INTRATRACHEAL INOCULATION OF THE GUINEA PIG IS AN IMPROVED MODEL FOR BRUCELLOSIS

A Dissertation

by

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ABSTRACT

B. melitensis is considered the most virulent of the *Brucella* species, and a need exists for an improved laboratory animal model of infection that mimics natural transmission and disease. Previous research has shown that guinea pigs are highly susceptible to infection with *Brucella* spp. and develop a disease syndrome that mimics natural disease after aerosol inoculation. Former aerosol studies utilized the Henderson apparatus, which is a device that fits over the head of the guinea pig to delivery an aerosol cloud. However, this route depends on the respiratory rate of the guinea pig and could lead to transfer of bacteria across mucosal membranes of the conjunctiva or oral cavity. In contrast, intratracheal inoculation is a means of generating aerosols that targets the respiratory tract and does not depend on the ventilation rate of the animal. As a targeted means of delivery, this route allows for the delivery of a defined dose to the lung with minimal losses. Female, Hartley guinea pigs were infected via intratracheal inoculation with 16M *B. melitensis*. Guinea pigs developed fever between 12-17 days post-inoculation and had recoverable bacteria from the spleen, liver, lymph nodes, lung, and uterus with corresponding pathologic changes consistent with human brucellosis. Next, pregnant guinea pigs were inoculated with $1x10^7$ CFU *B. melitensis* IT, which demonstrated that in addition to fever, guinea pigs develop stillborn fetuses and abortion. Finally, guinea pigs were vaccinated with either S19 or 16M*ΔvjbR* to evaluate the pregnant guinea pig as a model for vaccine efficacy. This study demonstrated that offer an improvement over the mouse because clinical signs and systemic colonization can be evaluated. The work in pregnant guinea pigs indicate that they offer an improved animal model to investigate the reproductive pathogenesis of disease and are a valuable model to evaluate promising vaccine candidates.

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Contributors

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1. INTRODUCTION

1.1. Brucellosis

Brucellosis is one of the most commonly reported zoonotic disease in humans with a worldwide distribution (1). Twelve species of *Brucella* have been identified, but only four are considered zoonotic: *Brucella abortus*, *B. suis*, *B. melitensis*, and *B. canis* (2). *Brucella* species were previously designated by the World Health Organization (WHO) as neglected zoonotic agents, which are disease entities that affect a resource limited population and have a low political profile with correspondingly limited investment by governments and communities (3). Neglected zoonotic agents are often endemic in areas with multiple disease etiologies that have a similar clinical presentation thus leading to misdiagnosis or under diagnosis of a particular agent (3). Even though *Brucella* spp. were removed from the WHO list, brucellosis remains endemic in many parts of the world and continues to cause morbidity in both animals and humans.

Of the twelve species, *B. melitensis* is considered the most virulent and is associated with the majority of human cases (2). *B. melitensis* is a reproductive pathogen of sheep and goats, which results in spontaneous mid-gestational abortion and placentitis in females and epididymitis in males (4). Aborted placentas and gestational products are a critical source of infectious materials for humans, and certain populations have a higher risk such as laboratorians, veterinarians, farmers, and abattoir workers (5, 6). As a primary pathogen of small ruminants, the bacteria is endemic in regions of the world with a large population of sheep and goats such as the Middle East, Africa, and Central and South America (4). *Brucella* spp. can also be shed in milk, and ingestion of unpasteurized milk or milk products are common sources of infection (4). The acute symptoms include non-specific, flu-like symptoms such as undulant fever, malaise, and anorexia. Clinically, acute infection is secondary to colonization of the spleen and lymph nodes by bacteria

that manifests as splenomegaly and lymphadenomegaly (6). *Brucella* spp. are also able to persist in reticuloendothelial organs, and periodic bacteremia can result in "chronic fatigue syndrome" and/or osteoarticular disease including spondylitis, peripheral arthritis, bursitis, or osteomyelitis (5).

Vaccination of domestic animals is the most important control strategy to prevent transmission to people in endemic countries since a vaccine is not available for human vaccination (7). The currently approved vaccines for use in livestock species are live-attenuated strains of *Brucella* spp., which are still capable of inducing abortion in livestock and disease in humans through accidental needle sticks (6). Attempts to develop a safe vaccine candidate to inoculate humans against brucellosis have not been successful. For the past thirty years, vaccine development has primarily utilized the mouse model to evaluate novel candidates and determine the immune response to vaccination (8). However, during that time, no vaccine candidate has been able to move from the mouse model to other species and demonstrate protection greater than the commercially available vaccines. This suggests that another animal model may be needed to evaluate vaccine candidates. Guinea pigs were the primary animal model utilized at the time of the development of S19, RB51, and Rev. 1 (9).

1.2. Reproductive brucellosis

A less recognized but more serious complication of brucellosis is reproductive failure in pregnant women based on reports of adverse pregnancy events and infertility during acute brucellosis (6, 10, 11). Countries of the Middle East, South America, and Africa where *Brucella* spp. are endemic in animal populations have a correspondingly higher number of human cases. For example, in Saudi Arabia, a retrospective analysis of hospital records for pregnant women with acute brucellosis identified a spontaneous miscarriage rate of 52% to 64% during the first

and second trimesters, respectively (10). During this same period, the rate of spontaneous miscarriage in the general population was 2.8% (10). Other reported complications from pregnancy associated brucellosis in women include premature delivery and vertical transmission to the fetus resulting in congenital brucellosis of the neonate (11, 12).

The underlying mechanism of *B. melitensis* reproductive pathology that contributes to infertility and pregnancy loss is unknown. A now disproven hypothesis stated that erythritol, a four carbon alcohol in the fetal fluids of ruminants, contributed to reproductive tropism and pathogenesis (13). However, studies have demonstrated that erythritol deficient *Brucella* strains such as S19 still retain virulence in the mouse model, and vaccination of pregnant animals with S19 can result in spontaneous abortion in vaccinated animals (14). Furthermore, *Brucella* species are capable of causing adverse pregnancy events in animal species that do not have a high concentration of erythritol in the placenta, and so some other factor must be driving the tropism (11).

1.3. Animal models

Mice, non-human primates (NHPs), rats, rabbits, and guinea pigs have been used as surrogates for understanding aspects of *Brucella* pathogenesis in humans (15). Few studies have been conducted in rats and rabbits due to low disease susceptibility and transient nature of infection (15, 16).

The pathogenesis of reproductive disease has been investigated utilizing pregnant animal models including the host species (small ruminants, cattle, and suids) as well as in laboratory models for human disease (mice, guinea pigs, nonhuman primates) (15, 16).

1.3.1. Mouse

Aside from studies in the natural hosts for *Brucella*, mice have been the most commonly utilized laboratory animal model for studying comparative pathology (17). The mouse model is most commonly used due to the ease of housing large numbers of animals and the ready availability of reagents for evaluating the immune response to infection. The mouse model has been used extensively for pathogenesis studies and for vaccine development. However, the majority of the studies utilize intraperitoneal (i.p.) inoculation, which is an artificial means of inoculation and the immune response to i.p. is skewed towards a Th2 response (18). This is in contrast to aerosol models, which have demonstrated a Th1 response to infection (18, 19). However, as a reproductive model the mouse has limitations including a short gestation time, and the response to infection is dissimilar from what is seen in people (17). Mice that are inoculated on day 4.5 will experience fetal resorptions, but this species does not abort regardless of dose or timing of inoculation (20). Additionally, mice are often inoculated intraperitoneally with *Brucella* spp., which is not a natural route of transmission, and studies have shown that the route of inoculation is important to determining the immune response to infection (18).

1.3.2. Guinea pigs

An alternative to the mouse model is the guinea pig, which has previously been used to investigate the pathogenesis of infection and for safety and efficacy studies to develop vaccines (9, 21-27). Several studies have demonstrated that guinea pigs are highly susceptible to infection with multiple species of *Brucella* including *suis*, *melitensis*, *abortus*, and *ovis* (15, 28). Infection of the guinea pig results in common disease features of human brucellosis: fever, weight loss, and listlessness (26, 29, 30). As an animal model, one of the intriguing aspects of the guinea pig is development of fever secondary to infection because this is not an aspect of infection that is

replicated in the mouse model (26, 31). In addition to clinical signs, guinea pig models replicate the key pathology of infection such as splenomegaly and hepatomegaly with granulomatous inflammation (21, 22, 29, 32). More importantly, guinea pigs can be infected by an aerosol route of transmission and develop clinical disease (31, 33, 34).

As a reproductive model, advantages to the guinea pig include comparative placentation coupled with a relatively longer length of gestation (~65 days) so the effect of infection on the fetus and dam can be studied (35, 36). Furthermore, guinea pigs could be a useful model for investigating the events that occur during pregnancy that lead to spontaneous pregnancy loss. A study by Bosseray and Diaz found that guinea pigs inoculated via intramuscular injection at mid-gestation with *B. abortus* 544 experienced stillbirths, spontaneous abortions, and vertical transmission to the fetuses (37).

1.4. Aerosol models of inoculation

While the respiratory tract is a common portal of entry, pulmonary pathology and respiratory disease are not typical features of *Brucella* spp. infection (38, 39). In the rare cases in which respiratory disease is reported, the common presentations include pneumonia, bronchopneumonia, pleural effusion, and dry coughing (39). Respiratory signs rarely occur in isolation, and patients often have concomitant disease such as hepatitis or spondylitis supporting the role of the lung as a portal of entry rather than a primary target (38, 39). Clinical signs in mice with respiratory infection have not been reported (40).

Several aerosol models have been developed including the Henderson aerosol apparatus, the dual-sided Class III aerosol exposure glovebox, the Madison Chamber, intranasal, and intratracheal (33, 34). The Henderson aerosol apparatus was first developed to inoculate guinea pigs with infectious aerosols and consists of a cone that fits snuggly around the animal's head to

contain the flow of the aerosol (33, 34, 41, 42). These studies found that guinea pigs developed splenomegaly and hepatitis following aerosol inoculation, but these studies were conducted in male guinea pigs (33, 34, 43). The dual-sided Class III aerosol exposure glovebox and the Madison Chamber utilize total body exposure by generating an aerosol that is contained within a pressurized chamber that then acts as a nebulizer (40). Total body exposure chambers require a large dose, depend on the animal's respiration rate, and pose a significant risk to the researcher who opens the chamber (44, 45). In addition, total body exposure offers the potential for the animal to be inoculated across mucous membranes or through ingestion during normal grooming activities.

