Effects of partial deletion of the *wzm* and *wzt* genes on lipopolysaccharide synthesis and virulence of *Brucella abortus* S19

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Abstract. Brucellosis is a worldwide human and animal infectious disease, and the effective methods of its control are immunisation of animals by vaccination and elimination. *Brucella abortus* S19 is one of the popular vaccines with virulence in the control of cattle Brucellosis. In the present study, allelic exchange plasmids of *wzm* and *wzt* genes and partial knockout mutants of *wzm* and *wzt* were constructed to evaluate the resulting difference in virulence of *B. abortus* S19. PCR analysis revealed that the target genes were knocked out. The mutants were rough mutants and they could be differentiated from natural infection by the Rose Bengal plate and standard agglutination tests. The molecular weights of lipopolysaccharides of the *Δwzm* and *Δwzt* mutants were clustered between 25 and 40 kDa, and 30 and 35 kDa separately, and were markedly different from those in *B. abortus* S19. The virulence of *B. abortus Δwzm* and *Δwzt* was decreased compared with that of *B. abortus* S19 in mice. All these results identified that there were several differences between the *wzm* and *wzt* genes on lipopolysaccharide synthesis and on the virulence of *B. abortus*.

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Introduction

Brucella species are facultative intracellular bacteria that cause brucellosis in humans and animals. *Brucella* invades phagocytic and non-phagocytic cells and then survives inside the host cells (1,2). The control of the infection is managed by vaccination against animal brucellosis. Human brucellosis has also been controlled by vaccination as well as culling within animals (3,4).

The effective vaccines currently used for livestock are *B. abortus* S19 and RB51 for cattle, and *B. melitensis* Rev1 for small ruminants (5). However, these stems are infectious for humans and cause abortion in pregnant animals. *B. abortus* S19 has been widely used to prevent cattle brucellosis, while it usually has low virulence (6). *B. abortus* S19 is able to induce the production of antibodies to the O-polysaccharide (PS), which is difficult to distinguish from that resulting from natural infection (7-9). Developing a safe and efficacious vaccine and overcoming the serological obstacle is likely to have a broad impact on public health.

Lipopolysaccharides (LPS) provide bacterial resistance to anti-microbial attacks and modulates the host immune response, which makes it a significant virulence factor for its survival and replication in the host cell (1,10). *Brucellae* without O-PS are termed as a rough or 'R' strain. *R. brucella* species or mutants lacking antigenic O-PS do not reduce levels of anti-O-PS antibodies and do not react with antibodies of this specificity (11,12). Thus vaccination with rough strains is distinguished from wild infection according to serological tests (13). It has been observed that *Brucella* R mutants are attenuated, and therefore, they are potential vaccines (14,15).

Several genes of *Brucella melitensis* 16M associated with LPS synthesis were analyzed and the integral membrane protein (wzm) and the adenosine triphosphatase (ATPase) domain (wzt) of the ATP-binding cassette (ABC)-type transporters were putative components of the ABC transporter system. Mutations

Table I. Bacteria strains and plasmids.

Strain or plasmid	Phenotype and/or genotype	Source
Strain		
Escherichia coli DH5α	F-, *decR*, *recA1*	Takara Co. D9057A
Brucella abortus S19	Vaccine strain, smooth	IVDC
Brucella abortus Δwzm	*Δwzm* gene partial deleted	Present study
Brucella abortus Δwzt	*Δwzt* gene partial deleted	Present study
Plasmid		
pBKCMV	Kanamycin resistance	Stratagene
pIBP279	Provided *sacB* gene	NJAU

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in the *wzm*/*wzt* genes was proved to lead to the absence of the O-side-chains on the bacterial surface (16,17).

In order to investigate the virulence and characteristics of the rough mutants of S19, mutants with partial deletion of the *wzm* and *wzt* genes with no DNA marker addition were constructed to estimate their effect on LPS synthesis, survival *in vivo* and the serological response. Finding were asperated to enhance the understanding of the effect of the *wzm* and *wzt* genes on LPS synthesis and on the virulence of the S19 vaccination strain, and provide valuable information for the construction of a vaccine based on a Brucella rough mutant.

