriction enzymes digestion; 3-8; the lanes of PCR, 5, 6; Negative control. M: 200 bp DNA maker

gene (Fig. 1b) and had right sequence. SDS-PAGE analysis of the cell lysate of induced *E. coli* BL21 (DE3) harboring pET-VirB8 showed the expression of about 30 kDa recombinant protein. After being purified by affinity chromatography with Histrap FF crude, it can react with anti-histidine monobody in Western Blot assay (Fig. 2a).

Preparation and identification of hyperimmune sera: Two healthy adult rabbits (New Zealand White) were injected intramuscularly with purified rVirB8 (100 μg) for the preparation of anti-VirB8 hyperimmune serum. A booster was given on the 14th and 28th days. The rabbits were bleed for serum on 42nd day. In Western Blot analysis, the serum diluted by 1:100 as first antibody can react specifically with purified rVirB and disintegrated S2

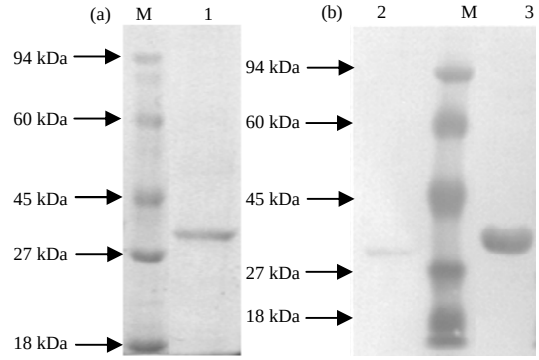

Fig. 2: Identification rVirB8 protein and rabbit hyperimmune sera by Western blot assay; a) Identification of rVirB8 (Lane 1) with anti-histidine antibody; b) Identification of rabbit hyperimmune sera with disintegrated cells of S2 (Lane 2) and the purified VirR8 protein (Lane 3). M: Molecular weight Marker

(Fig. 2b). It illustrated that the rVirR8 had a good antigenicity to induce the rabbits to produce the specific antibody.

Humoral immune response: Indirect ELISA was used to measure the titers of VirB8 antibodies in serum from mice immunized separately with pCDNA-VirB8 and pCDNA3.0. Mice vaccinated with pCDNA-VirB8 developed significant and steady increasing antibodies to rVirB8. OD value of the antibody to VirR8 varied form 0.17 on 14th day to 0.57 on 42nd day (Fig. 3a). The OD unit of IgG in the pCDNA-VirB8 group was significantly higher ($p<0.002$) than it in the pCDNA3.0 group.

Subtype (IgG1 and IgG2a) analysis of these antibodies indicated that the anti-VirB8 antibodies detected in pCDNA-VirB8-immunized mice were predominantly IgG2a at 42nd day post-vaccination. TheOD value of the specific IgG2a were significant higher than that of the specific IgG1 and the ratio of IgG2a/IgG1 is above 5 (Fig. 3b). On the contrary, the ratio in pCDNA3.0 group is about 1.5 without markedly difference.

Lymphocyte proliferation response: To evaluate the proliferative response of the rVirR8 protein, lymphocytes from mice immunized with pCDNA-VirR8 and pCDNA3.0 were stimulated with the protein. The result indicated the lymphocytes had an increasing proliferative response to pCDNA-VirB8 after every inoculation. At 14th day, the lymphocytes from mice of pCDNA-VirR8 group showed slight proliferative response to rVirR8 compared to

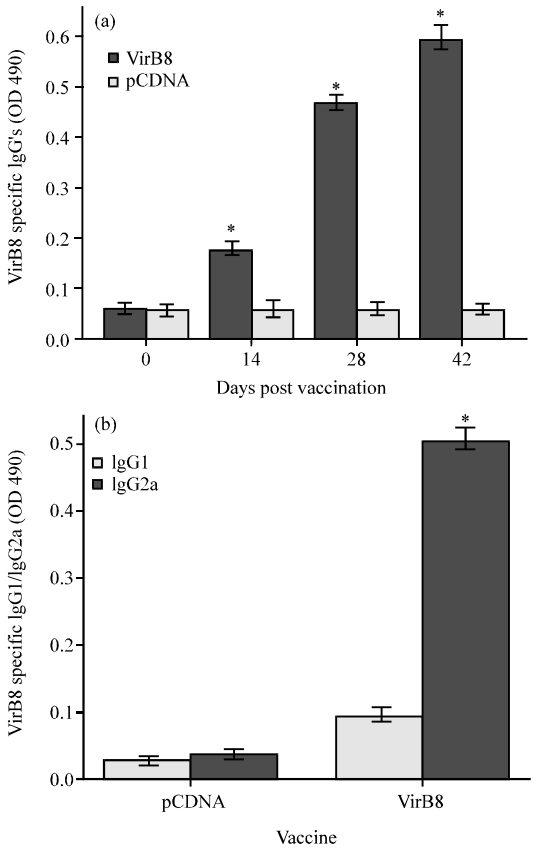

Fig. 3: ELISA detection of antibody levels of different groups; a) Anti-VirB8 IgG antibody levels in immunized mice; b) Antibody subtype profile of Anti-VirB8 IgG antibody of mice on 42nd day after inoculation. Each bar represents the mean titer±SD (error bars) of antibodies in six animals

pCDNA3.0 group whereas after 2 weeks post-booster, the lymphocytes of pCDNA-VirR8 group showed significant proliferative response compared to pCDNA3.0 group ($p<0.003$). The stimulation index at 42nd day post vaccinization reach 2.87 at a highest value (Fig. 4).

