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Deletion of the type IV secretion system promoter *VirB* in *Brucella abortus* A19 strain attenuated the virulence of the bacteria and promotes autophagy

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Running title: VirB deletion and *Brucella* virulence

Abstract

Brucella abortus is a Gram-negative intracellular parasite bacteria causing serious health hazards in humans and animals. The type IV secretion system (T4SS), encoded by the *virB* promoter, has been identified as an important virulence factor for *Brucella abortus*, but the impact on *Brucella abortus* A19 remains unclear. In this study, the T4SS of *Brucella abortus* A19 was inactivated by deleting the *virB* promoter, resulting in a mutant strain A19 Δ *virB*. Real-time PCR and Western-blotting analysis demonstrated that T4SS-related proteins were not expressed after *virB* promoter deletion. Moreover, the survival rate of A19 in high salt and strong acidic environments was decreased after *virB* promoter deletion. Compared to the parental strain A19, the A19 Δ *virB* mutant strain showed reduced growth rate in TSB, decreased invasion ability to macrophages and dendritic cells, and reduced virulence of the mutant strain in macrophages, dendritic cells and mice. In addition, the A19 Δ *virB* mutant strain showed enhanced autophagy on macrophages and dendritic cells compared with A19, and the A19 Δ *virB* mutant strain was able to upregulate IL-6 and downregulate IL-10 in macrophages. These data help us to better understand the T4SS of the A19 vaccine strain and contribute to our efforts to improve *Brucella* vaccines.

Keywords: *Brucella abortus* A19; *virB* T4SS; virulence; autophagy; cytokine

1. Introduction

Brucellosis is a zoonotic disease characterized by abortion and fever caused by *Brucella*, which poses a great threat to human health and the breeding industry (Prajapati et al. 2020).

To prevent and control brucellosis, *Brucella abortus* A19 (*B. abortus* A19) attenuated vaccine has been widely used for vaccination in China since 1956 (Zhao et al. 2020). The A19 strain has high structural similarity with the internationally used *Brucella abortus* S19 vaccine genome. However, there are some differential genes between A19 and S19, such as *eryC*, *eryD* and *eryF*. In addition, the outer membrane proteins, 2-isopropylmalate synthase, citrate synthase, GntR family transcriptional regulators and ABC transport proteins are different between A19 and S19 (Wang et al. 2020). Studies have reported that *Brucella* attenuated vaccine is able to cause abortion in pregnant animals and exposure to infection in occupational populations (Lalsiamthara and Lee 2017). Currently, no mechanism of attenuated virulence has been reported for the A19 vaccine strain. Therefore, there is a need to study the virulence of A19 attenuated vaccines.

It has been reported that *Brucella* A19 is incapable of escaping lysosomal degradation to establish a replicative niche in the host cell and fails to establish a chronic infection (Cheng et al. 2021). Interestingly, a recent case of *Brucella abortus* infection caused by *Brucella* A19 leakage was reported in China, where some individuals developed clinical symptoms such as headache, weakness, loss of appetite, fatigue, sweating and joint pain (Baoshan et al. 2021). In addition, the study reported that S19 was able to infect mice and persist in mouse tissue for 8 weeks (Jacob and Curtiss 2021). This suggests that although A19 is unable to escape lysosomal degradation, it is still able to survive in the host for a period of time to exert virulence. The *Brucella* type IV secretion system (T4SS), a key virulence factor encoded by the *virB* operon, is located on *Brucella* chromosome II and consists of 12 genes (*virB1-12*) that play an important role in regulating intracellular survival and manipulating the host's immune response to infection (O'Callaghan et al. 1999). Studies have shown that

Brucella invades the organism and targets mainly macrophages and embryonic trophoblast cells, but can also target dendritic cells (DCs) (Billard et al. 2007; de Figueiredo et al. 2015). *Brucella* enters host cells through many effector proteins secreted by T4SS, which modify the transport of pathogenic bacteria within these infected cells, avoiding eventual killing of *Brucella* and evading the immunosurveillance function of infected cells, maintaining the persistence of *Brucella* within the cells (De Jong and Tsolis 2012). Currently, there is no literature reporting the function of T4SS in A19.

Here, we constructed the mutant strain A19 Δ *virB* by deletion of the *virB* promoter of *Brucella* A19 T4SS. The growth state and virulence of the A19 Δ *virB* strain were investigated, and the survival rate and autophagy levels of A19 Δ *virB* in macrophages and dendritic cells were assessed. These data help us to better understand the T4SS of the A19 vaccine strain and contribute to our efforts to improve *Brucella* vaccines.

2. Materials and methods

2.1 Ethics

The female BALB/c mice of Six-week-old were purchased from the Experimental Animal Center of Zhengzhou University (Henan, China). All experimental procedures and animal care are carried out in accordance with institutional animal care regulations. During the breeding period, these animals have received humane care and health conditions, and comply with institutional animal health guidelines.