Materials and methods

Bacterial strains and growth conditions. The *Escherichia coli* DH5α strain was grown on Luria-Bertani broth (LB) agar at 37˚C. The *Brucella* strains *B. abortus* S19, *Δwzm* and *Δ*wzt were grown on tryptic soy broth (TSB, Sigma Co., St. Louis MO, USA) agar at 37˚C (Table I). In total, 100 *μ*g/ml ampicillin and 50 *μ*g/ml kanamycin were added for plasmid screening if it was considered necessary. A total of 7% sucrose in TSB medium was prepared for screening the allelic-exchange mutants.

Construction of allelic exchange plasmids. The allelic exchange plasmids were constructed by pBKCMV (kanamycin resistance, kan^r) with a *sacB* gene and fragments upstream and downstream of the target genes. The *sacB* gene along with its promoter was amplified from pIBP279 (presented by Nanjing Agricultural University) by polymerase chain reaction (PCR; Thermal Cycler Px2 PCR amplifier, Thermo Fisher Scientific Inc., Rockford, IL, USA) using the following primers: *sacB* forward, 5'-gtcgacACTCAGTAC ATAATAAAGGAGACAT-3' and reverse, 3'-ggatccTGGGATTCACCTTTATGTTGATAA G-5'. The PCR conditions were: 95˚C for 3 min; 95˚C for 30 sec, 56˚C for 30 sec, 72˚C for 90 sec, 30 cycles; 72˚C for 10 min. Next, it was ligated into pBKCMV for constructing plasmid pBKsacB using the following primers: *wzmf* forward, 5'-ggatccTTTCATTTGAGGAGCCGGAGTA-3' and reverse, 3'-ctcgagGCCCACGTAAATCAGACATTGAAAG-5'; *wzmr* forward, 5'-ctcgagGGCAGGGTGGATTGAATGCATTCG T-3' and reverse, 3'-cccgggGCGTCGCAACCGCAATCTTAT CAAT-5'; *wztf* forward, 5'-ggatccGCGATGAAGTCATTGT ACCGACCTT-3' and reverse, 3'-ctcgagGGCGTTTACTAG AGTTTTGACTGA GC-5'; *wztr* forward, 5'-ctcgagATAGGT GCAGGTGATGCGGCATTCA-3' and antisense 3'-tctagaT-GCCGAGTTCGCTCAGACAATCAAC-5'. The PCR conditions were: 95˚C 3 min; 95˚C 30 sec, 62˚C 30 sec, 72˚C 120 sec, 30 cycles; 72˚C 10 min. These were amplified and ligated into the pBKsacB plasmid to construct pBKsacBwzm and pBKsacBwzt.

Preparation of competent cells and electroporation. *B. abortus* S19 was cultured in TSB for 24 h until it reached ~10^8 cells/ml. The cells were prepared for electroporation by pelleting and washing, first with 1/2 volume of 10% ice-cold glycerol twice and then treated with 1/10 volume of 10% ice-cold glycerol. Finally, the sample was resuspended with 1/200 volume of ice-cold 10% glycerol and stored at -80˚C until further use.

In total, 30 ng/*μ*l pBKsacBwzm or pBKsacBwzt plasmid DNA was added to the competent cells (10-100 *μ*l) and electroporated at 1,500 kV (1 mm bottom; BTX, Holliston, MA, USA). Super Optimal Broth (1ml; 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, the cells were grown with agitation at 28˚C for 24 h and then plated on TSB agar (with 50 *μ*g/ml kanamycin) and cultured for 96 h at 28˚C.

Mutant screening. The colonies on the TSB (Kan^r) plates were inoculated in TSB liquid medium separately and cultured for 36 h at 28˚C, and then the cultures were plated on 7% sucrose TSB agar medium and cultured at 37˚C for 96 h. The colonies grown were picked in 96-well plates with TSB medium and incubated for 48 h at 37˚C and then detected by *wzm* and *wzt* primers as follows: *wzm* forward, 5'-catatg-GTGAGACGATTT CGTATGATATCGT-3' and reverse, 3'-ctcgagTCATAGGTA AAAAATGGCTCTCTTCTCC-5', *wzt forward,* 5'-catatgATG ATCCAGCCATCGATTACC CTGT-3' and reverse, 3'-ctcgag TCATGCTATAGCTCCCAT TCCCGAG-5'. The colonies in which the *wzm* or *wzt* fragment was altered, termed *Δwzm* and *Δwzt* positive mutants, were confirmed in TSB (Kan^r). The Kan^r-negative strains were inoculated on TSB agar medium as candidate mutant strains for next-cycle screening.

