Immunogenic response induced by *wzm* and *wzt* gene deletion mutants from *Brucella abortus* S19

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Abstract. Brucellosis is an infectious disease affecting humans and animals worldwide. Effective methods of control include inducing immunity in animals by vaccination and elimination. *Brucella abortus* S19 is one of the popular vaccines for control of cattle brucellosis, as it has low virulence. In this paper, allelic exchange plasmids of *wzm* and *wzt* genes were constructed and partially knocked out to evaluate the effects on the induction of immunity to *Brucella abortus* S19 mutants. Cytokine secretion *in vitro*, INF-γ induction *in vivo* and antibody dynamics were evaluated. These data suggested that the immunity-eliciting ability of the *wzm* and *wzt* gene deletion mutants was similar, although reduced compared with the S19 strain. The results demonstrated that the *wzt* gene may be more important in the regulation of the induction of immunity than the *wzm* gene.

Introduction

Brucella species, the cause of brucellosis in humans and animals, are facultative intracellular bacteria. *Brucella* invades phagocytic and non-phagocytic cells and survives inside the host cells (1,2). The properties of the intracellular lifestyle of *Brucella* limit the number of antibiotics that are effective against these organisms once they form *Brucella*-containing vacuoles (BCVs) (3). Under most conditions, control of brucellosis in animal reservoirs is achieved via vaccination. Human brucellosis has also been controlled by immunization and culling within cattle, goat and sheep herds (4,5).

Currently there are no vaccines for humans and the useful vaccines for livestock are (*Brucella abortus) B. abortus* S19 and RB51 for cattle and *Brucella melitensis* (*B. melitensis)* Rev1 for small ruminants (6,7). *B. abortus* S19 has been widely used to prevent cattle brucellosis, as it usually has low virulence. However, it is infectious in humans and always causes abortion when used in pregnant animals (8,9). Since *B. abortus* S19 induces antibodies to the O-polysaccharide, it is difficult to distinguish from wild-type infection. The relevant diagnostic antigen is the smooth lipopolysaccharide (LPS) present in field strains, as well as in *B. abortus* S19 and *B. melitensis* Rev 1 (10-12). The development of a safe and efficacious vaccine that conquers this serological obstacle may have a broad impact on public health.

LPS provides bacterial resistance to antimicrobial attacks and modulates the host immune response, which makes it an important virulence factor for survival and replication in the host cell (1,13). It provides *Brucella* with resistance to innate immunity antibacterial responses by inhibiting complement and antibacterial peptide attacks, and by preventing the synthesis of immune mediators (14-16). The O-chain appears to help *Brucella* to invade cells in the early entry stage (17).

Brucellae without O-side chains are termed as rough or 'R' strain. R *Brucella* species or mutants lack the antigenic O-side chain and they do not induce anti-O-side chain antibodies. Currently, vaccinated hosts are difficult to distinguish from wild-type-infected hosts by common serological tests. It has been shown that *Brucella* R mutants are attenuated; therefore, R mutants have potential for use as vaccines (18-22). Several genes of *B. melitensis* 16M LPS synthesis were analyzed and ABC-type transporter (integral membrane protein, Wzm) and ABC-type transporter (ATPase domain, Wzt) were determined to be putative components of the ABC transporter system. The *wzm*/*wzt* mutant was proven to lead to the absence of the O-side-chain on the bacterial surface (23,24).

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The *wzt* mutant of *B. melitensis* 16M was evaluated and determined to have virulence-reducing potential in a mouse model (25). In recent years, LPS has been shown to interfere with major histocompatibility complex (MHC)-II presentation, which inhibits peptide presentation in cells (13,16). In the present study, we constructed *wzm* and *wzt* gene partial deletion mutants with no DNA marker addition, in order to estimate the survival *in vivo* and the serological response. This may provide us with an improved understanding of the effect of *wzm* and *wzt* genes on the induction of immunity caused by the S19 vaccine strain.