Detection of gamma-Interferon (IFN-γ) and Interlukin-4 (IL-4) level in immunized mice: IFN-γ and IL-4 level in culture supernatant of splenocytes culture of the mice from each group were determinated following *in vitro* stimulation with rVirR8. Supernatants of spleen cell cultures from pCDNA-VirR8-immunized animals contained high levels of IFN-γ which is steadily increasing and reaches the peak (2.48 ng mL^{-1}) of the 42nd day post inoculation compared to the pCDNA3.0 control groups ($p<0.002$) (Fig. 5a). The rVirB8 protein only induced low level of production of IL-4 which has no significant

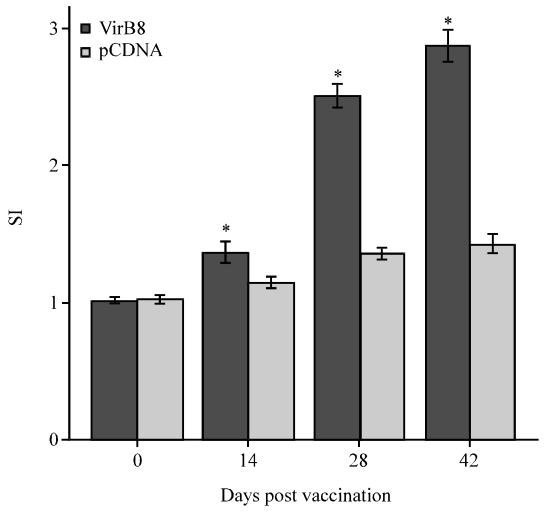

Fig. 4: Lymphocyte proliferation assay. Lymphocytes from each group (4×10^5 cells per well) were stimulated *in vitro* with purified rVirB8 (0.8 μg mL^{-1}). The data are average of SI of triplicate cultures of cells from six individual mice of each group after 72 h

difference between pCDNA3.0 group and pCDNA-VirB8 group ($p<0.09$) (Fig. 5b). In a word, the results indicate that immunization with pCDNA-VirR8 induces a specific Th1-type immune response in mice.

Protection against *B. suis* stain 2 challenge in immunized mice: To determine the protective efficacy of pCDNA-VirR8 vaccine, immunized mice were sacrificed on 14th day post challenge. Protection was defined as a significant reduction in the number of bacteria in the spleen from immunized mice compared to the PBS control mice receiving PBS. The vaccine efficacy was calculated as the log units of protection.

Results showed that VirB8 DNA vaccine can significantly reduce the CFU of S19 in mice spleens than that in pCDNA3.0 group. Average clearance observed in mice of pCDNA-VirR8 group was 0.863 which is above 0.117 of pCDNA3.0 group ($p<0.001$) and <1.415 of S2 control group ($p<0.002$). No significant difference was seen between the groups injected with pCDNA3.0 and PBS ($p>0.05$) (Table 1). These results indicate that pCDNA-VirB8 vaccine afforded a significant degree of protection against Brucella infection.

Up to now, the most successful way for preventing infectious diseases in animals and humans is still vaccination. But several live attenuated vaccines available for control of animal brucellosis have certain drawbacks (Kahl-Mcdonagh and Ficht, 2006; Perkins *et al*., 2010), the