2.2 Bacterial strain, plasmids and cell line

Brucella abortus A19 was obtained from the Xinjiang Tiankang Animal Biotechnology Co. Ltd., China. Studies have reported that *Brucella* infection in mice induces migration

and maturation of splenic dendritic cells (DC), and that *Brucella* interacts differently with different DC subpopulations (Papadopoulos et al. 2016). Therefore, mouse dendritic sarcoma cell line derived from the mouse spleen was chosen for our study. All *Brucella* was cultured on tryptic soy agar (TSA) or tryptic soy broth (TSB) (Difco, MI, USA) at 37 °C. *Escherichia coli* (*E. coli*) strain DH5 α and BL21 was used in generation of A19 Δ *virB* mutants, and grown on Luria–Bertani (LB) medium (Difco, MI, USA) at 37° C. Appropriate antibiotics (100 μ g/ mL ampicillin for A19 and 50 μ g/ mL kanamycin for *E. coli*) were supplemented in the medium as necessary. Plasmid pGEM-7Zf + was purchased from Promega (Madison, Wisconsin, USA). The murine macrophage RAW264.7 line (RAW264.7, TIB-71) and Murine dendritic cell sarcoma (DCS) cell line (obtained from Cell Resource Center, IBMS, CAMS/PUMC, Beijing, China) was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies, Rockville, MD, USA) at 37° C with 5% CO₂. Work on all *Brucella* strains was done in a biosafety level 3 laboratory.

2.3 Construction of the A19 Δ *virB* deletion mutants

Construction of A19 Δ *virB* deletion mutants according to previously disclosed methods (Li et al. 2015; Zhang et al. 2019). firstly, the sequence upstream (493 bp) of *virB* was amplified from the *Brucella abortus* A19 genome using the primer pairs *virB*-S-F and *virB*-S-R (Table 1). The sequence downstream (585 bp) of *virB* was amplified from the *Brucella abortus* A19 genome using the primer pairs *virB*-X-F and *virB*-X-R (Table 1). Then, the two homologous sequences of A19 *virB* gene were cloned into pMD19-T Simple Vector (Takara, Japan) and sequenced. After correct sequencing, the recombinant plasmid was digested with *Sal* I and *Eco*R I, the amplified fragment was

introduced into pGEM-7Zf⁺ to generate the suicide plasmid pGEM-7Zf⁺-*virB*. A pair of primers *kan*-F and *kan*-R (Table 1) was designed to amplify a *kan* DNA fragment (1093 bp) as a selectable marker gene. The *kan* DNA fragment was introduced into plasmid pGEM-7Zf⁺-*virB* to generate the plasmid pGEM-7Zf⁺-*virB*-*kan*. finally, pGEM-7Zf⁺-*virB*-*kan* was introduced into electrocompetent cells of A19 electroporation. The potential *virB* deletion mutant A19Δ*virB* was selected as 100 µg/mL ampicillin in the first round of screening and 100 µg/mL kanamycin in the second round. The deletions of *virB* gene in A19 were further verified by PCR and RT-PCR as described previously (Zhang et al. 2019) (Table 1,2), and primers *virB*-Q-F and *virB*-Q-R were designed for the detection of the deletion mutant.

2.4 Growth kinetics assay of A19Δ*virB* mutant

Monitor the growth of *Brucella* strains A19 and A19Δ*virB* mutants on normal TSB agar plates. Briefly, *Brucella* cultures were inoculated into 20 mL *Brucella* broth and cultured at 37°C (concentration adjusted to 0.2 OD). The optical density at 600 nm was measured every 2 hours for 24 hours using a Nanodrop 2000 (ThermoFisher).

2.5 Assessment of external environmental stress in A19 and A19Δ*virB* mutants

To investigate the effect of *virB* deletion on the ability of *Brucella* A19 to adapt to its environment, we chose extreme environments (1.5 M NaCl, pH 11.5 or 2.5, 58 degrees), as described previously (Shengnan et al. 2021). We incubated A19 and A19Δ*virB* in normal TSB medium or other different TSB medium at 37°C, the different culture medium includes 1.5 mol/L NaCl high-salt environment, pH 11.5 strong alkali environment, pH 2.5 strong acid environment and 58 °C high-temperature environment, and then incubated on a shaker at 180 rpm for 30 minutes and centrifuged to collect

bacteria. Use the plating on the TSA plate to count and calculate the live bacteria. The counting and calculated of viable bacteria was performed by plating on TSA plates.

2.6 Evaluation of invasion ability of A19 Δ *virB* attenuation in RAW264.7 macrophages and Murine dendritic cell sarcoma

The invasion ability of A19 Δ *virB* and the parent strain A19 were evaluated using murine macrophages RAW264.7 and murine dendritic cell sarcoma (DCS). Briefly, 1×10^6 cells/well of macrophages and dendritic cells were inoculated into 6-well plates and cultured for 24 h at 37°C in a cell culture incubator containing 5% CO₂, followed by infection with *Brucella abortus* A19 and mutant *Brucella abortus* A19 at a 100:1 multiplicity of infection (MOI). At 30, 45, and 60 min post-infection (pi), the cell plate were centrifuged at 350 g for 5 minutes at room temperature and washed three times with sterile PBS, and then the cells were incubated in standard medium (Invitrogen, Carlsbad, CA, USA) containing 50 µg/mL gentamicin to kill the remaining extracellular bacteria. The viable bacteria were counted and calculated by spreading the plates on TSA plates.