Materials and methods

Bacterial strains and growth conditions. *Escherichia coli* DH5α strain was grown on Luria-Bertani broth (LB) agar at 37˚C. Brucella strains *B. abortus* S19, Δwzm and Δwzt were grown on tryptic soy broth (TSB, Sigma, St. Louis, MO, USA) agar at 37˚C. Ampicillin (100 μg/ml) and kanamycin (50 μg/ml) were added for plasmid screening if required. TSB medium (7% sucrose) was prepared for screening of allelic-exchange mutants.

Construction of wzt and wzm mutants. The allelic exchange plasmids were constructed by pBKCMV (kan^r) with a *sacB* gene and fragments upstream and downstream of target genes. The *sacB* gene along with its promoter was amplified from pIBP279 (provided by Nanjing Agricultural University) using PCR methods and ligated into *pBKsacB* to construct *pBKsacBwzm* and *pBKsacBwzt* (Table I). Competent cells of *B. abortus* S19 were prepared and the constructed plasmid was electroporated into the cells at 1,500 kV [1-mm gap cuvette; BTX, Harvard Apparatus, Inc, Holliston, MA, USA]. Subsequently, 1 ml SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $CaCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added and cells were grown under agitation at 28˚C for 24 h and then plated on TSB agar (kan^r) and cultured for 96 h at 28˚C. The mutants were confirmed using PCR. The phenotype of the mutants was then determined by agglutination with acriflavine at a dilution of 1:100 (26).

Animals. The 4-6-week-old female specific pathogen-free (SPF) BALB/c mice were provided by the animal centre of Jilin University (Changchun, China). Mice were bred in the animal facilities with filtered air in a restricted-access room and under pathogen-limited conditions. Mice were acclimatised for a minimum of one week prior to the experiment and water and food was provided *ad libitum*. The animal experiments were approved by the Center of Laboratory Animals, Jilin University, China.

Serological test and antibody dynamics. Female BALB/c mice of 6-8 weeks of age were housed with water and food. Animals were randomly allocated to groups and acclimatised for 1 week prior to the initiation of experiments (n=5). To prepare the inoculated samples, bacteria were suspended in PBS and adjusted to the appropriate 10^8 CFU/ml in the same buffer. Blood samples from BALB/c mice were collected and allowed to clot for 12 h at 4˚C and centrifuged. Serum was divided into Eppendorf tubes (Eppendorf, Hamburg, Germany), and stored at -80˚C. The Rose Bengal plate agglutination test (RBPT, Harbin Pharmaceutical Group Bio-vaccine Co., Harbin, China) was carried out by mixing 30 μl serum and 30 μl antigen, and the reaction was observed after 4 min.

Table I. Bacteria and plasmids.

Strain or plasmid	Phenotype and/or genotype	Source
Strains		
B. abortus S19	Vaccine strain, smooth	IVDC
B. abortus Δ*wzm*	*B. abortus* S19 Δ*wzm*	This study
B. abortus Δ*wzt*	*B. abortus* S19 Δ*wzt*	This study
Plasmid		
pBKCMV	Kanamycin	Stratagene
pIBP279	With *sacB* gene	NJAU

IVDC, China Institute of Veterinary Drug Control, Beijing, China; NJAU, Nanjing Agricultural University, Nanjing, China.

The IgG antibody titer was estimated by indirect enzyme-linked immunosorbent assay (ELISA). The 96-well plates were coated by diluted *Brucella* antigens S19, *Δwzm* and *Δwzt* (pH 9.6, 0.05 M carbonate buffer 100 μl each well, antigen concentration 10^8 pfu), at 4˚C overnight. The plate was washed with 200 μl PBST buffer (pH 7.4, 0.01 M PBS: Tween-20, 1:1,000) three times, for 3 min each time, and then blocked by 100 μl 1% BSA (pH 7.4) and incubated at 37˚C for 1 h followed by three washes with PBST. The sera samples diluted from 1:100 to 1:3,200 were added and incubated at 37˚C for 1 h. After three washes with PBST, enzyme-labeled goat anti-mouse IgG (1:5,000) was added and incubated at 37˚C for 1 h and then washed with PBST three times. TMB substrate solution (100 μl) was then added and incubated at 37˚C for 10 min (in light). Then, 50 μl 2 M sulfate buffer was added to stop the reaction, followed by detection of optical density (OD) at 490 nm. The antibody titer was described as the diluted ratio controlled by the S/N ratio. If the S/N ratio was ≥2.1, the sample of serum was considered positive. The S/N ratio was calculated as: (sample - blank)OD_{490}/(negative - blank)OD_{490}.