Mutant detection and acriflavine agglutination. The mutants were inoculated for 30 generations, assessed by PCR and the

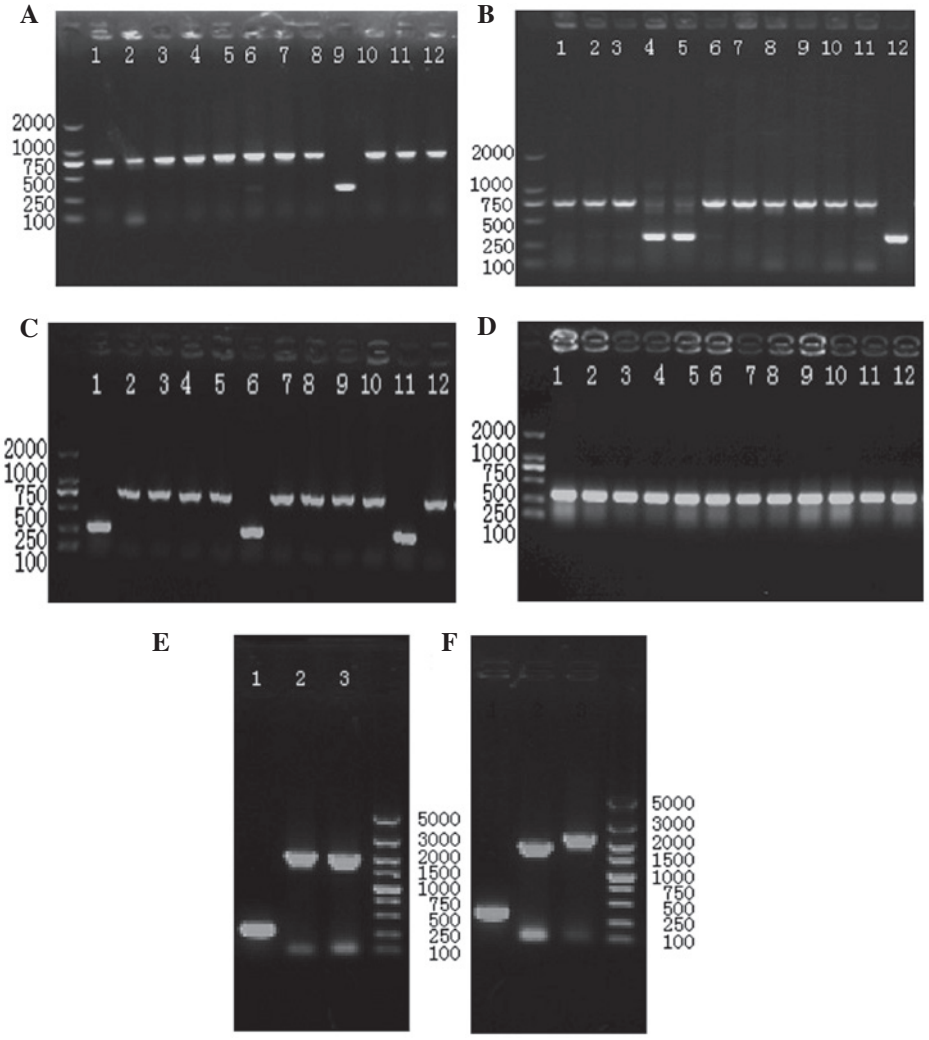

Figure 1. PCR detection of mutants. (A) Results of the fourth screen of *Δwzm*, lane 6 is a false positive mutant, and lane 9 is a putative positive mutant. (B) Results of the fourth screen of *Δwzt*, lanes 4 and 5 are false positive mutants and lane 12 is a putative positive mutant. (C) Second cycle screen of *Δwzm*. Lanes 1, 6, 11 are from putative positive mutants and lanes 2-5, 7-10 and 12 are false positive mutants. (D) Second cycle screen of *Δwzt* of a putative mutant. (E) PCR detection of target gene (lane 1, 280 bp) and upstream (lane 2, 2,000 bp) and downstream (lane 3, 1,900 bp) fragments *Δwzm*. (F) PCR detection of target gene (lane 1, 300 bp) and upstream (lane 2, 1,800 bp) and downstream (lane 3, 2,100 bp) fragments *Δwzt*. PCR, polymerase chain reaction.

sequences of the *wzm*, *wzt*, *wzmf*, *wzmr*, *wztf* and *wztr* fragments were analyzed. The phenotype of the mutants was further determined by agglutination with acriflavine at 1:100 (18).