2.7 Evaluation of A19 Δ *virB* attenuation in RAW264.7 macrophages and Murine dendritic cell sarcoma

The viability of A19 Δ *virB* and parental strain A19 was assessed in Murine macrophage RAW264.7 and Murine dendritic cell sarcoma (DCS), as previously described (Zhang et al. 2020). Briefly, macrophages and dendritic cells of 1×10^6 cells/well were inoculated into 6-well plates for 24 h in a cell culture incubator containing 5% CO₂ at 37° C and then infected with *brucella abortus* A19 and mutated *brucella abortus* A19 at a multiplicity of infection (MOI) of 100:1. Briefly, 1×10^6 cells/well of macrophages

and DCS were inoculated into 6-well plates and incubated for 24 h at 37°C in a cell culture incubator containing 5% CO₂. The plates were then infected with *B. abortus* and mutated *B. abortus* A19 at a multiplicity of infection (MOI) of 100:1. After post-infection (pi) for 45 min, then centrifuge at 350 g for 5 minutes at room temperature. Cells were washed three times with sterile PBS and incubated in standard medium (Invitrogen, Carlsbad, CA, USA) containing 50 µg/mL gentamicin for 45 min to kill the remaining extracellular bacteria.

The cultures were then placed in standard medium and incubated at 37°C. These infected cells were collected at 0, 4, 8, 12, 24, and 48 h pi and washed with sterile PBS, then lysed with phosphate-buffered saline (PBS) containing 0.1% Triton X-100. The viable bacteria were counted and calculated by spreading the plates on TSA plates.

2.8 Evaluation of A19Δ*virB* mutant strain virulence in mice

To determine the virulence of *Brucella* in BALB/c mice, 1.0×10⁹ CFU of A19, A19Δ*virB* and PBS were injected intraperitoneally (ip) into BALB/c mice (5 mice per group, 2 ml per mouse). Mice were euthanized at 7 and 15 days after post-infection, then spleens were aseptically removed and weighed and homogenized in sterile PBS, dilute 1:100 to 1:10000, and plated on the TSA plates. The plate was incubated at 37°C for 72 h and colonies were counted. The bacterial load per gram of spleen (CFU/g) was calculated for each BALB/c mouse and averaged, SPSS 11.5 software, and statistically analyzed by t-test ($P < 0.05$).

2.9 Evaluation of A19Δ*virB* mutant on autophagy levels in RAW264.7 macrophages and DCS dendritic cells

Mouse macrophages RAW264.7 and DCS dendritic cells were used to assess the level of autophagy in A19 Δ *virB* and parental strain A19. Mouse macrophages RAW264.7 and DCS dendritic cells were infected with the previous described method, and at 4, 8, 12 and 24 hours post-infection, the infected cells were washed with PBS and lysed by trizon, and then RNA was extracted from the infected cells with an RNA kit, reverse transcription of RNA was performed and transcript levels of autophagy-related genes LC3-I and LC3-II were measured by real-time quantification. The relative transcriptional levels were determined by the methods of $2^{-\Delta\Delta C_t}$ as described previously (Livak and Schmittgen 2001). β -actin was used as a reference gene and all assays were performed 3 times.

To further verify autophagy, Mouse macrophages RAW264.7 and DCS dendritic cells were infected with *Brucella abortus* A19 and A19 Δ *virB* respectively. Cells were fixed with 4% paraformaldehyde for 20 min after 12 h of infection. Cells were washed with PBS containing 2 mg glycine for 5 min and blocked with PBS containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 for 1 h. Cells were washed 3 times with PBS and LC3 primary antibody (Abcam, USA) was added. The cell cultures were then placed at 4°C overnight. Cells were washed 3 times with PBS and incubated with fluorescently labeled secondary antibody (Bioss, China) for 1 hour at room temperature. Subsequently, the fluorescence was observed by confocal microscopy (ZEISS, Germany).

2.10 Evaluation of A19 Δ *virB* mutant on IL-6 and IL-10 in RAW264.7 macrophages and DCS dendritic cells

Mouse macrophages RAW264.7 and DCS dendritic cells were infected with *Brucella* parental and A19 Δ *virB*, and cell supernatants were collected at 4, 8, 12, and 24 h post-

infection, followed by determination of cytokines IL-6 and IL-12 using the Mouse Quantikine ELISA kit according to the manufacturer's instructions. all tests were completed in triplicate and repeated at least three times.

2.11 Western blotting

The autophagy LC3-I and LC3-II protein of *Brucella* infected mouse macrophages RAW264.7 and DCS dendritic cells was analyzed by Western blot. At 4, 8, 12, and 24 h after post-infection, the cells were collected and cell lysates were added to collect proteins, The protein concentration was determined by BCA protein assay (Thermo Fisher), and then the equal amount of protein were subjected to 12% SDS-PAGE, and then transferred to nitrocellulose membrane by electroblotting in a Trans-blot cell (Bio-Rad, Richmond, Calif, USA) and blocked with blocking solution (5% skim milk powder in Tris-buffered saline Tween-20 (TBST)) overnight at 4°C. The membranes were then incubated with Rabbit Anti-LC3 primary antibody (1:2000 dilution) for 2 hours at room temperature. After washing 3 times with TBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG Ab (Abcam) for 1 hours at room temperature. ECL Western Blotting Substrate (Thermo Fisher) was used for detected immunoreactivity according to the manufacturer's instructions. However, the detection of antibodies in mouse serum was carried out by the method described by Zhang (Zhang et al. 2020), and the Recombinant VirB1, VirB5, VirB8 and L7/L12 were used as detection proteins, and antibodies against VirB1, VirB5, VirB8 and L7/L12 were detected by Western blot.

2.12 Statistical analysis

CFU data from A19 Δ *virB* attenuation, growth curve and mouse virulence determination were transformed into loga-rithmic numbers. All data were expresse presented as mean $\pm$ SEM (SD). SPSS V17.0 statistics software was used to performed Statistical analysis, $P < 0.05$ was defined as statistically significant. GraphPad Prism software was used to draw a dose-response curve of the obtained data results.