Lymphocyte proliferation. After injecting antigens for 4 weeks, the spleens of mice were removed under sterile conditions, put in a sterile Petri dish with 4 ml lymphocyte separation liquid (Dakewe Biotech Co., Shenzhen, China) and then ground with a disposable syringe core in a 200-mesh nylon sieve (74 μm pore diameter). The spleen cell suspension was then added to a sterile centrifuge tube and 500 μl of RPMI-1640 medium was gradually added. The cells were then centrifuged for 10 min at 300 x g. The lymphocyte layer was placed into new centrifuge tubes, resuspended in 10 ml of RPMI-1640 and centrifuged for 2 min at 1,500 rpm. Subsequently, 5 ml of 0.15 M Tris-NH_4Cl solution was added to the cells and the cells were centrifuged at 200 x g for 5 min after 5 min incubation at room temperature. The supernatant was removed and the cells were washed twice with RPMI-1640. The lymphocytes were resuspended by

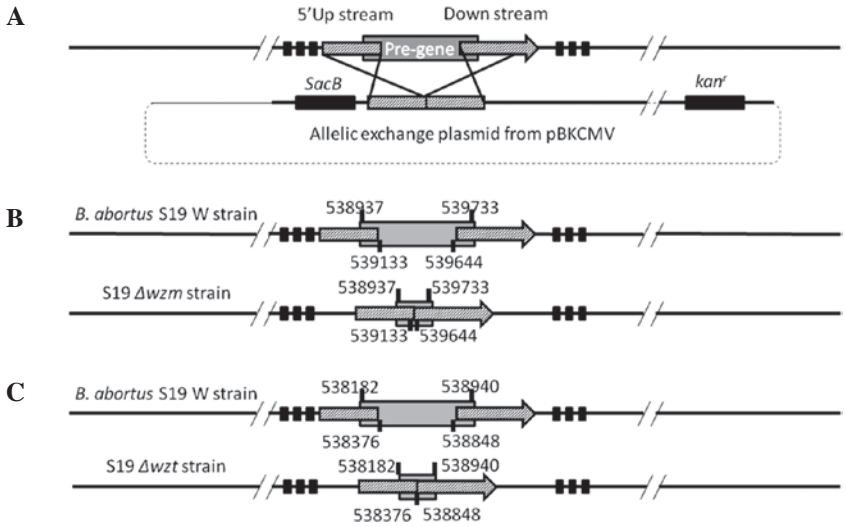

Figure 1. The construction of allelic exchange plasmids, and the mutant site descriptions at *Brucella abortus* (*B. abortus*) S19 chromosome. (A) Allelic exchange diagram of constructed plasmids and chromosome of S19. (B) Constructs of *B. abortus Δwzm*. (C) Constructs of *B. abortus Δwzt*. The number is the locus of the target genes on the chromosome from NCBI.

RPMI-1640 with 5% FBS and the cell concentration was adjusted to $2x10^6$/ml.

The lymphocytes ($1x10^4$ per well in triplicate) were incubated with corresponding antigens (multiplicity of infection of 200 CFU/cell; PBS in RPMI-1640 medium as negative control) in 96-well plates at 37˚C in an atmosphere containing 5% (v/v) CO_2. After 24 h of culture, 20 μl MTS solution was added to each well and the cells were incubated for 4 h at 37˚C in an atmosphere containing 5% (v/v) CO_2. The absorbance (optical density, OD) at 490 nm was recorded. Lymphocyte proliferation ratio (IS) was calculated as: $(OD\text{-}OD_{1640})/(OD_{PBS}\text{-}OD_{1640})$.