In contrast, intranasal or intratracheal aerosol inoculation offer a more direct route of inoculation (45, 46). For intranasal, drops of infectious agents are applied to the external nares and the animal breathes in the liquid (46). For intratracheal, a blunt tipped needle/probe is inserted into the trachea to deliver and infectious dose (45-47). Several studies in the mouse model have demonstrated both intratracheal and intranasal routes are effective in establishing systemic infection (19, 48-50). Intratracheal inoculation (IT) has been evaluated in guinea pig models for *Coxiella burnetti* (Q fever) and *Prescotella equi* (47, 51). However, the intratracheal route has not been used to infect guinea pigs with *Brucella* spp.

1.5. Goals and Experimental Approach

The goal of this study was to evaluate intratracheal inoculation, a targeted means of delivering an infectious aerosol, in the guinea pig. As part of the pilot study to evaluate IT inoculation, the kinetics of extrapulmonary dissemination were determined with an emphasis on the impact of infection on the reproductive tract.

After characterization of intratracheal inoculation in the non-pregnant guinea pig is complete, the next step was to use this route of inoculation of pregnant guinea pigs to determine if aerosol inoculation results in adverse pregnancy events. The literature regarding the use of female guinea pigs as models for *Brucella* spp. is sparse. A study from 1918 found rare colonization of the ovary and uterus in response to i.p. inoculation of female guinea pigs with *B. abortus* from aborted bovine fetuses and placentas (29). In 1974, Bosseray and Diaz used intramuscular injection to inoculate 14 pregnant guinea pigs with $5x10^4$ *B. abortus* 544, which resulted in spontaneous abortions and stillbirths (37). This was intriguing because it mimicked what has been documented in the case reports of *Brucella*-associated adverse pregnancy outcomes in women (6, 37). However, similar to the mouse, the first studies investigating reproductive disease in guinea pigs used artificial means of inoculation thus the intratracheal route provides a more physiologic inoculation method.

Finally, the pregnant guinea pig was evaluated as a model for vaccine safety and efficacy studies for *Brucella* vaccine candidates. Guinea pigs were utilized extensively during the 1950s during the development and testing the safety and efficacy of *Brucella* spp. vaccines candidates such as the *B. melitensis* mutant Rev. 1 and *B. abortus* S19 vaccines (52, 53). Guinea pigs were also used to compare the protection provided by the various vaccine strains and antigens such as S19, Rev. 1, 45/20 bacterins, etc. against field isolates and were used to determine if vaccination against one strain offered cross protection (23, 25, 27, 54-57). Further demonstrating the usefulness and value of the guinea pig, the World Animal Health Organization (OIE) lists guinea pigs as a suitable animal model to test master seed virulence, safety of S19 and Rev. 1, and toxicity of brucellin prior to their use in domesticated animals (58).

The most commonly utilized vaccines against *Brucella* spp. are live-attenuated vaccines (LAV) including *B. abortus* S19 and *B. melitensis* Rev 1, which are preferentially used over other vaccine types such as subunit or DNA vaccines because they provoke a strong immune response that persists (7). However, an unfortunate consequence of the LAV is that they can cause disease symptoms including abortion in pregnant animals that limits their use to non-pregnant females and the vaccine strain could potentially be shed in milk (59, 60). 16M*ΔvjbR* is being developed as a vaccine candidate based on studies which indicated that deleting the *vjbR* gene resulted in an attenuated mutant in the both cell lines and a mouse model (61, 62). Further studies of 16MΔ*vjbR* in immunocompromised mice demonstrated that the vaccine was safe and reduced colonization following challenge with a virulent species (63). Studies comparing the safety and efficacy of 16M*ΔvjbR* versus the commercially available vaccine, Rev. 1, in pregnant small ruminants have shown that the vaccine is safer than Rev. 1 (fewer abortions) while providing a similar level of protection (64). In order to improve the efficacy of 16MΔ*vjbR*, Quil A adjuvant was used to boost the immune response to vaccination. It is important to note that Quil A is on the list of approved adjuvants for use in human vaccines and thus if a vaccine candidate proves efficacious in the pregnant guinea pig, it could serve as a model for vaccine candidates that are in the pipeline.

The overall goal of this project is to develop the guinea pig as an animal model for reproductive pathogenesis studies and to demonstrate the utility of the pregnant guinea pig as a model for vaccine development. The mouse model has allowed researchers to make great strides in *Brucella* spp. pathogenesis and vaccinology; however, the guinea pig offers an exciting model to expand upon this work and move the field forward.

2. CHARACTERIZATION OF AN INTRATRACHEAL AEROSOL CHALLENGE MODEL OF BRUCELLA MELITENSIS IN GUINEA PIGS*

2.1. Summary

B. melitensis is considered the most virulent of the *Brucella* species, and a need exists for an improved laboratory animal model of infection that mimics natural transmission and disease. Guinea pigs are highly susceptible to infection with *Brucella* spp. and develop a disease syndrome that mimics natural disease after aerosol inoculation. Intratracheal inoculation is a targeted means of generating aerosols that offer advantages over aerosol chamber delivery. To establish this delivery method, female, Hartley guinea pigs were infected via intratracheal inoculation with PBS or 16M *B. melitensis* at low dose (10^1 to 10^3) or high dose (10^6 to 10^8) and monitored for 30 days for signs of disease. Guinea pigs in the high dose groups developed fever between 12-17 days post-inoculation. Bacteria were recovered from the spleen, liver, lymph nodes, lung, and uterus at 30-days post-inoculation and demonstrated dose dependent mean increases in colonization and pathologic changes consistent with human brucellosis. To study the kinetics of extrapulmonary dissemination, guinea pigs were inoculated with 10^7 CFU and euthanized at 2-hours post inoculation and at weekly intervals for 3 weeks. $5.8x10^5$ to $4.2x10^6$ CFU were recovered from the lung 2 hours post-inoculation indicating intratracheal inoculation is an efficient means of infecting guinea pigs.

*Reprinted from "Characterization of an intratracheal aerosol challenge model of *Brucella melitensis* in guinea pigs" By Hensel ME, Garcia-Gonzalez DG, Chaki SP, Samuel J, Arenas-Gamboa AM. *PloS one*. 2019;14(6):e0218065.

Starting at 1-week post inoculation bacteria were recovered from the aforementioned organs with time dependent mean increases in colonization. This data demonstrates that guinea pigs develop a disease syndrome that models the human manifestation of brucellosis, which makes the guinea pig a valuable model for pathogenesis studies.

2.2. Introduction

Brucellosis is a disease caused by a gram-negative coccobacillus of the genus *Brucella* and is a zoonotic pathogen that has a worldwide distribution (65). Of the twelve currently recognized *Brucella* species, *Brucella melitensis* is considered the most virulent (5). The natural hosts of *B. melitensis* are sheep and goats (5). The primary clinical presentation in affected small ruminants are abortion, stillbirths, and decreased fertility; bacteria are shed in large numbers after abortions in the placenta or through secretory products like milk (5). People are commonly exposed through aerosols or by ingestion of unpasteurized milk or milk products (5). In humans, clinical brucellosis typically manifests as relapsing periods of fever, malaise, and inappetance (5). More severe complications such as disease of the reproductive, osteoarticular, cardiovascular, or nervous systems are also possible (5, 66).

Aerosols are a common means of transmission in people and animals and inhalation of bacteria leads to colonization of the reticuloendothelial organs such as the spleen, liver, and lymph nodes (5). Certain occupations are at a greater risk of exposure due to close proximity with animals including veterinarians, farmers, and abattoir workers (5). Humans who are exposed to aerosols generated following an animal abortion event are often exposed to up to 10^9 colony forming units (CFU), but a dose of 10-100 CFU is reported to generate disease (2, 5). Due to the ease of aerosolization and the low infectious dose, *B. melitensis* could potentially be

weaponized and is designated a Category B agent by the Centers for Disease Control and Prevention (2).

Animal models utilized to study human brucellosis include mice, guinea pigs, rabbits, rats, and nonhuman primates (16). Mice are currently the most commonly used model for brucellosis due to the ready availability of many genetic and immunologic tools (16). A drawback to murine research is the large number of infectious organisms required to induce disease, which is well above the dose required to cause infection in people, and mice do not develop fever (17, 40). Additionally, the most common means of inoculating mice with *Brucella* is intraperitoneal injection, which is not a means of natural transmission and thus the results of these experiments may not be as relevant. Guinea pigs were the animal model of choice to study the pathogenicity of *Brucella* species from the early 1900s to 1960 but were supplanted by the mouse model (15, 30, 67). Similar to mice, guinea pigs can be infected by a variety of routes including intraperitoneal, intramuscular, subcutaneous, and inhalation. In contrast to mice, guinea pigs not only develop systemic disease but also demonstrate clinical signs of infection that include fever (26). Previous studies utilizing aerosol chambers to infect guinea pigs with *Brucella* spp. resulted in systemic disease, indicating that guinea pigs could be used to model aerosol transmission (33, 34, 43). However, by using an aerosol chamber, guinea pigs in these early experiments were exposed via the conjunctiva and mucosal membranes as well as by ingestion during grooming after exposure. A need exists for an animal model that can be infected via a targeted aerosol transmission method to fully study the disease syndrome that arises from aerosol infection.

The experiments described herein represent a novel approach to understand the pathogenesis of aerosol transmission in a guinea pig model including the dose response to

infection, kinetics of dissemination after aerosol exposure, and macroscopic and microscopic pathologic findings. Previous studies have indicated that guinea pigs are a physiologically relevant model and with an updated approach to inoculation, the guinea pig could be used to evaluate vaccine candidates or therapeutics.

2.3. Materials and methods

2.3.1. Ethics statement

This study includes the use of guinea pigs. This study was carried out in an approved facility in strict accordance with all university and federal regulations. All guinea pig experimentation was reviewed and approved by the Texas A&M University Laboratory Animal Care and Use Committee (protocol: 2015-0036). The protocol was approved and is in accordance with the Institutional Animal Care and Use Committee (IACUC) policies of Texas A&M University. Texas A&M is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC).

2.3.2. Animal husbandry

Outbred Harley female guinea pigs (n=44) weighing approximately 300-350 g were obtained from Charles River Laboratories and housed individually in microisolator caging in a biosafety level three facility. Guinea pigs were acclimated to the facility for 5 days prior to infection and were on a 12-hour—12-hour light-dark cycle with ad libitum access to pelleted food, Timothy hay, and water. A modified Karnofsky performance status scoring system was used to evaluate the guinea pigs daily to determine if early removal from the study was required.