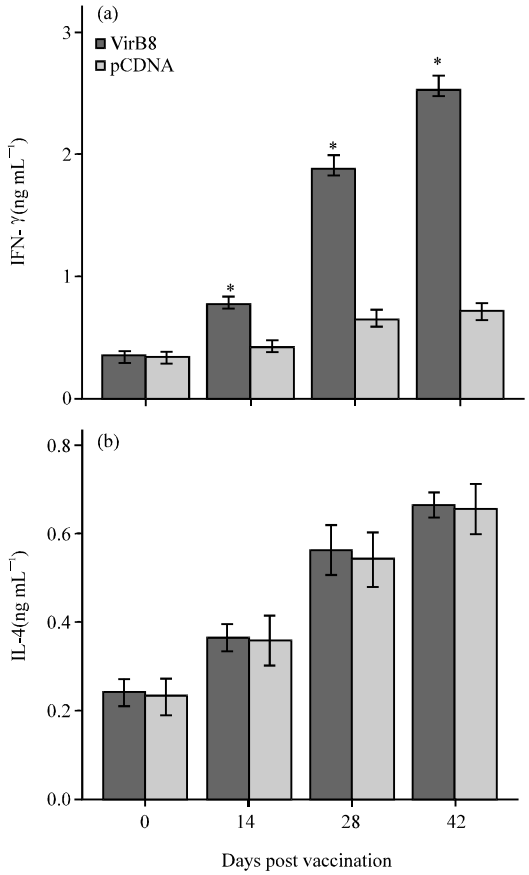

Fig. 5: Quantitative ELISA analysis of cytokines. a) IFN-γ and b) IL-4; secreted by splenocytes upon stimulation with rVirB8. Spleen cells (4×10^5 mL^{-1}) from mice vaccinated with pCDNA3.0 or pCDNA-VirB8 were stimulated with rVirB8 (0.8 μg mL^{-1}) for 72 h. Each bar represents the geometric mean±SD (error bars) of the responses in spleen cells from six individual mice from each group

Table 1: Protection of mice against challenge with S19 after immunization with DNA vaccine pCDNA-VirR8

Groups	Mean log10 CFU±SD in spleen	Log units of protection
PBS	2.972±0.074[a]	0.000
pCDNA3.0	2.855±0.062[a]	0.117
pCDNA-VirR8	2.109±0.050[b]	0.863
B. suis stain 2	1.557±0.056[c]	1.415

[a-c]Means bearing different superscripts in a column which differs significantly ($p<0.05$)

development of new-generation vaccine to prevent brucellosis is needed. Because Brucella is an intracellular pathogen and protection against its infection requires the generation of a Th1-type immune response over time (Golding *et al*., 2001), DNA vaccines is very promising due to its ability of inducing a broad range of immune responses including antibody production, CD8 Cytotoxic T cells (CTLs) and CD4 T helper cell activation after inoculated naked DNA naturally processed and presented on Major Histocompatibility Complex (MHC) class I and II molecules (Perkins *et al*., 2010; Donnelly *et al*., 1997).

Brucella type IV secretion system VirB is a specialized multiprotein complex in the cell envelope for the delivery of effector molecules into host cells that subvert varied host cellular processes and is importent to subvert lysosome fusion and build up a replicative vacuole associated with the endoplasmic reticulum of the host cell (Carle *et al*., 2006; Paredes-Cervantes *et al*., 2011).

VirB8 participating in multiple protein-protein interactions to mediate assembly of the translocation machinery is a essential components of T4SSs in brucella (Den Hartigh *et al*., 2008). Furthermore, VirB8 protein production in the vaccine strains was markedly different from that in the wild-type strains at pH 7 (Rouot *et al*., 2003).

However, all the studies about VirB8 focus on the interaction of it and other protein. No research about the DNA vaccine of VirB8 was reported. In the present study, the immunogenicity and protective efficacy of the VirB8 DNA vaccine was evaluated. The result was exciting VirB8 DNA vaccine can elicit high humoral immune reaction in BALB/c mice. Specific IgG in mice inoculated with the plasmid pCDNA-VirB8 increased rapidly from first to third injection. Moreover, it can also induce the cultured lymphocytes of the immunized mice to produce a high proliferative response and a high level of IFN-γ. Because IFN-γ is secreted by the Th1 of the CD4+cells, VirB8 DNA vaccine induces a Th1 cellular response. It was also in accordance with the predominance of IgG2a over IgG1.

Finally, researchers tested the protective efficacy of the pCDNA-VirB8 vaccine against experimental infection by measuring reduction of bacterial burden in spleen of the immunized mice. Due to the rigidity of the bacteria and limit of the bio-safety laboratory level 3, S19 which has a stronger virulence than S2 (Arenas-Gamboa *et al*., 2009; Xin, 1986) was used as a challenger in this study. Result showed that the mice inoculated with *B. abortus* S2 gave highest protection of 1.415 log units at 2 weeks post challenge followed by mice vaccinated with pCDNA-VirB8 at 0.863. On the contrary, mice inoculated with pCDNA3.0 did not show significant protection compared to control mice (Table 1). The facts that the protective effect of S2 is better than that of rVirB8 DNA vaccine probably result from the complex components of the S2. At meantime, the cause protective effect of live vaccine is below the value of present reports is probably led to by the way of intramuscular inoculation and the virulence of the challenger (Kaushik *et al*., 2010; Onate *et al*., 2003; Yu *et al*., 2007b). The decision of intramuscular injection

based on the fact that it is a natural way of infection to brucellosis rather than intraperitoneal inoculation. Because S19 still can express VirB8 and translocate into the spleen of the inoculated mice as well as other wild brucella, the present study can embody the protective efficacy of VirB8 DNA vaccine in some extent. As for its protective efficacy against the wild brucella, researchers suggest further protection studies conducted in qualified laboratory.

CONCLUSION

The result of the present study shown that the *VirB8 DNA* gene can elicit both antibody and CMI responses of Th-1 type and confers protection against S19 challenge. VirB8 is a promising candidate for studies of DNA vaccine against brucellosis in future.

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