Cytokine induction in vitro and in vivo. The lymphocyte cells ($1x10^5$ per well in triplicate) were incubated with corresponding antigens (multiplicity of infection of 200 CFU/cell; PBS in RPMI-1640 as negative control) for 24 h in 24-well plates at 37˚C in an atmosphere containing 5% (v/v) CO_2. The cell culture supernatants were collected and stored at -80˚C. The cytokine levels were analyzed using R&D Quantikine TNF-α, INF-γ, IL-2, IL-4 and IL-10 kits according to the manufacturer's instructions (Minneapolis, MN, USA). The INF-γ levels of serum were estimated using the same method.

Statistical analysis. The data were analyzed using Original 7.5 software and presented as the means ± standard deviation (mean ± SD). Significant differences between the groups were identified by one-way ANOVA (significant difference, $P<0.01$ and $P<0.05$).

Results

Screening of mutant strains. In order to obtain partial mutants of *wzm* and *wzt* genes, the plasmids pBK*sacBwzm* and pBK*sacBwzt* were constructed as shown in Fig. 1A-C. The plasmid was then electroporated into *B. abortus* S19 cells. The transformed samples were plated on TSB agar medium (Kan^r) for the first screen. The colonies were added to TSB medium and detected by PCR with *sacB* primers for the second screen. The positive culture was spread on 7% sucrose TSA medium for the allelic exchange screen (27). The colonies from 7% sucrose TSB agar medium were inoculated into TSB medium and screened by pre-gene primers (*wzm* or *wzt* gene) for the fourth screen. If the cells were mutants, the target gene was found to be shorter than the pre-gene. The positive mutants were those with only one band ~300 bp after the screening process (Fig. 2). The putative-positive mutants were inoculated into TSB medium (Kan^r) to remove the false-positive mutants.

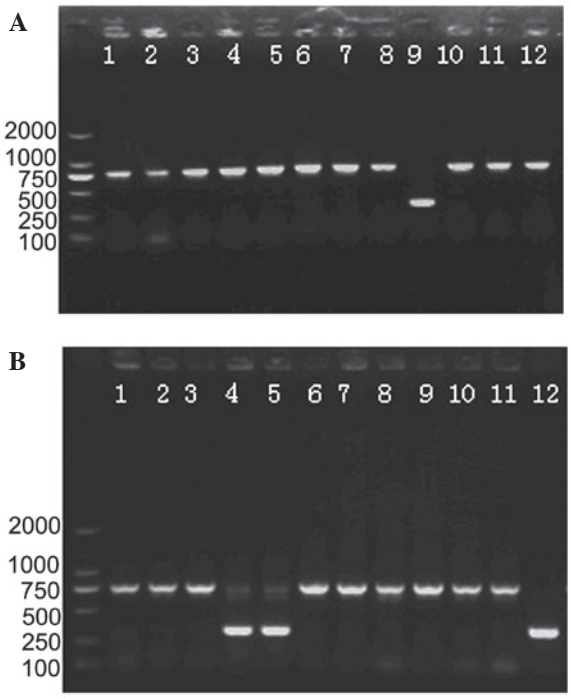

Figure 2. PCR detection of mutants. (A) The results of the fourth screen of *Δwzm*. Lane 6 is the false positive mutant and lane 9 is the putative positive mutant. (B) The results of the fourth screen of *Δwzt*. Lanes 4 and 5 are false positive mutants, and lane 12 is the putative positive mutant.

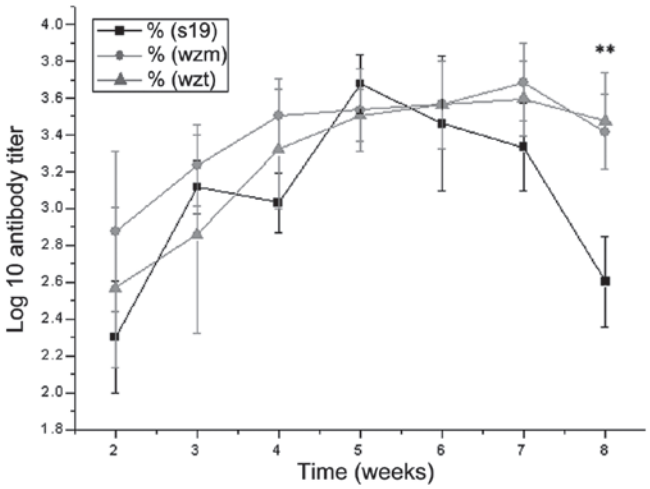

Figure 3. Antibody dynamic of positive control (S19), *Δwzm* and *Δwzt* mutants (n=5). **P<0.01 compared with the S19 group.