2.3.3. Bacteriology

Brucella melitensis 16M wild-type strain, originally acquired from an aborted goat fetus, was routinely grown on tryptic soy agar (TSA) (Difco Laboratories) at 37°C in an atmosphere

containing 5% (vol/vol) CO_2 for 72 hours (68). Bacteria were harvested into phosphate-buffered saline (PBS) (pH 7.4; Gibco) to obtain the final concentration needed for each experiment, as estimated turbidometrically using a Klett meter. Serial dilution was performed to accurately determine the number of organisms in the inoculum. To determine if passage through the MicroSprayer® affected the inoculum dose, 100 µl of the inoculum was passed through the MicroSprayer® and collected in the microcentrifuge tube containing 900 µl PBS for serial dilution and culture on TSA in duplicate.

2.3.4. Animal experiments

2.3.4.1. Dose titration

Guinea pigs were randomly divided in 7 groups (n=4). These 7 groups were further subdivided as low dose (10^1, 10^2,10^3), high dose (10^6, 10^7, 10^8), or control (PBS) groups. Guinea pigs were anesthetized with ketamine/xylazine (50mg/kg;5mg/kg) and a subcutaneous IPTT-300 microchip was placed to monitor temperature throughout the study (Bio Medic Data Systems). The 50 µl doses of *B. melitensis* 16M were prepared from cultures resuspended into PBS and serially diluted to obtain the dose groups. The inoculum was administered into the proximal trachea and lungs using the PennCentury™ MicroSprayer I-1C device (Penn Century Inc.). Animals were monitored daily for 30 days for changes in body temperature, respiratory pattern and effort, and weight. Temperatures of ≥39.5°C were defined as fever. At 30-days post-inoculation, animals were euthanized by intraperitoneal injection of sodium pentobarbitol (Beuthanasia) followed by cardiac exsanguination. Samples of lung, liver, spleen, cervical lymph node, tracheobronchial lymph node, and uterus were aseptically collected into 1 ml PBS, homogenized, serially diluted, and 100 µl of each dilution was plated in duplicate onto Farrell's

medium (TSA plus *Brucella* Oxoid supplement, equine serum, and 50% dextrose) and incubated at 37°C in an atmosphere containing 5% (vol/vol) CO_2 (40). Bacterial colonies were enumerated after 72 hours to quantify tissue colonization. Spleen and liver were weighed at necropsy, and the aforementioned tissues were collected and fixed in 10% neutral buffered formalin for evaluation by light microscopy.

2.3.4.2. Kinetics of infection

Guinea pigs were divided into four groups (n=4) and were infected via intratracheal inoculation with 50 µl of $1x10^7$ CFU *B. melitensis*. The endpoints were 2-hours post-inoculation and at weekly intervals thereafter for three weeks. To determine the actual number of infectious organisms delivered by intratracheal inoculation, 4 animals were euthanized 2-hours post-inoculation, and the lung was divided into four quarters (left and right, cranial and caudal), collected into 1 ml PBS, homogenized, and serial dilutions plated on Farrell's medium. Spleen, liver, CLN, TBLN, and uterus were collected for culture and histology at each of the time points, as described in experiment 1.

2.3.5. Anti-*Brucella* specific IgG ELISA

300 µl of blood was collected into serum separator tubes from the lateral saphenous vein at day 14 and from the heart at day 28 following euthanasia. Blood was centrifuged at 1000 x g for 5 minutes, and the serum was collected for anti-*Brucella* specific immunoglobulin G (IgG) indirect enzyme linked immunosorbent assay (iELISA). 96 well plates were pre-coated with 25 µg/well of *Brucella abortus* 2308 heat killed lysate and held overnight at 4°C. Plates were washed three times and then blocked with 3% skim milk for 2 hours at room temperature. Guinea pig sera samples were diluted in blocking buffer (0.25% [wt/vol] bovine serum albumin) to 1:1000 and incubated at 37°C for 1 h. Plates were washed five times and then peroxidase

labeled goat anti-guinea pig IgG (KPL) was added at 1:2000, followed by incubation at 37°C for 1 hour. After a final washing step, horseradish peroxidase substrate (Sigma) was added and plates were protected from light and incubated for 30 m at 37°C. Absorbance was measured at 450 nm. All assays were performed in triplicate, and the results are presented as the mean value for the three wells.

2.3.6. Histopathology

Spleen, liver, lung, uterus, CLN, and TBLN were collected at necropsy and fixed in 10% neutral buffered formalin for a minimum of 48 h. Tissues were routinely processed and embedded, sectioned at 5 μm, and stained with hematoxylin and eosin. Sections from spleen, liver, lung, and uterus were graded in a blinded fashion by a board-certified veterinary pathologist (MH) on a scale of 0-4 for inflammation type, necrosis, and severity (Figure A1). The mean total score for each tissue was compared between groups.

2.3.7. Immunohistochemistry

Unstained slides from spleen, uterus, liver, and lung were adhered to positively charged glass slides for immunohistochemistry. Slides were deparaffinized and rehydrated through a series of xylene and ethanol steps before antigen retrieval was performed using 1:10 EMS Solution A (Electron Microscopy Services) in a 2100 Antigen Retriever (Aptum Biologics Ltd.), according to manufacturer protocol. Endogenous peroxidases were blocked by 10 m incubation with Bloxall Blocking Solution (Vector Laboratories) followed by 20 m blocking with normal goat serum (Vector). After each step slides were washed with PBS plus 0.5% tween for 5 minutes. Primary incubation was overnight at 4° C with *Brucella* polyclonal rabbit antibody (Bioss) at 1:600. Negative control tissues were incubated with rabbit nonimmune serum diluted in PBS. A Vectastain ABC and Betazoid DAB chromagen kits (Biocare Medical) were used

following primary incubation according to the manufacturer's instructions. The slides were counterstained with Meyer's hematoxylin III.

2.3.8. Statistical analysis

Analysis was performed using the GraphPad Prism 6.0 Software. The difference between group means was analyzed using a one-way analysis of variance (ANOVA) repeated-measures test, and Dunnett's multiple comparisons was used to generate *P* values for selected mean comparisons. Tukey's multiple comparison was used to generate *P* values to compare mean IgG values.

2.4. Results

Our first objective was to determine if passage of the inoculum through the MicroSprayer® affected the bacterial viability. Bacterial suspensions of each dose were sprayed through the device and collected into 900 µl of PBS, serially diluted, and plated on TSA in duplicate to calculate the number of viable bacteria. Bacterial viability was minimally affected by passage through the device. As an example, the original inoculum for guinea pigs in the 10^7-group contained $4.4x10^7$ CFU/50 µl and after passage through the MicroSprayer® $4.1x10^7$ CFU/50 µl was recovered (Table 2.1). This study proves that the device is a reliable means of generating an infectious aerosol and passage through the MicroSprayer® does not adversely affect the viability of the bacteria.

Having established that the MicroSprayer® does not adversely affect bacterial viability, we next evaluated the ability of the device to inoculate guinea pigs with low doses (10^1, 10^2,10^3) or high doses (10^6, 10^7, 10^8) of *B. melitensis* 16M. After intratracheal inoculation with *B. melitensis*, guinea pigs were monitered for signs of clinical disease including fever, loss of appetite, respiratory disease (ocular discharge, increased respiratory effort), and lethargy.

Brucellosis is a disease of high morbidity but low mortality and, as expected, intratracheal inoculation did not result in any deaths in any dose group despite evidence of systemic infection. However, guinea pigs in the 10^8 group had more severe clinical signs including roughened hair coat, ocular discharge, and lethargy. Body weight was not affected by infection in any dose group, and all guinea pigs continued to gain weight throughout the study period. Guinea pigs inoculated with PBS or the low doses (10^1, 10^2,10^3) of *B. melitensis* did not develop fever or other clinical signs of brucellosis at any time point. In the high dose groups, the onset of fever (temperature ≥39.5°C) developed at day 16 to 18 post-infection (Fig 2.1). In the 10^6 and 10^7 groups 3/4 animals in the developed fever. Based on the kinetics study, the earliest onset of fever appears to be 12-days post-inoculation (Figure A2). The average daily temperature was significantly increased (P <0.05) in the 10^6 and 10^7 groups between days 16 to 24 compared to the uninfected control group (Figure A3). The guinea pigs in the 10^8 group did not develop fever to the same level, but 2 animals had a single episode of fever. We ascribe the lack of fever response in the 10^8 group to overwhelming disease that resulted in a sepsis-like condition.

A hallmark of brucellosis in natural hosts and humans is splenomegaly. Previous aerosol studies with guinea pigs demonstrated the development of splenomegaly after infection (34). In response to infection, spleen weight was significantly increased ($p<0.0001$) in the high dose group (10^6, 10^7, 10^8) compared to the uninfected controls (Fig 2.2 panel A). The average spleen weight in the 10^6, 10^7, and 10^8 group was 3.45 g, 2.96 g, and 3.33 g, respectively compared to 0.6 g in the control group. Spleen weight continuously increased over a four-week course of infection (Fig 2.2 panel B). Similarly, the liver weight was not significantly different between dose groups or time points in guinea pigs.

To determine colonization following intratracheal inoculation, the spleen, liver, lung, cervical lymph node (CLN), tracheobronchial lymph node (TBLN) and uterus were collected for culture. Guinea pigs inoculated with either PBS or 10^1 and 10^2 CFU doses of *B. melitensis* did not result in colonization of any tissue examined. Animals in the 10^3 and high dose groups (10^6, 10^7, 10^8) demonstrated dose-dependent mean increases in CFU recovered per gram of the spleen, liver, lung, cervical lymph node, tracheobronchial lymph node, and uterus at 30-days post-inoculation (Fig 2.3 panels A-F). Following intratracheal inoculation, bacteria are rapidly disseminated to the spleen, draining lymph nodes, and uterus within 2-hours post-inoculation and could be recovered from the lung, CLN, and TBLN in 100% of the animals (Fig 2.4 panels C-F). The inoculum was evenly distributed throughout all lung lobes indicating that intratracheal inoculation generates a particle size that is able to reach the terminal airways (Figure A4). Peak replication occurred at 3-weeks post-inoculation in the spleen, liver, and uterus (Fig 2.4 panels A,B,D). Replication continued to increase in the CLN and TBLN for the entire study period (Fig 2.4 panels E-F).

The earliest gross lesions developed 2-weeks post-inoculation and included nodular lymphoid hyperplasia in the spleen, perinodal hemorrhage around the CLN, multifocal random 1-2 mm pale foci in the liver, and consolidation of the cranioventral lung lobes with multifocal 1-3 mm depressed gray foci scattered throughout the pulmonary parenchyma. A single animal (1/4) in the 10^7 group had a splenic abscess. No gross or microscopic lesions consistent with brucellosis were observed in any organ in the PBS control, 10^1, or 10^2 groups or at 2-hours post-inoculation.