3. Results

3.1 Construction and Characterization of the *Brucella abortus* A19 Δ *virB* mutants

The *Brucella abortus* vaccine strain A19 Δ *virB* mutants were successfully obtained. A 314 bp DNA fragment was amplified from A19 strain with primers *virB*-Q-F and *virB*-Q-R. However, the primers *virB*-Q-F and *virB*-Q-R did not amplify anything from A19 Δ *virB* (Figure. 1), indicating that *virB* was knocked out in A19 Δ *virB*, and the successful knockout of *virB* was reconfirmed by sequencing. RT-PCR showed that *virB* T4SS-related gene *virB1*, *virB5*, and *virB8* showed low transcript levels in A19 Δ *virB* (Figure. 2), and Western blotting showed that VirB T4SS-related proteins VirB1, VirB5 and VirB were not expressed in A19 Δ *virB* (Figure. 8), indicating that our constructed A19 Δ *virB* mutant is unable to produce effector proteins to function.

3.2 The *virB* gene regulates the growth of *Brucella abortus* A19

To study the role of *virB* in the growth of *Brucella*, A19 Δ *virB* and A19 were cultured in TSB medium for 24 h. At 8 hours, the OD600 of the two strains grown in TSB medium was not significantly different. At 10 hours, the OD600 of the A19 strain grown in TSB medium was about 1.3 times that of the A19 Δ *virB* mutant. At 12 hours, the OD600 of the A19 strain was about 1.8 times that of the A19 Δ *virB* mutant. From the overall level, the growth of A19 strain in TSB medium was higher than that of

A19 $\Delta virB$ mutant strain (Figure. 3). These results indicate that the *virB* gene regulates the growth of *Brucella abortus* A19.

3.3 Decreased A19 $\Delta virB$ resistance to external environment

We investigated the viability of *virB* mutants under different environmental conditions. In a high-salt environment, the survival rate of A19 $\Delta virB$ was 30% lower than that of A19. In a strong alkali environment, the survival rate of A19 $\Delta virB$ was 28% lower than that of A19. In a high temperature environment, the survival rate of A19 $\Delta virB$ was 3% lower than that of A19 (Figure. 4). These results indicate that the *virB* mutant reduces the survival rate of *Brucella* under different environmental conditions.

3.4 Attenuation of A19 $\Delta virB$ mutants invasion ability in RAW 264.7 murine macrophages and Murine dendritic cell sarcoma

To evaluate the effect of *virB* gene mutation on *Brucella* invasion ability, RAW 264.7 murine macrophages and dendritic cell sarcomas (DCS) were infected with *Brucella abortus* A19 and A19 $\Delta virB$ mutants. The results showed that the number of A19 $\Delta virB$ mutants in DCS was 0.21-fold, 0.2-fold and 0.18-fold lower than that of the parent strain after 30 minutes, 45 minutes and 60 minutes of post-infection. At 45 minutes of infection, the number of A19 $\Delta virB$ mutants in RAW 264.7 macrophages was 0.4 times lower than the parent strain. However, there was no significant difference in the invasive ability of the A19 $\Delta virB$ mutant and the parental strain of *Brucella abortus* A19 ($P>0.05$) (Figure. 5). These results showed that knock out of *virB* gene reduced the invasion ability of *Brucella abortus* A19 to DCS.

3.5 Decreased survival of A19 Δ *virB* mutant in RAW264.7 murine macrophages and murine dendritic cell sarcoma

RAW 264.7 murine macrophages and dendritic cell sarcoma (DCS) were infected with *Brucella abortus* A19 and A19 Δ *virB* mutants to determine the survival and replication capability of A19 Δ *virB* mutants in macrophages and DCS. At 0 h post-infection, the A19 Δ *virB* mutant and the parent strain A19 had the same survival capability in RAW 264.7 macrophages (Figure. 6A), but the survival capability of the A19 Δ *virB* mutant in DCS was lower than that of the parent strain A19 (Figure. 6B). From 4 h to 48 h, the survival capability of the A19 Δ *virB* mutant in RAW 264.7 macrophages decreased and was lower than that of the parent strain A19 (Figure. 6A), but the survival capability of the A19 Δ *virB* mutant in DCS first decreased and then increased (Figure. 6B), and the survival capability was lower than that of the parent strain A19 except for 12 h (Figure. 6B). These results show that *Brucella abortus* A19 and A19 Δ *virB* mutants have different survivability in RAW 264.7 macrophages and DCS. *virB* is involved in the intracellular replication of *Brucella*. The deletion of the *virB* gene reduces the survival capability of *Brucella* in RAW 264.7 macrophages and DCS.

3.6 Attenuation of A19 Δ *virB* mutants survival ability in mice

To study the role of *virB* gene in *Brucella* virulence, BALB/c mice were injected with 1×10^9 CFU of *Brucella abortus* A19, A19 Δ *virB* and PBS (negative control). At 7 and 15 days post-infection, *Brucella* colonies in mouse spleen were counted. Compared with the A19 group, the number of A19 Δ *virB* decreased by 1.1-log at 7 day post-infection (Figure. 7A), and the number of A19 Δ *virB* decreased by 0.9-log at 15 day post-infection (Figure. 7A). The study of the spleen weight index showed that the weight of the spleen of the mice in the A19 Δ *virB* group was lower than that of the A19

group (Figure. 7B), indicating that the injection of the A19 Δ *virB* mutant could reduce inflammation in the mice. These results indicate that the virulence of the A19 Δ *virB* mutant is lower than that of the A19 strain and the A19 Δ *virB* mutant is highly attenuated in mice.