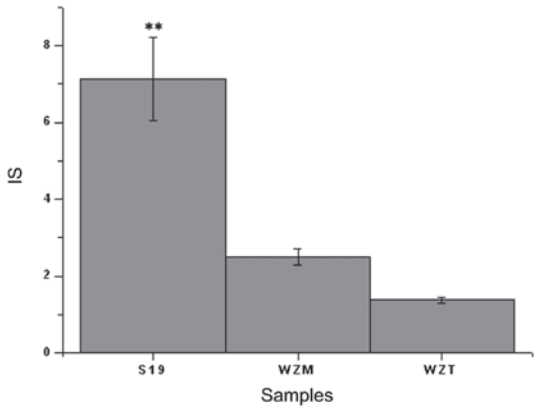

Figure 4. Lymphocyte proliferation (IS) of S19, *Δwzm* and *Δwzt* mutants by the MTS method (n=5). **P<0.01 vs. the wzm and wzt groups, respectively.

Mutant strains were rough mutants. After 30 passage cultures for genetic stability, the mutants were detected by PCR using target gene primers and upstream and downstream fragment primers, and the sequences were analyzed. The mutants were prepared for acriflavine agglutination. The *Δwzm* and *Δwzt* mutants were positive and the S19 strain was negative for acriflavine agglutination.

The results of the Rose Bengal plate agglutination test (RBT) showed positive and negative serum for the S19 group and the *Δwzm* and *Δwzt* groups, respectively. Therefore, *Δwzm* and *Δwzt* mutants did not elicit the antibody response to O-antigen in the host. These results indicated that the mutants were rough mutants.

Antibody dynamics. The IgG antibody changes of *Δwzm* and *Δwzt* mutants and S19 are shown from the second to the ninth week (Fig. 3). The antibody titer of *Δwzm* induced in mice was higher than S19 strain before the fourth week and after the sixth week. Particularly at the eighth week, the log10 antibody titer of mutant strains (S/N value of *Δwzm* and *Δwzt* mutants was 3.42±0.20 and 3.48±0.26, respectively) was significantly higher than the S19 strain (S/N=2.60±0.25, P<0.01). These results indicated that the rough mutants may induce higher antibody titers than the S19 strain.

Lymphocyte proliferation. Lymphocyte proliferation is an important stage of the immune response. The results showed (Fig. 4) that the IS of S19 (7.13±1.09) was significantly higher than that of *Δwzm* (2.48±0.21) and *Δwzt* (1.38±0.07) mutants (P<0.01), which indicated that S19 induced higher lymphocyte proliferation. The IS of the *Δwzm* mutant was approximate to that of the *Δwzt* mutant (P>0.05). The disruption of *wzm* and *wzt* genes caused significantly decreased lymphocyte proliferation ability compared with the wild-type strain S19.

Cytokine secretion. The important immune-related cytokines INF-γ, IL-2, IL-4, IL-10 and TNF-α were detected *in vitro* (lymphocytes cultured in 24-well plates). The TNF-α, INF-γ, IL-2, IL-4 and IL-10 results showed that the cytokines induced by the *Δwzm* and *Δwzt* rough mutants were decreased and significantly lower than the S19 parent strain (P<0.01, Fig. 5A-E). IL-4 and TNF-α levels induced by *Δwzt* mutants were lower than those induced by *Δwzm* mutants (P<0.01) and INF-γ, IL-10 and IL-2 levels induced by *Δwzt* mutants were approximate to those induced by *Δwzm* mutants (P>0.05).