A grading system was developed to assess microscopic findings in the spleen, liver, lung, and uterus (Figure A1). Application of the grading system demonstrated a significant increase

(P<0.0001) in lesion severity based on average histologic score as the dose increased between the uninfected controls and high dose groups.

Sections were graded by a board-certified veterinary pathologist (MH). Lesions in all organs in the high dose groups (10^6, 10^7, 10^8) increased in number, size, and severity by 30-days post-inoculation in a dose dependent manner. Histologic evaluation of the spleen revealed an inflammatory infiltrate of predominantly epithelioid macrophages with fewer neutrophils that effaced the normal architecture (Fig 2.5). Similarly, the earliest lesion at 1-week post-inoculation were small foci of epithelioid macrophages in the red pulp that increased in size and number at 2 and 3-weeks post-inoculation. The cortex and medulla of the lymph node were also expanded by a large number of epithelioid macrophages. The liver lesion was characterized by variably sized random foci of liquefactive and coagulative necrosis surrounded by neutrophilic and histiocytic inflammation and multifocal random microgranulomas composed of accumulations of histiocytes (Fig 2.6). Portal areas were expanded by lymphocytes and plasma cells. In addition, guinea pigs had foci of necrosis surrounded by macrophages and neutrophils.

The earliest lesion in the lung at 1-week post-inoculation included expansion of the bronchus-associated lymphoid tissue (BALT), congestion of the alveolar walls, and edema. By 2-weeks post-inoculation, alveolar walls were thickened by an inflammatory infiltrate of macrophages and neutrophils surrounded by lymphocytes and plasma cells. At 3 to 4-weeks, the inflammatory infiltrate had coalesced into variably sized nodules of histiocytic and neutrophilic inflammation (Figure A5).

Interestingly, at 2-weeks post-inoculation, the endometrial stroma was variably expanded by edema, and endometrial glands were distended by an inflammatory infiltrate of intact and degenerate neutrophils and macrophages. The lesion progressed in severity and by 3 and 4-weeks

post-inoculation, foci of histiocytic inflammation were developing in the myometrium (Fig 2.7). A single animal in the 10^8-dose group had histiocytic salpingitis. No lesions were identified 1-week post-inoculation in the uterus.

To further support the CFU data that the lesions in the liver, spleen, and uterus were due to *Brucella* infection, IHC was performed to colocalize *Brucella* antigen within foci of inflammation. *Brucella* antigen was detected within epithelioid macrophages in the spleen, liver, and uterus by IHC further corroborating the etiology of the lesion (Figs 2.5-7). Antigen was also detected intracellularly within macrophages in the lung (Figure A5), CLN, and TBLN.

Guinea pigs develop a humoral response (anti-*Brucella* specific IgG) to infection with *Brucella melitensis* delivered via intratracheal inoculation. No change in IgG level was noted in the PBS, 10^1, or 10^2 groups. Only guinea pigs in the high dose groups (10^6, 10^7, 10^8) were capable of mounting a humoral response against *B. melitensis*. The increase in IgG level was statistically significant in the 10^7 and 10^8 groups at 4-weeks post-inoculation ($P < 0.01$) (Fig 2.8 panel A). Levels of *Brucella*-specific IgG antibodies increased starting 1-week post-inoculation and increased throughout the study period (Fig 2.8 panel B).

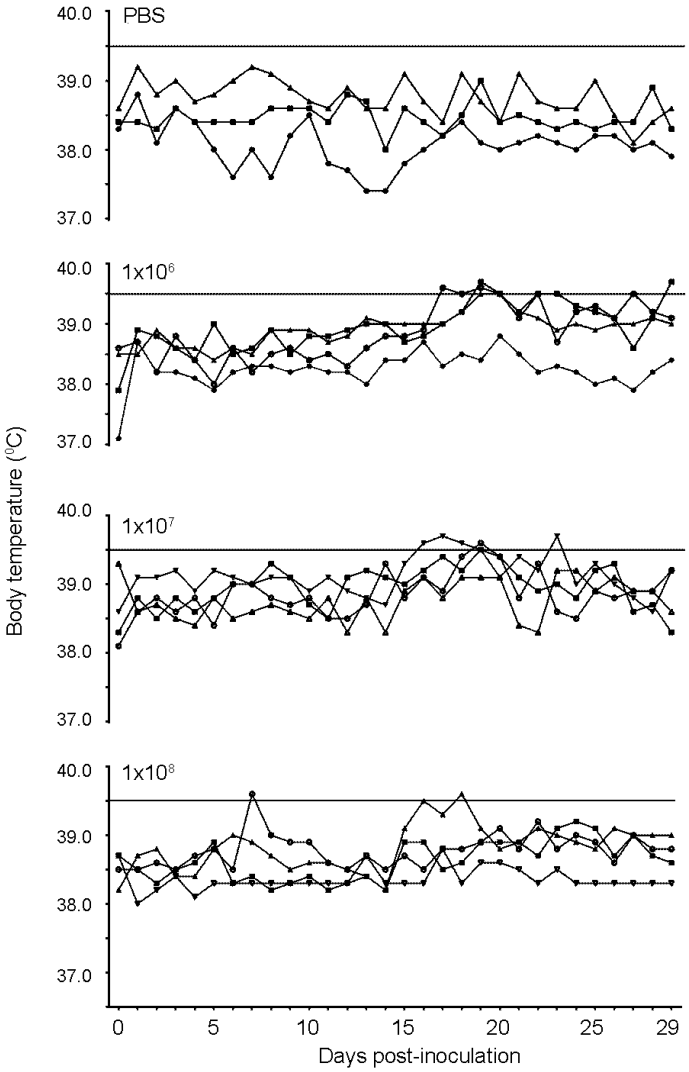


Figure 2.1 Body temperature changes in guinea pigs after intratracheal inoculation with PBS, 10^6, 10^7, and 10^8 *B. melitensis* 16M.

The solid line at 39.5° C indicates the threshold for fever. Guinea pigs in the 10^6 and 10^7 groups developed fever beginning at day 16 to 18 post-infection.

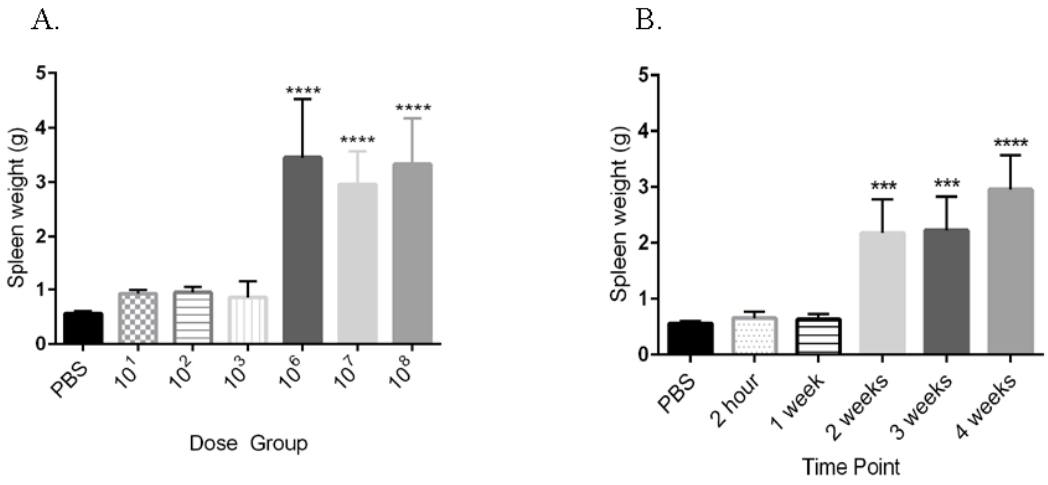


Figure 2.2 *Brucella* induces splenomegaly in guinea pigs.
Splenic weights in guinea pigs (n=4) inoculated with *B. melitensis* 16M or PBS. Splenomegaly was induced by high doses (10^6, 10^7, 10^8) of *B. melitensis* by 30-days post-inoculation (A). Splenomegaly was detected as early as 2-weeks post-inoculation with $1x10^7$ CFU and increased through the study period (B). Data bars represent the mean spleen weight plus the standard deviation for all guinea pigs in each dose group. Mean spleen weight from each dose group (n=4) or time point (n=4) was compared to mean spleen weight of the uninfected control guinea pigs (n=3) and statistical significance was determined by ANOVA followed by Dunnett's multiple-comparison test. Three asterisks, $P < 0.001$. Four asterisks, $P < 0.0001$.