3.7 Increased autophagy levels of A19 Δ *virB* mutants in RAW264.7 macrophages and DCS cells

RAW 264.7 murine macrophages and dendritic cell sarcoma (DCS) were infected with *Brucella abortus* A19 and A19 Δ *virB* mutants to determine the autophagy capability of A19 Δ *virB* mutants in macrophages and DCS. LC3 is a crucial gene involved in autophagosome formation. When autophagy is formed, cytoplasmic LC3 enzyme cleaves off a small segment of LC3 polypeptide to form LC3-I, which is converted to LC3-II by binding to phosphatidylethanolamine (Guo et al. 2012). In addition, it has also been reported that LC3-II is a gene that promotes autophagy and LC3-I is a gene that inhibits autophagy, and *Brucella*-induced autophagy can be measured by the level of LC3II and the LC3-II/I ratio as previously described (Sengupta et al. 2009; Zhang et al. 2019). At 4, 12 and 24 h post-infection, the data showed that LC3-II and LC3-II/I were higher in A19 Δ *virB*-infected macrophages than in A19-infected macrophages (Figure. 9A, C). However, at 4, 8, 12 and 24 h post-infection, the LC3-II and LC3-II/I were significantly higher in A19 Δ *virB*-infected DCS than in A19-infected DCS (Figure. 9B, D). Western blotting also showed the same results (Figure. 10). In addition, to further verify autophagy, we determined the number of LC3 puncta by fluorescence microscopy as described previously (Yoshii and Mizushima 2017). We found that 12 hours after *Brucella* infection of mouse macrophages RAW264.7 and DCS dendritic cells, LC3 puncta were significantly higher in the A19 Δ *virB* and A19 groups than in

the control group, and the number of LC3 puncta in the A19 Δ *virB* group was significantly higher than that in the A19 group (Figure. 11). This indicates that the A19 Δ *virB* mutant has a higher level of autophagy in macrophages and DCS than A19.

3.8 IL-6 enhancement and IL-10 attenuation of RAW264.7 Macrophages and DCS are infected with A19 Δ *virB* mutant

IL-6 contributes to host resistance against *Brucella* infection by controlling *Brucella* activity of Macrophages and CD8⁺T Cell Differentiation (Hop et al. 2019). Mariana N Xavier's research shows that IL-10 contributes to avoid the phagolysosome fusion of intracellular *Brucella*, resulting in persistence of *Brucella* in macrophages (Xavier et al. 2013). Therefore, we evaluated the expression levels of IL-6 and IL-10 in A19 Δ *virB* mutants infected RAW264.7 macrophages and Murine dendritic cell sarcoma (DCS). The data showed that the expression level of IL-6 was higher in A19 Δ *virB*-infected DCS than in A19-infected DCS at 4, 8, 12 and 24 h post-infection (Figure. 12A), indicating that the A19 Δ *virB* mutant contributes more to resistance to *Brucella* infection than *Brucella* A19. In contrast, the expression level of IL-10 was lower in A19 Δ *virB*-infected DCS than in A19-infected DCS (Figure. 12C), indicating that *Brucella* abortus A19 is more beneficial than A19 Δ *virB* mutant for survival in DCS. However, the IL-6 and IL-10 expression levels of A19 Δ *virB* and A19 were essentially the same in macrophages (Figure. 12B, D).

4. Discussion

As the promoter encoding the type IV secretion system (T4SS) of *Brucella*, the correct expression of *virB* is a prerequisite for the induction of T4SS to exert virulence. It has been shown that deletion of the *virB* promoter decreases *Brucella* virulence and

viability, while affecting the establishment of *Brucella* metabolism, regulation, and infection processes (Boschiroli et al. 2002; Ke et al. 2015). In this study, We first demonstrated that T4SS lost its function after *virB* promoter deletion and that the growth status of *Brucella* abortus A19 was reduced (Figure. 3), which may be due to the loss of *virB* affecting *Brucella* metabolism and thus reducing *Brucella* growth on TSB. The pathogenesis of *Brucella* is mainly based on the ability of *Brucella* to adapt to the harsh environmental conditions encountered during its infection of the host (Köhler et al. 2003). When we simulated the host intracellular harsh environment in vitro, we found that the survival of the A19 Δ *virB* mutant strain was significantly reduced in strongly acidic, alkaline, and high-salt environments (Figure. 4), indicating that *virB* promoter deletion reduced the ability of *Brucella* to adapt to harsh environments. We all know that the adhesion and invasion of pathogenic bacteria to host tissues is a necessary stage for bacterial colonization and the basis for the bacterial establishment of infectious diseases, while for *Brucella* abortus, adhesion, invasion, immune escape, and intracellular transport is a complex process (Basyony et al. 2018; Castañeda - Roldán et al. 2006). Our study found for the first time that the invasion ability of *Brucella* abortus A19 to macrophages was almost unchanged when the virulence factor T4SS lost its function. However, the invasion ability to dendritic cells was attenuated (Figure. 5). Moreover, it was further demonstrated by mouse experiments that the virulence of the A19 Δ *virB* mutant was highly attenuated in mice (Figure. 7). Studies have reported that A19 is virulent to humans and can cause abortions in pregnant females (Lalsiamthara and Lee 2017), however, attenuated *Brucella* melitensis lacking *virB2* does not settle in fetal tissue or cause abortions in pregnant goats (Kahl-Mcdonagh et al. 2006). Our study indicated that the virulence of

Brucella attenuated vaccine A19 was significantly higher than that of the A19 Δ *virB* mutant and that the virulence of attenuated vaccine A19 in humans may be due to T4SS.