LPS extraction and analysis. The extraction process of LPS was performed using the LPS Extraction Kit (no. 17141; iNtRON, Seongnam-Si, Korea) according to the manufacturer's instructions. The extracted LPSs from S19, *Δwzm* and *Δ*wzt were subjected to 12% SDS-PAGE. Silver nitrate staining was processed following the method described by Tsai and Frasch (19). New Zealand white rabbits were immunized three times with *Brucella abortus* vaccine strain S19 by multi-point injection, and the injection interval was 4 weeks. Immunization was detected by ELISA. The ear blood was collected to prepare serum. The crude LPS samples were assayed by western blotting using rabbit serum containing antibodies.

Animals. The 4-6-week-old female specific pathogen-free 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 acclimatized for a minimum of one week prior to the experiments and water and food were provided *ad libitum* (14). All animal experiments were approved by the Center of Laboratory Animals in Jilin University (Changchun, China).

Survival of Δwzm and Δwzt strains in mice. Survival of the strains, *Δwzm* and *Δwzt*, were determined by quantitating the number of colony-forming units (CFU) of the strains in the spleens at different time periods. Female BALB/c mice of 6-8 weeks of age were housed with water and food. Animals were randomly allotted and acclimated for one week prior to the start of the experiments. To prepare the inoculated samples, bacteria were suspended in phosphate-buffered saline (PBS) and adjusted to the appropriate 10^8 CFU/ml in the same buffer. In all the experiments the number of CFU administered was determined by culturing triplicate aliquots. At 1, 2, 4 and 8 weeks animals were anaesthetized by ether inhalation and sacrificed; spleens were removed and homogenized in 10 mM

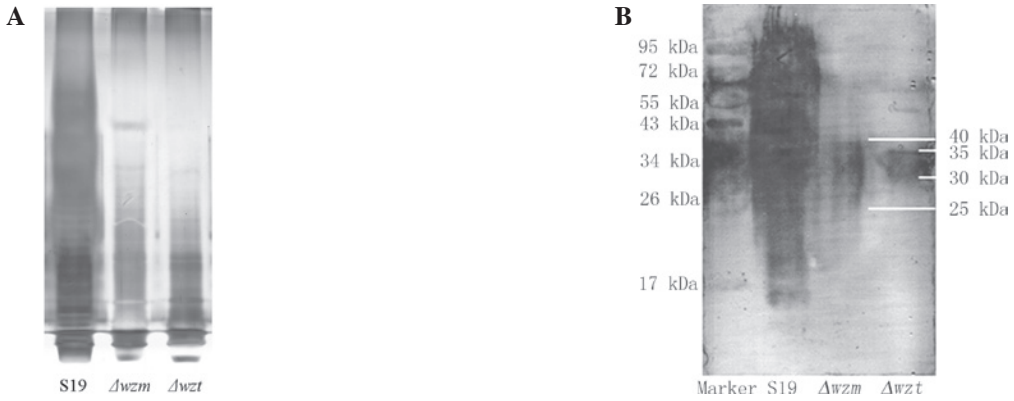

Figure 2. (A) Silver stain of crude extract from S19, *Δwzm* and *Δwzt* strains. The bands of polysaccharide of S19 had disappeared. (B) Western blot of crude extract of S19, *Δwzm* and *Δwzt* strains with S19 immuned mice serum, molecular weight of crude S19 LPS was broad (10-100 kDa), the *Δwzm* was 25-40 kDa and the *Δwzt* was 30-35 kDa.

PBS with 1% Triton-100. Tissue homogenates were serially diluted with PBS and plated onto TSB agar to determine the number of CFU per spleen by incubating for 72 h at 37˚C. The spleens were processed in order to calculate the mean and standard deviation (n=5) of the log10 of CFU per spleen (known as infection kinetics).