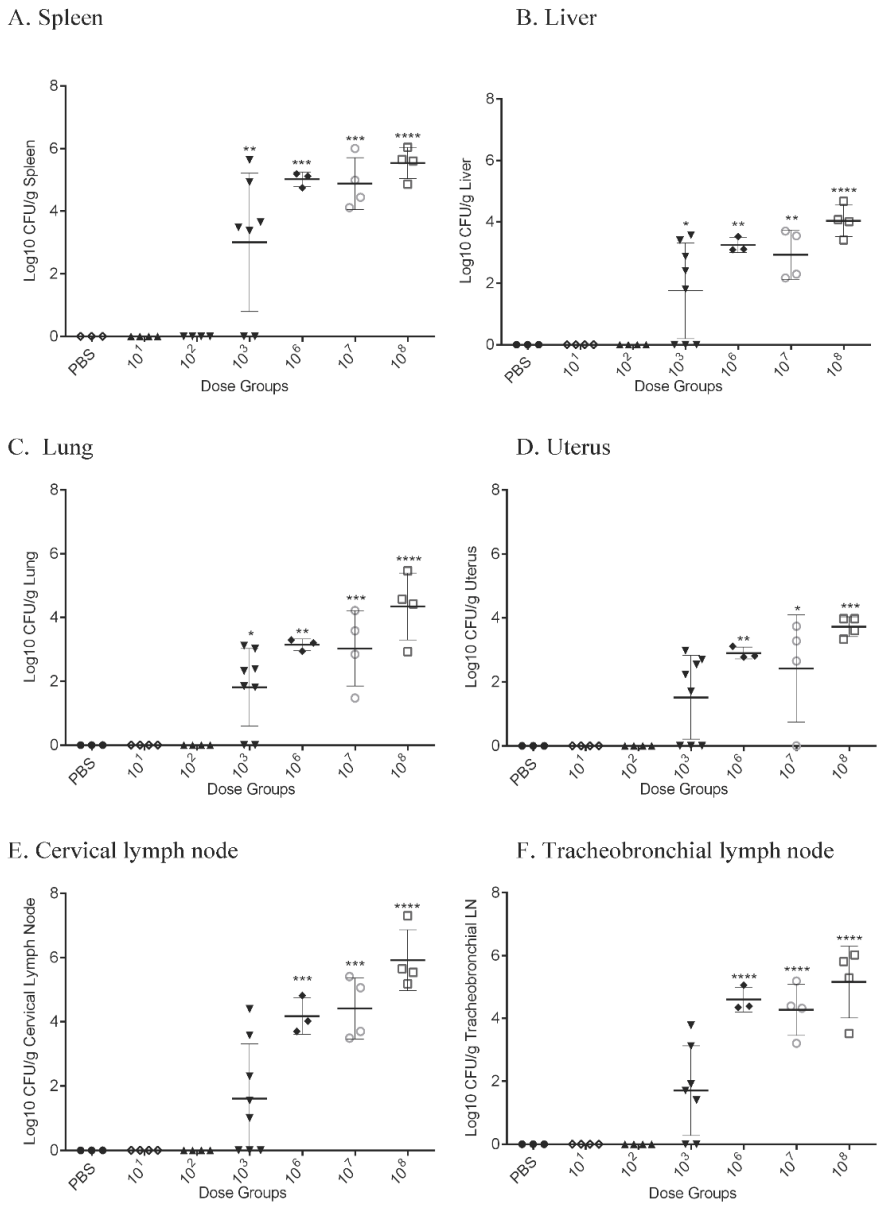


Figure 2.3. Intratracheal inoculation with *B. melitensis* 16M in female Hartley guinea pigs results in systemic infection.

Guinea pigs were divided in 7 groups (n=4) consisting of low dose (10^1, 10^2,10^3), high dose (10^6, 10^7, 10^8), or control (PBS) groups (n=3). Guinea pigs were inoculated using the MicroSprayer® Aerosolizer and were euthanized 30-days post-inoculation. Colonization was evaluated in the spleen (A), liver (B), lung (C), uterus (D), cervical lymph node (E), and tracheobronchial lymph node (F). The recovery of organisms is plotted as the total CFU/g (means ± standard deviation). Mean recovery per gram of tissue was compared between dose groups and uninfected control guinea pigs. Statistical significance was determined by ANOVA followed by Dunnett's multiple comparisons. One asterisk, $P < 0.05$. Two asterisks, $P < 0.01$. Three asterisks, $P < 0.001$. Four asterisks, $P < 0.0001$.

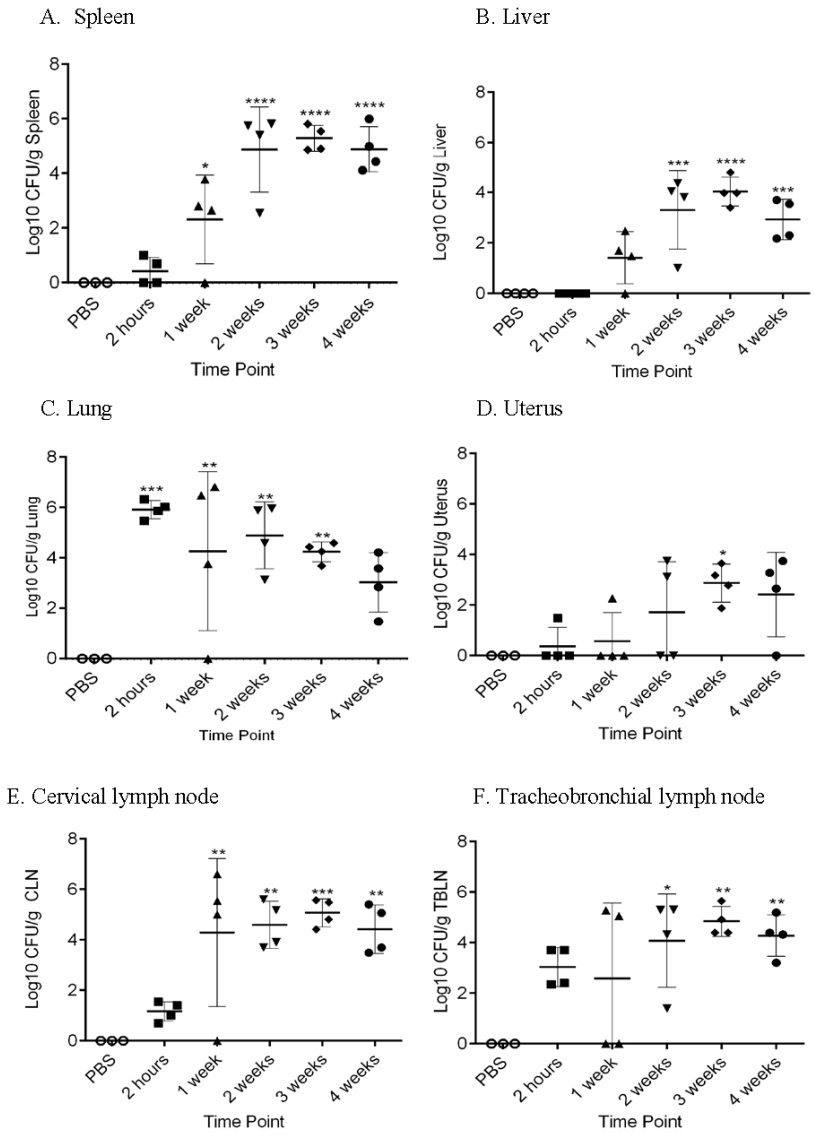


Figure 2.4. Kinetics of systemic infection of *B. melitensis* 16M in guinea pigs.
Four female Hartley guinea pigs per time point group were inoculated intratracheally with $1x10^7$ CFU/50 µl. The initial lung colonization was evaluated 2-hours post-inoculation to determine the inhaled dose. Guinea pigs (n=4) were euthanized at 1,2,3, and 4-weeks post-inoculation to determine the numbers of B. melitensis in the spleen (A), liver (B), lung (C), uterus (D), cervical lymph node (E), and tracheobronchial lymph node (F). Mean recovery per gram of tissue was compared between time points and uninfected control guinea pigs. Statistical significance was determined by ANOVA followed by Dunnett's multiple comparisons. One asterisk, $P < 0.05$. Two asterisks, $P < 0.01$. Three asterisks, $P < 0.001$. Four asterisks, $P < 0.0001$.

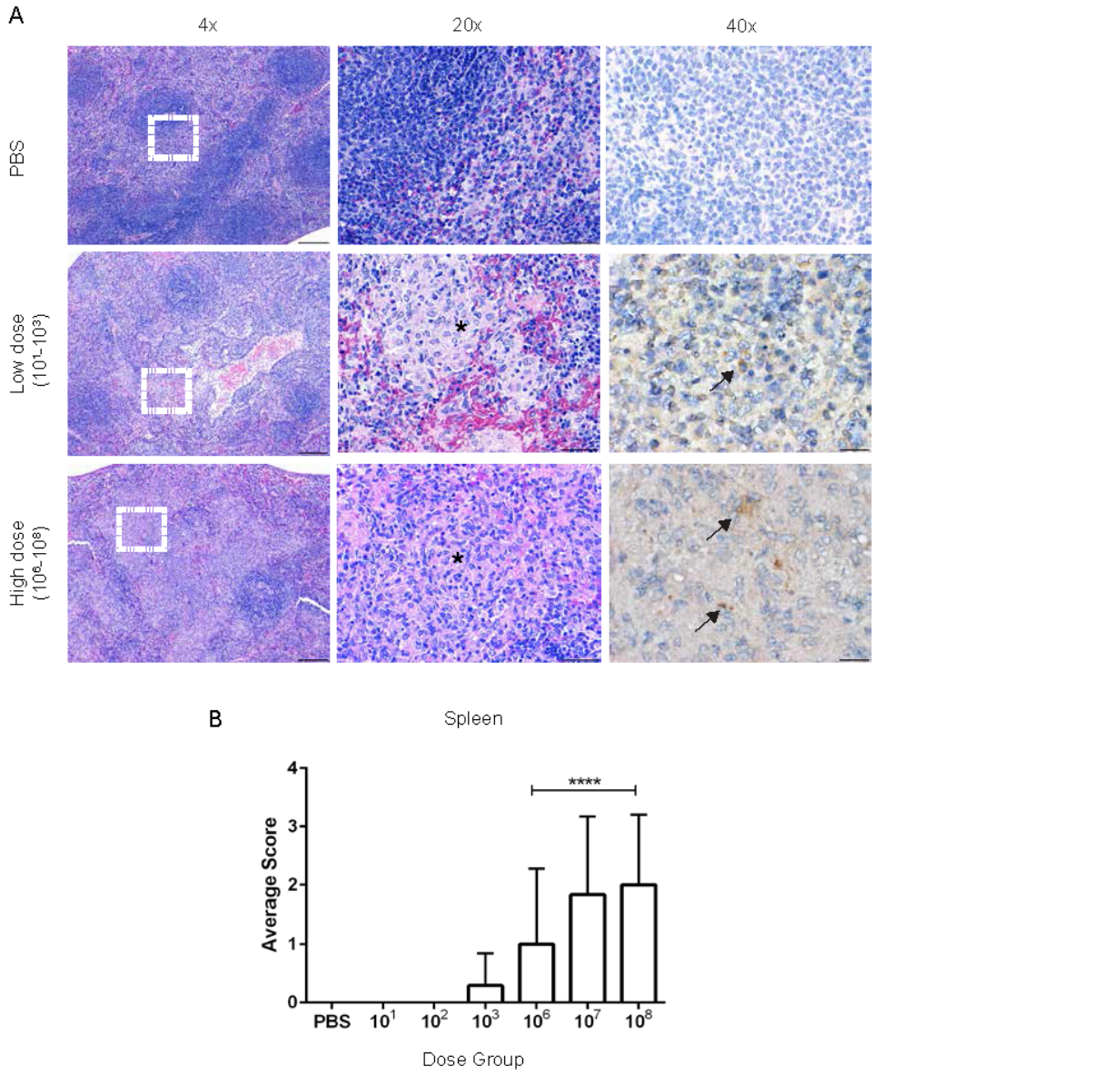


Figure 2.5. Histopathology of the spleen following *Brucella* infection.
Representative images of histopathology and immunohistochemistry of the spleen following intratracheal inoculation with PBS (top), *B. melitensis* 16M at low dose (middle), or high dose (bottom) at 30-days post-inoculation (A). Sections were scored for severity from 1-4 (Table S1) based on accumulation of epithelioid macrophages, neutrophils, and necrosis (B). The white dashed box in the left panel indicates the section highlighted for higher magnification in the middle and right panels. Infection with *B. melitensis* induces accumulation of epithelioid macrophages (*). *Brucella* antigen was detected within epithelioid macrophages by immunohistochemistry (arrows). Magnification 4x (left, H&E, bar= 200 μm), 20x (middle, H&E, bar= 50 μm), 40x (right, Anti-*Brucella* IHC, bar=20 μm).