Cellular infection and intracellular survival are key steps in the development of chronic infection by *Brucella*. Many intracellular bacterial pathogens use the type IV secretion system (T4SS) to deliver effectors and disrupt eukaryotic host cell defenses (Sieira 2013). When *Brucella* is internalized, the bacteria are encapsulated in a so-called vacuole to form a *Brucella*-containing vacuole (BCV), which rapidly acquires an early in vivo marker, contacts the endoplasmic reticulum (ER) membrane, and transiently interacts with lysosomes, inducing *virB* gene expression through signals provided by a limited fusion between BCV and lysosomes, seeking to respond to the bactericidal machinery of the host cell (Celli et al. 2003; De Jong et al. 2010). However, *Brucella* vaccine strain A19 could not escape lysosomal degradation and had difficulty in establishing invisible chronic infection in the target hosts (Cheng et al. 2021). It is suggested that the role of T4SS is different between *Brucella* strong virulent strains and vaccine strains. Currently, the role of T4SS in *Brucella abortus* A19 is still unknown. Our study revealed that the survival of *Brucella abortus* A19 in macrophages and dendritic cells was significantly reduced after the deletion of *Brucella* A19 T4SS promoter *virB* (Figure. 6), indicating that T4SS of A19 can still exert virulence on macrophages and dendritic cells. In addition, macrophages were able to prevent intracellular survival of *Brucella abortus* A19 better than dendritic cells when *Brucella abortus* lacked the virulence factor T4SS (Figure. 6). It is possible that macrophages prevented the activation of dendritic cells after capturing *Brucella*. Only when macrophages are depleted can dendritic cells enhance *Brucella* uptake and induce a stronger inflammatory response to the pathogen (Archambaud et al. 2010).

Autophagy is an effective way for the organism to combat pathogenic invasion, and many pathogens have evolved anti-autophagic strategies to resist innate and adaptive immunity, successfully surviving and replicating in macrophages and dendritic cells, such as *Coxiella*, *Brucella*, *Listeria*, *Salmonella* (Skendros and Mitroulis 2012). In addition, the presence of *virB* T4SS may help *Brucella* to manipulate host cell autophagy to protect its intracellular survival (Campoy and Colombo 2009). We found that both macrophages and dendritic cells showed increased levels of autophagy of *Brucella abortus* A19 after *virB* deletion (Figure. 10,11). This result is consistent with deletion of the T4SS effector *vceA* promotes autophagy (Zhang et al. 2019), suggesting that *Brucella abortus* A19 T4SS also plays an important role in autophagy compared to *Brucella abortus* S2308. Autophagy in macrophages has numerous roles, such as being responsible for xenophagy, phagocytosis associated with LC3, antimicrobial peptide synthesis and distribution, and inflammatory vesicle activation regulation (Wu and Lu 2020). In dendritic cells, autophagy is involved in every step of dendritic cell maturation, such as antigen presentation and cytokine secretion (Germic et al. 2019). We speculate that the significant increase in dendritic cell autophagy levels after *virB* deletion may be related to dendritic cell maturation. Interestingly, some articles have reported that *virB* T4SS does not participate in and control dendritic cell maturation and does not interfere with the Th1-type adaptive immune response (Billard et al. 2008). So far, there are relatively few research reports on T4SS and dendritic cell autophagy, and the related mechanisms need to be further clarified.

Brucella virB T4SS can activate inflammatory responses and immune responses (de Jong et al. 2013b). Studies have reported that patients infected by *Brucella*, IL-6 and IL-10 are significantly increased and that IL-10 contributes to decreased IFN- λ development and inhibition of macrophage function and is considered a risk factor for

brucellosis (Jin et al. 2020; Kazemi et al. 2016). When the *Brucella* T4SS promoter *virB* was deleted, we found that IL-6 increased and IL-10 decreased in dendritic cells, while IL-6 and IL-10 remained unchanged in macrophages (Figure. 12). Interestingly, IL-6 levels in macrophages were significantly decreased after *Brucella* 2308 *virB2* and *vceC* deletion respectively (de Jong et al. 2013a). This suggests that the ability of T4SS to induce inflammatory responses differs between *Brucella* strong strains and vaccine strains, and also in dendritic cells and macrophages.

In conclusion, this study constructed a novel *Brucella abortus* A19 mutant strain A19 Δ *virB*, in which deletion of the *virB* promoter resulted in the inability of T4SS to function virulently. The virulence of the A19 Δ *virB* mutant strain was significantly reduced in vivo and in vitro compared to the parental strain, and the A19 Δ *virB* mutant was more efficiently cleared by dendritic cells and macrophages. Besides, the A19 Δ *virB* mutant strain induced more significant autophagic and inflammatory responses in dendritic cells than in macrophages. Overall, our data suggest that A19 Δ *virB* is a promising *brucella* vaccine candidate. However, more studies are needed to explore the protective power of A19 Δ *virB* and whether it is pathogenic to humans. In addition, we need to characterize the relationship between T4SS-induced autophagy and inflammation in dendritic cells and macrophages and the difference between the two.