Serological test. 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) kit (Harbin Pharmaceutical Group Bio-vaccine Co. Ltd, Harbin, China) was performed with 30 μl serum and 30 μl antigen mixing and the reaction was observed to occur within 4 min. The positive samples were evaluated by a tube agglutination test. The sera were diluted from 1:12.5 to 1:400 with 0.85% sodium chloride solution, and 0.5 ml inactivated standard *B. abortus* broth was added in a 1:1 ratio. Sodium chloride solution (0.85%) was used as the negative control and standard positive serum and negative serum were from the National Institute for Communicable Disease Control and Prevention (Chinese Center for Disease Control and Prevention, Beijing, China). The sample tubes were maintained at 37˚C for 24 h. The positive samples were defined by a titer >1:100.

Statistics. Data were analyzed using Original 7.5 software (OriginLab Corporation, Northampton, MA, USA) and presented as the mean ± standard deviation. Differences between groups were identified by statistical tests using one-way analysis of variance, with $P<0.01$ indicating a statistically significant difference.

Results

Generation of mutant strains. In order to obtain partial mutants of the *wzm* and *wzt* genes, the plasmids pBK*sacBwzm* and pBK*sacBwzt* were constructed. The plasmids were electroporated into *B. abortus* S19 cells and the transformed samples were plated on TSB agar medium (Kan^r) for the first screening. The selected colonies were spread onto TSB medium and detected by PCR with *sacB* primers for the second screening. The positive culture was spread on 7% sucrose TSA medium for allelic exchange screening (20). The colonies from the

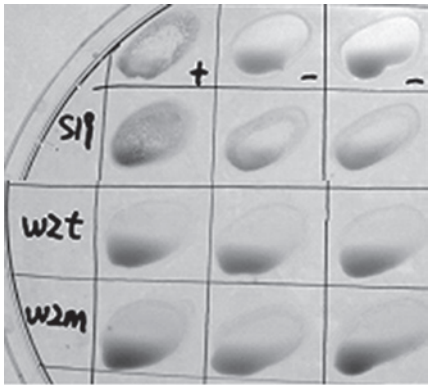

Figure 3. The Rose Bengal test results of serum from *B. abortus* S19, *Δwzm* and *Δwzt* mutants. '+' is positive serum result, '-' is negative serum result, three samples in line marked S19 are results of sera from S19 immunized, '*wzt*' is for sera from *Δwzt* mutant immunized, '*wzm*' is for sera from *Δwzm* mutant immunized.

7% sucrose TSB agar medium were inoculated into TSB medium and screened by pre-gene primers (*wzm* or *wzt* gene) for the fourth screening. The pre-gene in the mutant cells was expected to be shortened. The positive mutants had only one band at ~300 bp subsequent to the screening process. The putative positive mutants were inoculated into TSB medium (Kan^r) to remove any false positives.

The PCR results (Fig. 1A and B) of the third screening showed that the bands of 8.5% (8/94) of colonies exhibited transformed pBKsacBwzm and the bands of 19.6% (18/92) colonies exhibited transformed pBKsacBwzt (Fig. 2A and B). The positive mutant ratio of *Δwzm* was 1.0% (1/94) and that of *Δwzt* was 3.3% (3/92) (Fig. 1C and D).

Mutant strains were rough mutants. Subsequent to a 30 generation culture for genetic stability, the mutants were detected by PCR using target gene, upstream and downstream fragment primers, and the sequences were analyzed. The target gene contained only 300 bp. *wzmf* contained a 2.0 fragment and *wzmr* 1.9, wztf 1.8 and wztr contained 2.1 kb with stable sequences (Fig. 1E and F). The mutants were prepared for acriflavine agglutination. The *Δwzm* and *Δwzt* mutants were positive, and the S19 strain was negative for acriflavine agglutination.