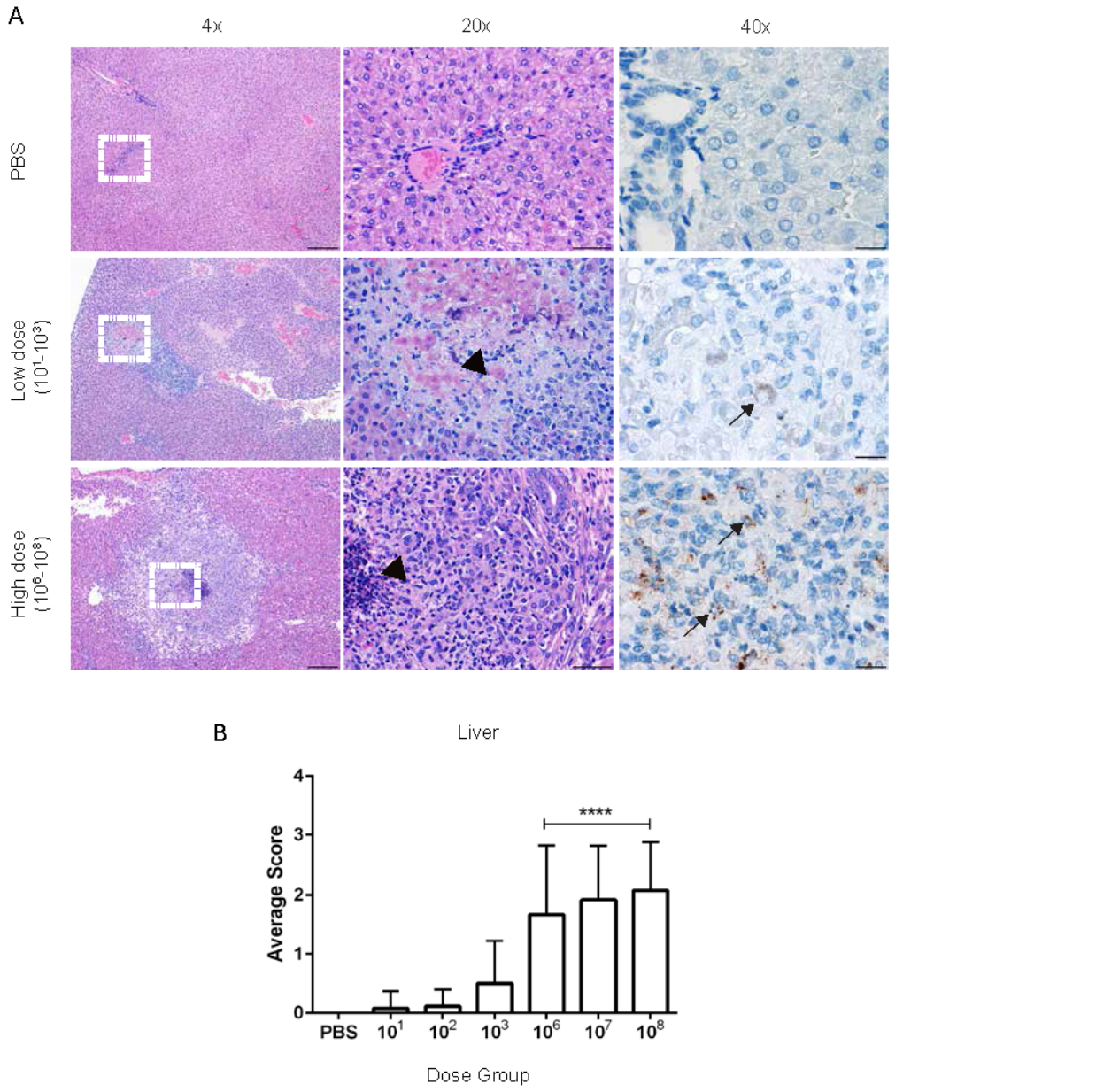


Figure 2.6. Histopathology of the liver following *Brucella* infection.
Representative images of histopathology and immunohistochemistry of the liver following intratracheal inoculation with PBS (top), *B. melitensis* 16M at low dose (middle), high dose (bottom) at 30-days post-inoculation. Sections were scored for severity from 1-4 (Table S1) based periportal inflammation, number and size of microgranulomas and necrosis. The white dashed box in the left panel indicates the section highlighted for higher magnification in the middle and right panels. Foci of necrosis were seen in the low and high dose groups (arrowheads), but the lesions were larger in the high dose group. *Brucella* antigen was detected within necrotic hepatocytes and macrophages in areas of necrosis by IHC (arrows). Magnification 4x (left, H&E, bar= 200 μm), 20x (middle, H&E, bar= 50 μm), 40x (right, Anti-*Brucella* IHC, bar=20 μm).

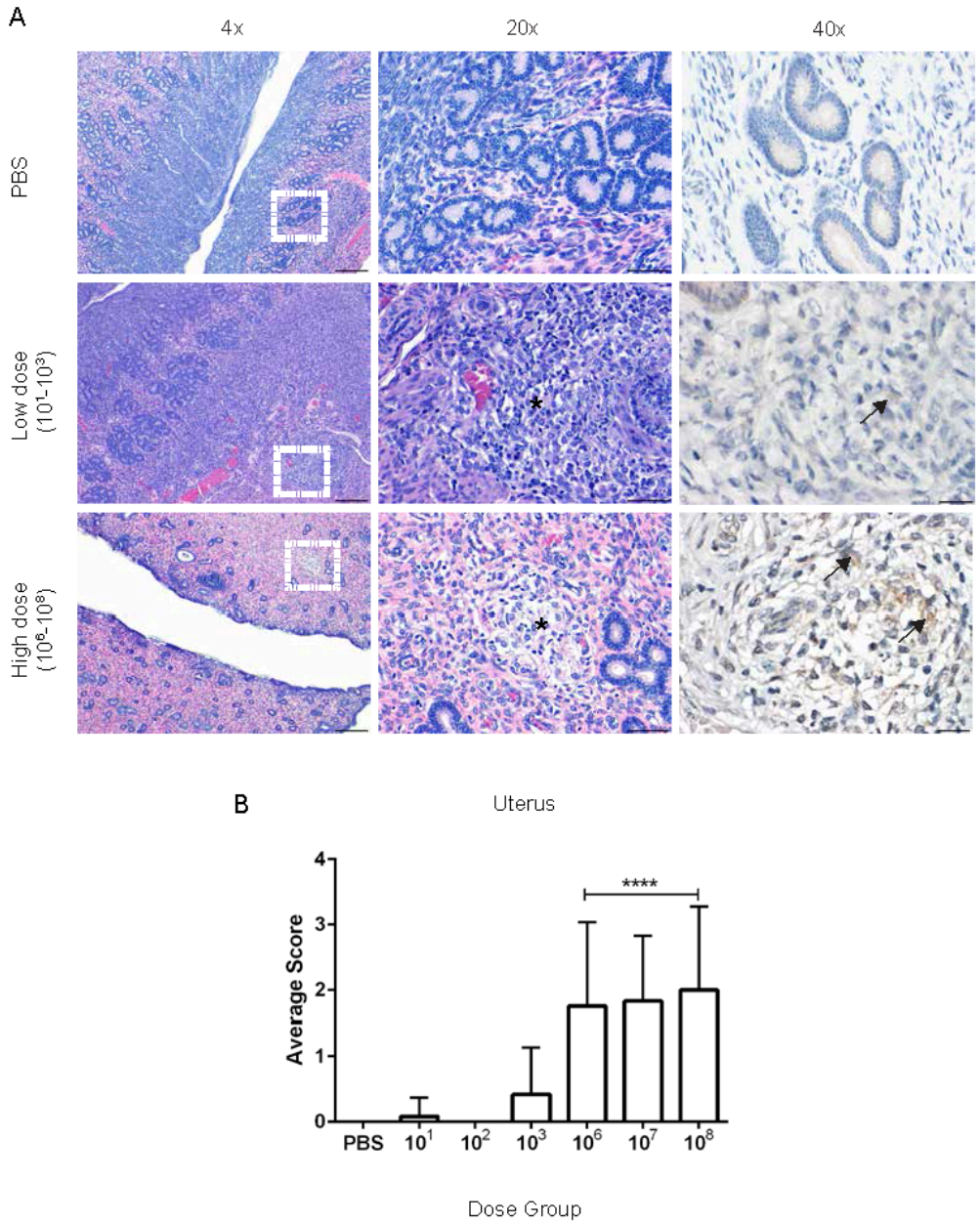


Figure 2.7. Histopathology of the uterus following *Brucella* infection.
Representative images of histopathology and immunohistochemistry of the uterus following intratracheal inoculation with PBS (top), *B. melitensis* 16M at low dose (middle), or high dose (bottom) at 30-days post-inoculation. Sections were scored for severity from 1-4 (Table S1) based on edema, endometrial neutrophilic inflammation, and myometrial inflammation. The white dashed box in the left panel indicates the section highlighted for higher magnification in the middle and right panels. The high dose group had increased numbers of neutrophils in the endometrium, foci of histiocytic inflammation within the myometrium (*), and *Brucella* antigen was detected intracellularly via IHC (arrows). Magnification 4x (left, H&E, bar= 200 μm), 20x (middle, H&E, bar= 50 μm), 40x (right, Anti-*Brucella* IHC, bar=20 μm).