In conclusion, this study constructed mutant strain A19 Δ *virB*, in which deletion of the *virB* promoter resulted in the inability of *Brucella abortus* A19 to produce the T4SS effector protein. Compared to the parents, the A19 Δ *virB* mutant was significantly less virulent in vitro and in vivo, the A19 Δ *virB* mutant had significantly reduced invasion ability to macrophages and dendritic cells, the A19 Δ *virB* mutant had significantly reduced survival in macrophages and dendritic cells, but the A19 Δ *virB* mutant had

enhanced autophagy in macrophages, and the A19 Δ *virB* mutant strain induced a significant autophagic and inflammatory response in dendritic cells. Currently, there are few reports on attenuated *Brucella* A19 vaccine, and our data will contribute to a more detailed understanding of the T4SS of attenuated vaccine A19.

Author contributions

YW and XD conceived the study; DX, YW and JH performed experiments; JY, HZ, YW, XD, JH, YW and QY analyzed data; JH and XD wrote the paper. All authors have read and approved the final version of the manuscript.

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Conflict of interest

The authors have declared no competing interests.

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Figure legends

Figure. 1. PCR verification results of A19 Δ *virB* mutant strains.

M:super DNA Marker; 1-3: Primers *virB*-S-F and *virB*-X-R are used to amplify suicide plasmid genes; 4: Primers *virB*-S-F and *virB*-X-R are used to amplify the A19 strain gene; 5: negative control; 6-8: Primers *VirB*-Q-F and *VirB*-Q-R are used to amplify the

internal genes of the A19 Δ *virB* mutant strain; 9, 10: Primers *virB*-Q-F and *virB*-Q-R are used to amplify the internal genes of the A19 strain; 11: negative control

Figure. 2. The mRNA transcription level of IV type secretion system *virB*-related gene. The RNA of *Brucella abortus* A19 and A19 Δ *virB* grown in the logarithmic phase was extracted and reverse transcribed into cDNA, and the mRNA transcription level of *virB1*, *virB5* and *virB8* was detected by RT-PCR. The data are represented with means $\pm$ SD by three independent experiments. * $P < 0.05$.

Figure. 3. Growth curve of *Brucella abortus* A19 Δ *virB* mutant strains.

Brucella abortus A19 and A19 Δ *virB* were grown in normal TSB. Nanodrop 2000 was used to measure the optical density at 600 nm every 2 hours for 24 hours. The data are represented with means $\pm$ SD by three independent experiments. * $P < 0.05$.

Figure. 4. Survival rate of *Brucella abortus* A19 and A19 Δ *virB* in different environments.

A19 and A19 Δ *virB* were cultured in 1.5 mol/L NaCl, pH 11.5, pH 2.5 TSB medium and 58°C environment. The bacteria were collected within 30 minutes and the survival rate was calculated. The data are represented with means $\pm$ SD by three independent experiments. * $P < 0.05$.

Figure. 5. The invasion capability of A19 Δ *virB* mutants in RAW264.7 macrophages and Murine dendritic cell sarcoma (DCS).

RAW264.7 macrophages and DCS were infected at an MOI of 100 with *Brucella abortus* A19 or the A19 Δ *virB* mutants. At 30, 45 and 60 min post-infection, infected

macrophages and DCS were lysed, and supernatants were diluted for CFU enumeration. Significant differences between the *Brucella* abortus A19 and A19 Δ virB mutants are indicated by * $P < 0.05$.

Figure. 6. Intracellular replication of A19 and A19 Δ virB mutants in RAW264.7 macrophages and Murine dendritic cell sarcoma (DCS).

(A) RAW264.7 macrophages and (B) DCS were infected with *Brucella* abortus A19 and A19 Δ virB mutant at an MOI of 100 for 45 minutes, and replaced with standard medium, and then these infected cells were collected at 0, 4, 8, 12, 24, and 48 h. These infected cells were lysed and spread on TSA plates for CFU counting. Significant differences between the *Brucella* abortus A19 and A19 Δ virB mutants are indicated by * $P < 0.05$.

Figure. 7. Determination of virulence of A19 and A19 Δ virB strains.

BALB/c mice were infected with 1.0×10^9 CFU of A19 and A19 Δ virB. At 7 and 15 days post-infection, the spleen was isolated, (A) and the amount of *Brucella* in each spleen and (B) Spleen weight index were assessed. The spleen weight index was calculated as the average spleen weight per gram (g) of mouse body weight. The data are represented with means $\pm$ SD by five independent mouse experiments. The difference in spleen bacterial content and weight between *Brucella* abortus A19, A19 Δ virB mutants and PBS groups are indicated by * $P < 0.05$.

Figure. 8. Detection of anti-VirB1, anti-VirB5 and anti-VirB8 in serum.

Serum from mice immunized with A19 and A19 Δ virB were collected, and antibodies against VirB1, VirB5, VirB8 and L7/L12 were detected by Western blot using

recombinant VirB1, VirB5, VirB8 and L7/L12 as detection proteins. No antibodies against VirB1, VirB5 and VirB8 proteins were detected in the serum collected from the A19 Δ virB group.