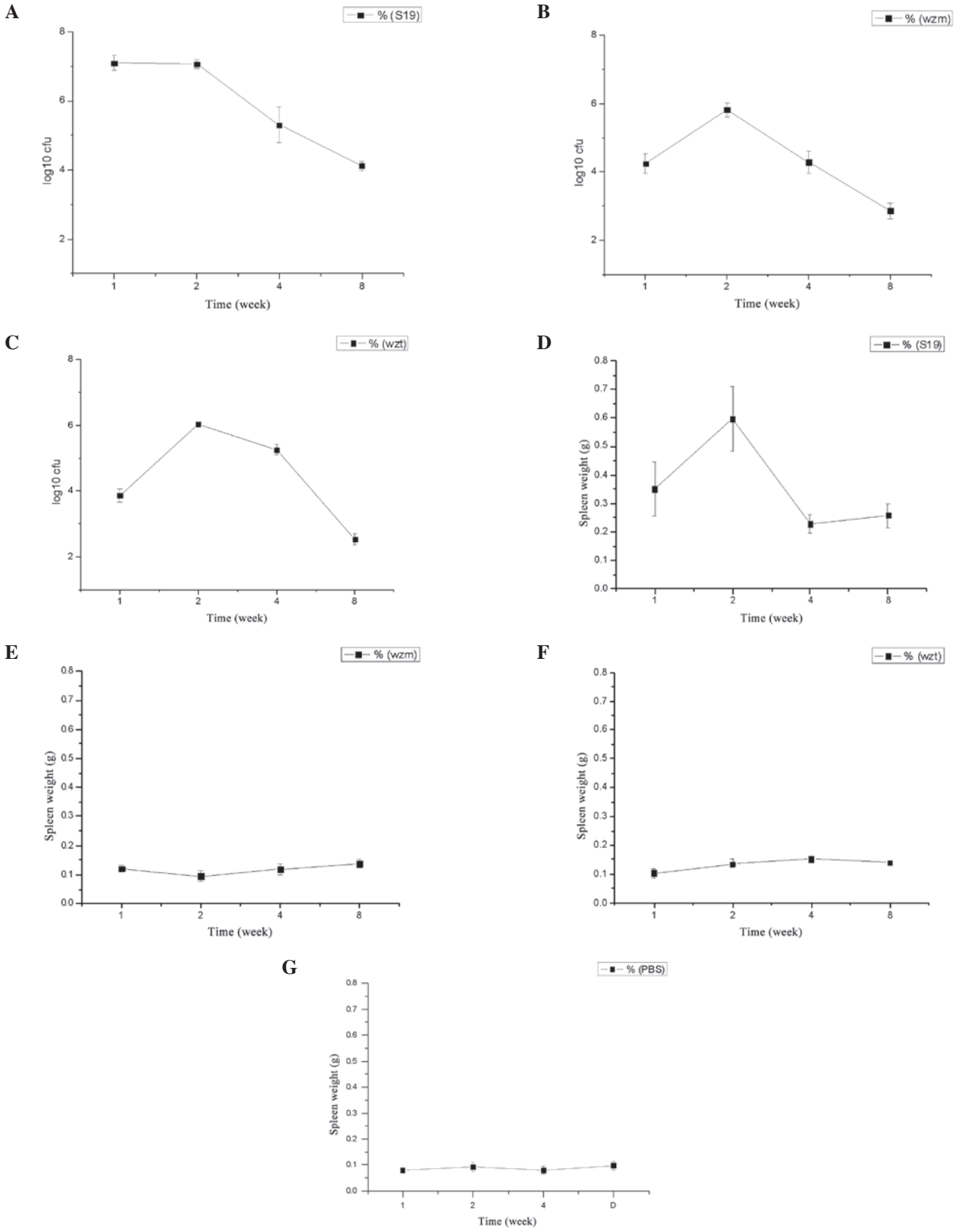

Figure 4. Infection kinetics and spleen weight changes of different mutants. (A-C) Mean of the log CFU/spleen shows the infection kinetics of S19, *Δwzm* and *Δwzt* mutants; (D-G) spleen weight changes of S19, *Δwzm* and *Δwzt* mutants, and PBS. CFU, colony-forming units; PBS, phosphate-buffered saline.

wzm and wzt mutation causes differences in LPS. The LPS of S19, *Δwzm* and *Δwzt* was extracted by kits. Fig. 2A shows that the crude LPS of mutants was significantly changed compared with the S19 strain. There was no detectable signal of extracted LPS from the *Δwzm* and *Δwzt* mutants. There was no difference between the *Δwzm* and *Δwzt* mutants. This indicated that the mutants of *Δwzm* and *Δwzt* may be able to interfere with LPS synthesis.

The western blotting results indicated that LPS in *Δwzm* and *Δwzt* mutants was significantly different from S19 (Fig. 2B).