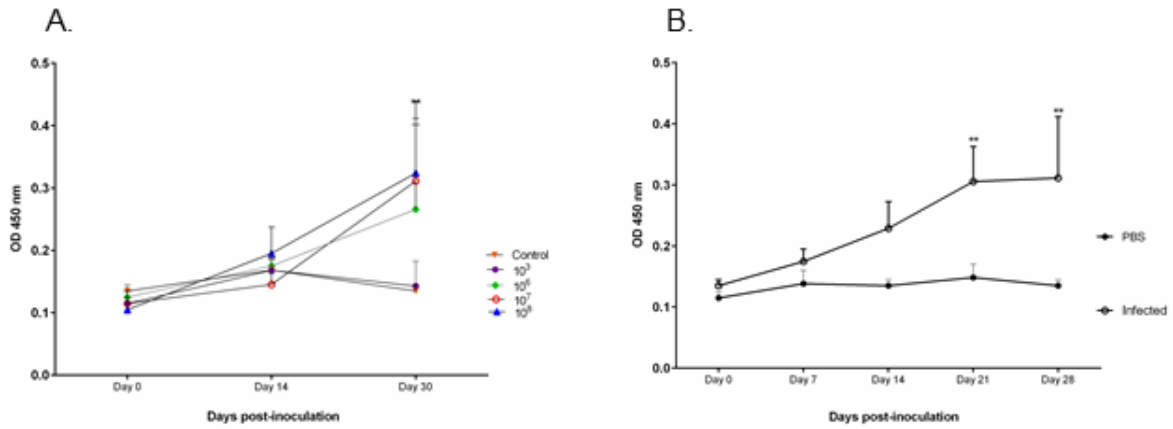


Figure 2.8. Humoral response to infection with *Brucella melitensis*.
Anti-*Brucella* specific IgG ELISA with sera from guinea pigs inoculated by intratracheal route with *B. melitensis* 16M at doses of 10^3, 10^6, 10^7, 10^8, or uninfected control (A) at day 0, 14, and 30 post-inoculation. The kinetics of the IgG response was evaluated at 1,2,3, and 4-weeks post-infection in guinea pigs (n=4) inoculated with $1x10^7$ CFU (B). Guinea pigs in the 10^7 and 10^8 groups developed a statistically significant humoral response to inoculation with *B. melitensis*. The results are expressed as the mean absorbance (± standard error). Statistical significance was determined by ANOVA followed by Dunnett's multiple-comparison of each group (n=4) to the uninfected controls (n=3). Two asterisks, $P < 0.01$.

2.5. Discussion

Brucella organisms can be easily aerosolized and inhalation of bacteria is a route of natural transmission in both animals and people (2). One of the limitations to developing stronger intervention measures such as a safe and efficacious vaccine has been the difficulty of replicating natural disease in a laboratory animal model. Mice are the most commonly utilized animal, but limitations to this model include lack of fever response, relatively high dose required to generate systemic infection, and an artificial route of inoculation that does not mimic natural transmission events (17). In contrast, guinea pigs develop key features of disease when

inoculated via an aerosol route, which closely mimics the naturally occurring disease process (33, 34, 43). Guinea pigs were used in the early twentieth century as the model of choice to evaluate the pathogenicity of *Brucella* species such as *B. abortus*, *B. suis*, and *B. melitensis* and could offer an improvement over the mouse model for vaccine and therapeutic development (21, 22, 26, 29, 30, 32-34, 43, 69-71).

This study utilized the PennCentury™ MicroSprayer because it is a targeted means of generating aerosols and has been used successfully to inoculate mice with bacterial pathogens (45). The MicroSprayer® device has not been previously used to inoculate guinea pigs, but it offers an improvement over aerosol chambers or aerosol devices like the Henderson apparatus because it allows for the direct inoculation of bacteria into the upper respiratory tract through the trachea. However, the MicroSprayer® Aerosolizer does bypass the nares, which would be a line of defense in the upper respiratory tract against natural transmission. Due to the guinea pig oral anatomy, the device is inserted into the proximal trachea at the level of the arytenoid cartilage. Microparticles are generated after passage through the MicroSprayer®, which then move by centripetal force through the trachea and into the lower airways. This is similar to the natural transmission in which inhaled particles must pass from the nares into the trachea and then into the bronchi and bronchioles. Particle size determines the site of deposition within the airway with larger particles (>15 μm) removed through the nares and sinuses while smaller particles (6-10 μm) deposit in the bronchi (72). The smallest particle size (≤5 μm) are able to deposit in the terminal bronchioles and alveoli (72). The MicroSprayer® generates a mean particle size of 8 μm, which allows for the particles to be deposited in the lower airways (73).

Recurrent or undulant fever is a hallmark of brucellosis in humans and is a feature of disease that is not replicated in the mouse model (5). The first study to document fever in guinea pigs used an intraperitoneal, intravenous, or subcutaneous route of inoculation. The severity of the temperature elevation was not reported, and it was further stated that fever developed in the acute stage of infection, described as 72 hours post-inoculation (26). In people, the onset of clinical symptoms such as fever tend to be insidious but likely develop between 6 to 90 days after exposure, and the temporality and undulant nature of the fever response suggests guinea pigs could be a biologically relevant model for future studies (2). The aerosol literature with *Brucella* spp. in guinea pigs did not evaluate body temperature, and thus it was previously unknown if aerosol inoculation would result in fever.

People can be infected with as few as 10-100 CFU of *Brucella* and thus this study evaluated the ability of low doses (10^1, 10^2,10^3) of *B. melitensis* 16M to infect guinea pigs (2). A high dose range (10^6, 10^7, 10^8) was also evaluated because many of the infectious aerosols that people are exposed to likely exceed the minimum dose estimated to generate infection (2, 5). The dose titration study indicated a dose of at least 10^6 CFU was required to induce temperature elevations although systemic infection developed in the majority of the guinea pigs inoculated with 10^3 CFU. Previous aerosol studies in guinea pigs delivered a dose of between $4.5x10^3$/ml to $5.0x10^5$/ml, which generated an estimated dose range of 48-2800 CFU (33, 34, 43, 74). The majority of the early aerosol studies utilized the Henderson apparatus for generating aerosols, which is a mask that fits over the head and neck of the guinea pig to create a small aerosol chamber (33, 34, 42, 43). As such, the guinea pigs were exposed not only through the respiratory tract, but bacteria were also likely deposited on mucous membranes of the conjunctiva and oral cavity and potentially ingested. The calculated dose did not account for these other potential

routes of exposure, which could have increased the dose inoculated. Furthermore, since the doses from the earlier aerosol studies also based inoculation dose on calculations of ventilation rate and respiratory tidal volume of the guinea pig, the dose could have been underestimated . These factors could explain the discrepancy between the dose reported in the literature and the higher dose required in this study to generate clinical disease. Alternatively, the previous studies evaluated infection by colonization of organs such as the spleen and liver, whereas this study used clinical parameters such as body temperature plus organ colonization to demonstrate infection.

While the respiratory tract is a common portal of entry, pulmonary pathology and respiratory disease are not atypical features of *Brucella* spp. infection (38, 39). In the rare cases in which respiratory disease is reported, the common presentations include pneumonia, bronchopneumonia, pleural effusion, and dry coughing (39). Respiratory signs rarely occur in isolation, and patients often have concomitant disease such as hepatitis or spondylitis supporting the role of the lung as a portal of entry rather than a primary target (38, 39). Clinical signs in mice with respiratory infection have not been reported (40). In the 10^8 group, two animals developed transient ocular discharge, which can be associated with respiratory disease in guinea pigs (75). Guinea pigs had a pattern of cranioventral lung lobe consolidation and embolic foci, which suggests a dual pattern of infection. The initial inoculation with *B. melitensis* via intratracheal delivery likely leads to the development of cranioventral consolidation as the site of initial deposition followed by an embolic pattern as the animals become bacteremic. A previous aerosol study in guinea pigs by Elberg and Henderson reported no *Brucella*-specific macroscopic or microscopic pulmonary pathology, and several other contemporary studies failed to evaluate the lung for lesions (33, 34, 43).

Brucella has a tropism for organs of the reticuloendothelial system including the spleen, lymph nodes, and liver (5, 6, 66, 76). Splenomegaly, lymphadenomegaly, and hepatitis are common macroscopic lesions in natural and experimental infection (66). The microscopic splenic lesion has not been well described in the medical literature but has been described as congestion, lymphoid hyperplasia, and histiocytic splenitis in mice (17). Guinea pigs develop splenic congestion and lymphoid hyperplasia with occasional necrosis and abscesses thirty days after receiving an aerosol dose of $2.16x10^3$ CFU of *B. abortus* and *B. melitensis* (34). Lymphadenomegaly is another well documented sequelae of infection with *Brucella* spp. in both people and guinea pigs (6, 33, 34, 43). An aerosol study by Elberg and Henderson noted the development of caseous abscesses in the cervical and tracheobronchial lymph nodes; however, *Brucella* was not cultured from the nodes so the etiology of the abscess cannot be definitively assigned to brucellosis (34). The final reticuloendothelial organ that is commonly affected during infection is the liver. While the liver is a frequent target of *B. melitensis*, infection is not associated with hepatomegaly in humans (76). A prospective study of patients with hepatitis due to *B. melitensis* found that disease is often subclinical but can cause mild derangements in hepatic enzymes such as alanine aminotransferase (ALT)(76). The acute lesion of brucellosis is described most frequently as lymphocytic portal to lobular inflammation with fewer cases diagnosed with noncaseating granulomas or microgranulomas (76). The range of morphologic diagnoses seen in guinea pigs is similar to those described in the liver of people infected with *B. melitensis* including lymphocytic portal hepatitis and microgranulomas. The foci of necrosis surrounded by macrophages and neutrophils seen in this study may correspond to the noncaseating granulomas described by Young (6).

Brucella spp. are best known as pathogens of the reproductive tract during pregnancy and cause a range of adverse events such as abortion, stillbirths, and infertility in small ruminants and people (5, 11). Less is known about the tropism of *Brucella* organisms for the non-gravid uterus. Reproductive studies in mouse models have not reported lesions in non-pregnant female reproductive organs (77). Researchers in the early twentieth century did not identify lesions in the reproductive tract of female guinea pigs, and thus it was assumed that females were not an appropriate animal model for use in reproductive pathogenesis investigations. Instead, the early studies focused on male guinea pigs and identified orchitis, epididymitis, and peri-orchitis subsequent to intraperitoneal, intratesticular, and aerosol inoculation (22, 26, 28, 30, 71). This study demonstrates that the non-gravid uterus can be a target of *Brucella* infection and could suggest that pregnancy is not required to generate tropism. Since infertility is also described in non-pregnant women infected with *Brucella* spp., it is possible that inflammation of the reproductive tract is a contributing factor (11). Furthermore, a study from 1974 demonstrated that when pregnant guinea pigs are inoculated at mid-gestation with 10^5 *B. abortus* 2308 via intramuscular injection, stillbirths, abortions, and vertical transmission occur(37). Thus, guinea pigs may be suitable models for future investigations into the pathogenesis and tropism of *Brucella* spp. for the gravid uterus.