The INF-γ levels in serum were also detected from weeks 2 to 9. The curve (Fig. 6) showed that S19 and *Δwzm* rough mutant induced higher INF-γ levels (S19, 127.6±1.1 pg/ml at the third week; *Δwzm* rough mutant, 67.6±6.8 pg/ml at the fourth week), while the INF-γ levels induced by *Δwzt* rough mutants were lower than those of S19 and *Δwzm* (17.8±14.7 pg/ml on fourth week). The peak time of the INF-γ induction of *Δwzm* rough mutants (the fifth week was the peak of the curve) was delayed compared with S19 (peak of the curve was evident at the fourth week), and the *Δwzt* rough mutants were even more delayed. The concentrations of INF-γ induced by rough mutants were lower than those of S19, and the concentrations induced by *Δwzt* rough mutants were the lowest.

Discussion

In the present study, knockout of *wzm* and *wzt* genes caused the rough mutant. The *wzm* and *wzt* genes are the membrane-spanning homologs and the ATP-binding homologs of ABC-transporters involved in transmembrane export for O-polysaccharide chain biosynthesis (28). Previous studies on *B. melitensis* 16M observed that the mutant of *wzm* or *wzt* gene was a rough mutant (23-25). The acriflavine agglutination results indicated that the *Δwzm* and *Δwzt* mutants were rough mutants (21). Smooth strains did not induce acriflavine agglutination, suggesting that vaccination with these mutant strains may allow for differentiation between vaccinated and wild-type smooth strain-infected animals.

The cytokine-inducing ability of rough mutants was reduced. INF-γ is one of the most important cytokines in resistance to *Brucella* invasion, which enhances the macrophage bactericidal activity (29). The INF-γ levels induced by the *Δwzm* and *Δwzt* rough mutants was lower than those of the smooth strain S19 *in vivo* although not significantly lower. By contrast, INF-γ levels induced by the *Δwzm* and *Δwzt* mutants were significantly lower as compared to S19. IL-2 was detected