The molecular weight of normal S19 LPS ranged from 10 to 100 kDa, whereas that of *Δwzm* and *Δwzt* mutants was clustered between 25 and 40 kDa and 30 and 35 kDa separately.

wzm and wzt mutants lack antigenicity to LPS antibodies. Smooth strains of brucella present O-polysaccharides on their surface. *B. abortus* S19 as a vaccine maintains the O-antigen, which causes difficulties in its diagnosis. The Rose Bengal plate agglutination test (RBPT) was performed (21,22). The results revealed that the serum of S19 was positive and *Δwzm* and *Δwzt* were negative compared with the positive and negative serum in regard to agglutination (Fig. 3). The tube agglutination tests revealed that the titer of the S19 serum was over 1:100 (++, positive ratio was 100%), while the titers of *Δwzm* and *Δwzt* were not detected (negative ratio was 100%). These results indicated that there were no effective LPS antibodies formed by *Δwzm* and *Δwzt* mutant infection in BALB/c mice.

wzm and wzt mutation reduces virulence. The infection kinetics in the spleens and the spleen weight of BALB/c mice inoculated with the mutants and *B. abortus* S19 are presented in Fig. 4A-C. The infection kinetics show that the $\log_{10}$ of the number of colony-forming units (CFU) of S19 was maintained at ~7.1 prior to the second week and after two weeks it was decreased to 4.1 until the eighth week. While the infection kinetics of *Δwzm* and *Δwzt* mutants were just about half of that of S19 following the first week, there was an increasing phase at the second week prior to a decrease in the $\log_{10}$ of CFU to 2.9 for *Δwzm*, and 2.5 for *Δwzt* (Fig. 4A-C). The survival rate of the mutants *in vivo* was lower than that for S19, which indicates that the virulence was decreased. The virulence of *Δwzm* and *Δwzt* was almost identical.

The spleen weight in mice injected with the S19 strain was significantly higher compared with that in mice injected with the mutants and the PBS-negative control ($P<0.01$) (Fig. 4D-F). In particular, after two weeks, the spleen weight showed a maximum following a decrease to a relatively stable weight. The weight of the *Δwzm* and *Δwzt* mutants was similar, and was increased compared with that in the PBS group; however, the difference was not significant.

Discussion

Knockout of the *wzm* and *wzt* genes resulted in rough mutants. The *wzm* and *wzt* genes are membrane-spanning and the associated ATP-binding homologues of ABC-transporters are involved in transmembrane export for O-polysaccharide biosynthesis (23). Mutantion of the *wzm* and *wzt* genes is expected to result in rough mutants, such as *B. melitensis* 16M (16,17,24). Acriflavine agglutination indicated that the *wzm* and *wzt* mutants are likely to be rough mutants (14). Smooth strains were not able to induce agglutination of acriflavine. Analysis of crude LPS extracts using western blot analysis with multiple antibodies and serological test results provided more evidence that the *Δwzm* and *Δwzt* mutants were rough mutants. They were able to be distinguished from S19.

The molecular weights of crude LPS profiles were evaluated using western blot analysis. The results revealed that the molecular weight of LPS in the mutants was significantly different compared with that in S19. This result may provide information on the O-LPS synthesis mechanism. The *wzm* and *wzt* genes are components of ABC transporters and are expected to have a similar function in LPS synthesis (16,17,24). The difference in the obtained results may be caused by the different effects of the *wzm* and *wzt* gene disruption process; however, more evidence is required.

Knockout of the *wzm* and *wzt* genes caused a reduction in virulence. LPS is one of the predominant virulence factors, which provides bacterial resistance to anti-microbial attacks and modulates the host immune response, making it a significant virulence factor for the survival and replication in the host cells. LPS may be the dominating factor of S19 virulence (10). *Wzm* and *wzt* genes are the putative genes of the ABC transporter system. Although there is no evidence in regard to Wzm and Wzt proteins structure and function in cells, evidence of exogenous transporter insertion in *wzm* and *wzt* genes causing *B. melitensis* virulence attenuation has been reported (23). Attenuation or optimization of S19 are likely to be required in order to develop a human vaccine strain (25). The virulence results indicated that the infectious ability of *Δwzm* and *Δwzt* mutants was lower, while there was no difference between them, and the knockout of *wzm* and *wzt* reduced the virulence of S19 in a similar manner to that reported for RB 51 and other rough mutant strains (12-14,24). These strains may be applicable for studies on the mechanism of LPS on S19 virulence.

Acknowledgements

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