Figure. 9. The mRNA levels of autophagy-related genes were detected by qRT-PCR. (A) RAW264.7 macrophages and (B) DCS were infected with *Brucella* abortus A19 and A19 Δ virB mutants at an MOI of 100. These infected cells were then collected at 4, 12, 24 and 48 h and total RNA was extracted and reverse transcribed into cDNA. mRNA levels of autophagy genes LC3-I and LC3-II were determined by qRT-PCR and then LC3-II versus LC3-I rates. * $P < 0.05$, ** $P < 0.01$ indicates significant differences between *Brucella* abortus A19 and A19 Δ virB mutants.

Figure. 10. The expression levels of autophagy-related proteins were detected by Western blotting.

(A)(C) RAW264.7 macrophages and (B)(D) DCS were infected with *Brucella* abortus A19 and A19 Δ virB mutants at an MOI of 100. These infected cells were then collected at 4, 12, 24 and 48 h and cell lysates were added to collect proteins, and the expression levels of LC3-I and LC3-II proteins were determined by Western blotting.

Figure. 11. Detection of *Brucella* abortus A19 and A19 Δ virB mutants infecting macrophages and dendritic cell autophagy by laser confocal microscopy.

RAW264.7 macrophages and DCS dendritic cells were infected with *Brucella* abortus A19 and A19 Δ virB mutant at an MOI of 100 for 12 h, and cells were incubated with antibodies and GFP-LC3 expression was examined under confocal laser microscopy.

(A)(C) Magnification of 200 $\times$, (B)(D) Magnification of 40 $\times$.

Figure. 12. Detection of IL-6 and IL-10 expression levels by ELISA.

RAW264.7 macrophages and DCS were infected with *Brucella abortus* A19 and A19Δ*virB* mutants at an MOI of 100, and cell supernatants were collected at 4, 12, 24 and 48 h post-infection , and the expression levels of IL-6 and IL-10 in DCS were determined by ELISA, (A)(C) and the expression levels of IL-6 and IL-10 in RAW264.7 macrophages were determined by ELISA. (B)(D) The expression levels of IL-6 and IL-10 in RAW264.7 macrophages were determined by ELISA. * $P<0.05$, ** $P<0.01$ indicates significant differences between *Brucella abortus* A19 and A19Δ*virB* mutants.

Table 1 Primer sequences for gene amplification and real-time quantification

Table 2 RT-PCR primers for VirB-related proteins

Figure. 1. PCR verification results of A19ΔvirB mutant strains.

820x458mm (59 x 59 DPI)

Figure. 2. The mRNA transcription level of IV type secretion system virB-related gene.

720x423mm (59 x 59 DPI)

Figure. 3. Growth curve of *Brucella abortus* A19ΔvirB mutant strains.

755x424mm (59 x 59 DPI)

Figure. 4. Survival rate of Brucella abortus A19 and A19ΔvirB in different environments.

652x408mm (59 x 59 DPI)

Figure. 5. The invasion capability of A19ΔvirB mutants in RAW264.7 macrophages and Murine dendritic cell sarcoma (DCS).

676x366mm (59 x 59 DPI)

Figure. 6. Intracellular replication of A19 and A19ΔvirB mutants in RAW264.7 macrophages and Murine dendritic cell sarcoma (DCS).

505x665mm (59 x 59 DPI)

Figure. 7. Determination of virulence of A19 and A19ΔvirB strains.

851x335mm (59 x 59 DPI)

Figure. 8. Detection of anti-VirB1, anti-VirB5 and anti-VirB8 in serum.

552x206mm (59 x 59 DPI)

Figure. 9. The mRNA levels of autophagy-related genes were detected by qRT-PCR.

991x581mm (59 x 59 DPI)

Figure. 10. The expression levels of autophagy-related proteins were detected by Western blotting.

1173x359mm (59 x 59 DPI)

Figure. 11. Detection of *Brucella abortus* A19 and A19ΔvirB mutants infecting macrophages and dendritic cell autophagy by laser confocal microscopy.

836x523mm (59 x 59 DPI)

Figure. 12. Detection of IL-6 and IL-10 expression levels by ELISA.

991x550mm (59 x 59 DPI)

Table 1 Primer sequences for gene amplification and real-time quantification

Primer	Sequence(5'-3')
VirB-S-F	GCACGACAACAATCGGCTTT
VirB-S-R	GACATTCATCCCAGGTGGCATGGTGCCATTCTTGTC
VirB-X-F	TCTGGGGTTTCGAAATGACCGCGCAGGACGGAAAGGA
VirB-X-R	CGATATGGATGGCAACGGC
Kan-F	GCCACCTGGGATGAATGTC
Kan-R	CGGTCATTTCGAACCCCA
VirB-Q-F	GATATCGGTCATTATTGGGTAGTGTT
VirB-Q-R	GCCTAATGGAGCAAGTTCGGTA
LC3-I-F	CCGACCGCTGTAAGGAGG
LC3-I-R	GCCGGATGATCTTGACCAAC
LC3-II-F	GAACAAAGAGTGGAAGATG
LC3-II-R	GCCGTCTGATTTATCTTGA

Table 2 RT-PCR primers for VirB-related proteins

Primer	Sequence(5'-3')
GAPDH-F	CAGCCTCAAGATCATCAGCA
GAPDH-R	TGTGGTTCATGAGTCCTTCCA
virb1-F	AAGCAATCACGACAGCACAG
virb1-R	CGGCGTAGTAACAGGAGAATG
virB5-F	GCATGTGAGGCTAAAGTGGTG
virB5-R	GGGTCTCCCGTCTTGTTGAT
virB8-F	GGGCTTTCGGCACCATTAC
virB8-R	AGCGTGTACCAGTCGTAGG