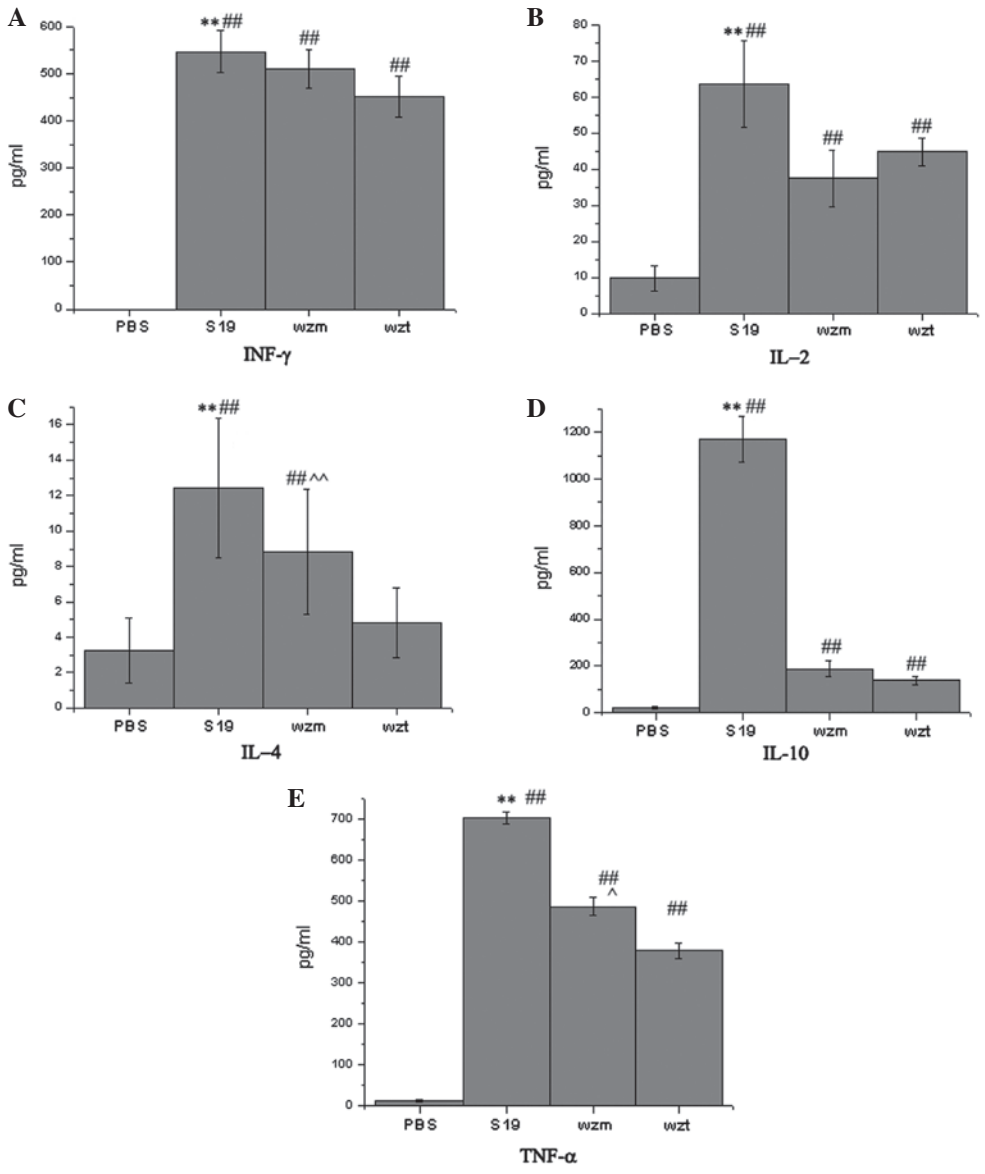

Figure 5. Cytokine concentrations of (A) INF-γ, (B) IL-2, (C) IL-4, (D) IL-10 and (E) TNFα (n=5). **P<0.01 compared with *Δwzm* and *Δwzt*, respectively; ##P<0.01 compared with negative control; ^^P<0.01, ^P<0.05 compared with *Δwzt*.

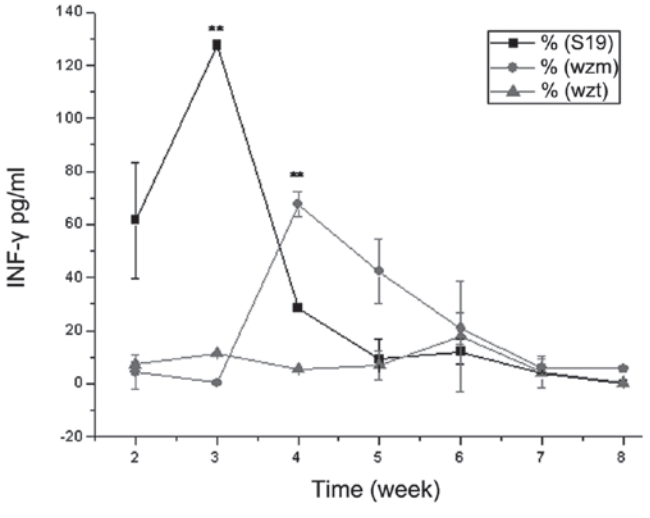

Figure 6. Changes of INF-γ levels of S19, *Δwzm* and *Δwzt* mutants in serum (n=5). **P<0.01 compared with *Δwzt* mutants group.

in low quantities and IL-4 was detected at very low levels. IL-10 levels induced by S19 were significantly higher than those induced by the rough mutants, while IL-10 levels induced by the *wzm* mutant were higher than those of the *wzt* mutant.

Secretion of the inflammatory molecule TNF-α was reduced by rough mutants although not significantly as compared to S19. LPS is considered to be the most important modulator of TNF-α that is required for host defense against intracellular pathogens (30,31). The results showed that the disruption of LPS caused a reduction in TNF-α levels and spleen weights, and the spleen kinetics caused by rough mutants were significantly lower than those of the S19 strain. The TNF-α levels induced by the *Δwzt* mutant were lower than those induced by the *Δwzm* mutant.

These data indicate that the *wzt* gene may affect the host immune response indirectly. As previously reported, the *wzm* mutant provides efficient protection against *Brucella* invasion compared with mutants (25). Wzt disruption may affect the

expression of more genes associated with the induction of immunity.

Acknowledgements

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