This study describes pathologic changes and the kinetics of infection following aerosol inoculation with a novel intratracheal method in the guinea pig and further supports the utility of the guinea pig as an appropriate animal model for brucellosis. Intratracheal inoculation of the guinea pig offers an intriguing model for the study of reproductive disease in addition to providing a reliable means of generating systemic and clinical brucellosis that can be used to evaluate vaccine candidates.

3. INTRATRACHEAL INOCULATION WITH *BRUCELLA MELITENSIS* IN THE PREGNANT GUINEA PIG IS AN IMPROVED MODEL FOR REPRODUCTIVE PATHOGENESIS AND VACCINE STUDIES

3.1. Summary

Reproductive failure is the hallmark of brucellosis in animals. An uncommon but important complication in pregnant women who become acutely infected with *Brucella melitensis* is spontaneous pregnancy loss or vertical transmission to the fetus. Unfortunately, the mechanism behind reproductive failure is still obscure, partially due to the lack of a proper study model. Recently, it was demonstrated that intratracheal inoculation (IT) of non-pregnant guinea pigs would replicate features of clinical disease in humans. To determine if IT would induce reproductive disease, guinea pigs were infected at mid-gestation and monitored them daily for fever and abortions. Fever developed between day 14 to 18 post-inoculation and by 3-weeks post-inoculation, 75% of pregnant guinea pigs experienced stillbirths or spontaneous abortions mimicking natural disease. Next, to investigate the guinea pig as a model for evaluating vaccine efficacy during pregnancy, non-pregnant guinea pigs were vaccinated with S19, 16M*ΔvjbR* + Quil-A, or 100 µl PBS + Quil-A. Guinea pigs were bred and vaccinated guinea pigs were challenged at mid-gestation with *B. melitensis* IT and monitored for fever and abortions. Vaccination with both vaccines prevented fever and protected against abortion. Together, this study indicates that pregnant guinea pigs are an appropriate animal model to study reproductive disease and offer an improved model to

evaluate the ability of vaccine candidates to protect against a serious manifestation of disease.

3.2. Introduction

Brucellosis is one of the most commonly reported zoonotic diseases with a worldwide distribution (1). Of the 12 recognized species, *Brucella melitensis* is considered the most virulent and is associated with the majority of human cases (2). In its natural hosts of sheep and goats, *B. melitensis*, infection results in spontaneous mid-gestational abortion and placentitis (4). Disease transmission to humans occurs after ingestion of unpasteurized dairy products or exposure to infectious aerosols (4). The acute illness manifests with non-specific flu-like symptoms including undulant fever, malaise, and anorexia. Alarmingly, recent epidemiological evidence also indicates that reproductive disease occurs in women who become infected during pregnancy and can result in first or second term spontaneous pregnancy loss or transmission to the fetus (10, 78-80).

The pathogenesis of reproductive brucellosis in natural host species as well as in humans is a subject of considerable interest. Reproductive disease during pregnancy has been investigated in the natural host (small ruminants, cattle, and suids) as well as in laboratory animal models (mice, guinea pigs, nonhuman primates) (15, 16). Utilizing the natural hosts to study *Brucella* pathogenesis presents numerous challenges as they require biosafety level 3-agriculture facilities (BSL-3Ag) and are more expensive and time-consuming due to large size of the animal and greater length of gestation. As an alternative, mice are commonly utilized for studying host-pathogen interactions and for

investigating vaccine candidates (17). The mouse model has outpaced the use of other animal models such as guinea pigs or nonhuman primates due to the ease of housing large numbers of animals and the ready availability of reagents for evaluating the immune response to infection. The mouse presents several limitations as a model for human reproductive disease such as a difference in placentation and failure to abort regardless of dose or timing of inoculation with *Brucella* spp. instead exhibiting fetal resorptions when infected at day 4.5 of gestation (20, 63).

Guinea pigs were used extensively in the past for pathogenesis investigations and to develop and evaluate vaccines for *Brucella* spp. (9). As a reproductive model, advantages to the guinea pig include similar placentation to humans and a relatively longer length of gestation (~65 days). Furthermore, a study found that when pregnant guinea pigs were inoculated via intramuscular (IM) injection at mid-gestation with $1x10^5$ CFU *B. abortus* 544, they experienced stillbirths and spontaneous abortions (37). While these results are intriguing, IM inoculation represents an artificial route of exposure for brucellosis. Intratracheal inoculation (IT) simulates aerosol exposure and is a more natural route of infection. Using IT inoculation, we have previously demonstrated that non-pregnant guinea pigs develop fever and systemic disease when inoculated with *B. melitensis* (81). In this study, we built upon this foundation by using intratracheal inoculation of pregnant guinea pigs to determine the effect upon reproductive success and to evaluate the pregnant guinea pig as an improved animal model for vaccine efficacy and safety.

3.3. Materials & Methods

3.3.1. Bacterial strains. *B. melitensis* 16M (isolated from the lung of an aborted goat fetus), *B. abortus* S19 (National Veterinary Services Laboratory [NVSL], Ames, IA), and *B. melitensis* 16M*ΔvjbR* (engineered for previous study) were used in this study (68, 82). Bacteria were cultured on tryptic soy agar (TSA; Difco, Becton, Dickinson) at 37°C with 5% (vol/vol) CO_2 for 72h. *B. melitensis* 16M (WT) was harvested from plates with phosphate-buffered saline (PBS; Gibco) and diluted to a final concentration of $1x10^7$ CFU/50 µl using a Klett colorimeter and standard curve. S19 and 16M*ΔvjbR* were similarly grown on TSA, harvested with PBS, and diluted to a final concentration of $1x10^9$ CFU/100 µl. Final concentrations were retrospectively verified through serial dilution and plating onto TSA medium in duplicate.

3.3.2. Animal research ethics statement. All studies were performed with the approval of the Texas A&M University's Institutional Animal Care and Use Committee (protocol: 2018-0046). Texas A&M University is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

3.3.3. Kinetics of intratracheal inoculation. Twenty-two multiparous pregnant Hartley guinea pigs at approximately 20-25 days of gestation (week 3 of gestation) were obtained from Charles River (Wilmington, MA) and housed individually in microisolator cages at an animal biosafety level 3 (ABSL-3) facility at Texas A&M University for the duration of the studies. After an acclimation period, animals were divided into four groups (n=4) and were anesthetized intraperitoneally (i.p.) with a cocktail of ketamine (50 mg/kg) and xylazine (5mg/kg). Implantable subcutaneous

IPTT-300 microchip (BioMedic Data Systems, Seaford, DE) were placed to monitor body temperature. At 30-35 days of gestation, animals were then inoculated with $1x10^7$ CFU/50 µl *B. melitensis* 16M using the PennCentury MicroSprayer™ Aerosolizer (Wyndmoor, PA) as previously described (81). Briefly, a small animal laryngoscope was used to visualize the larynx and the blunt end of the MicroSprayer™ was inserted into the proximal trachea to deliver a dose of 50 µl.

Guinea pigs were evaluated daily for fever (body temperatures of ≥39.5°C) using a DAS-7000 reader (BioMedic Data Systems)(83) and were monitored twice daily for signs of reproductive failure such as vaginal discharge or abortion.

At 7-day intervals representing gestation weeks 5 to 8, groups of pregnant guinea pigs (n=4) were euthanized i.p. with sodium pentobarbital (100 mg/kg) followed by cardiac exsanguination. One gram of spleen, liver, lung, left and right mammary gland, superficial inguinal lymph node, uterus, and placenta were collected into pre-sterilized 2 mL collection tubes containing 1 ml PBS and 1.47 g of ceramic beads (Omni International, Kennesaw, GA). Tissues were homogenized using a Bead Ruptor Elite Bead Mill Homogenizer (Omni International) for 30 sec, serially diluted, and cultured on Farrell's media [TSA plus Oxoid™ *Brucella* selective supplement (ThermoFischer Scientific, Waltham, MA), equine serum, and 20% dextrose] at 37°C with 5% (vol/vol) CO_2 (81). After incubation for a minimum of 72h, colonies were counted to determine CFU/g.

To evaluate the effect of infection on fetal development, fetuses were weighed and crown to rump (C-R) length was measured (84). Vertical transmission of infection from

the dam to the offspring was evaluated by collecting liver, spleen, lung, and stomach contents from each fetus. Tissues were homogenized as described above, serially diluted, plated on Farrell's media, and incubated at 37°C with 5% (vol/vol) CO_2 for 72h. Colonies were counted to determine CFU/g.

3.3.4. Vaccination study. Seventeen female Hartley guinea pigs at 6-7 weeks of age (Charles River) and six 450-500g, male, Hartley guinea pigs (Charles River) were obtained and segregated by sex. Males were group housed at a BSL-1 animal facility prior to breeding. Female guinea pigs were individually housed in microisolator cages at a BSL-2 animal facility. Following an acclimation period of 5 days, female guinea pigs were randomly assigned to groups and were vaccinated by subcutaneous injection in the right inguinal region with 100 µl of $1x10^9$ CFU/100 µl *Brucella abortus* S19 (n=6) or *Brucella melitensis* 16M Δ*vjbR* + 10 µg Quil-A (n=6). Control animals were sham vaccinated with 100 µl sterile PBS + 10 µg Quil-A (n=5).

3.3.5. Breeding. Approximately 1-month after vaccination, female guinea pigs were segregated by vaccine group and co-housed with males in a BSL-2 level facility in open-bank cages for breeding purposes at a ratio of 3:1. After 2-weeks, females were evaluated daily by abdominal palpation to detect pregnancy (84). When guinea pigs were at approximately 30-35 days of gestation, they were moved to the BSL-3 facility, individually housed, and acclimated for a minimum of 2-days prior to challenge (Figure B1).

3.3.6. Challenge of vaccinated pregnant guinea pigs with $1x10^7$ CFU *B. melitensis* at 35-days of gestation. Pregnant guinea pigs were anesthetized as described above,

implanted with subcutaneous IPTT-300 microchip (BioMedic Data Systems), and challenged with $1x10^7$ CFU *B. melitensis* 16M via IT inoculation. Euthanasia was performed 28-days post-inoculation (p.i.) or at the time of parturition by sodium pentobarbital (100 mg/kg) followed by cardiac exsanguination. One gram of placenta, uterus, liver, spleen, lung, superficial inguinal lymph node, and mammary gland were collected for bacterial culture on Farrell's media as previously described. Fetal spleen, liver, lung, and stomach contents were collected for bacterial culture on Farrell's media.

3.3.7. Evaluation of histopathological changes. From the adult guinea pigs, placenta, liver, spleen, lung, and left and right mammary gland were collected at the study endpoints and fixed in 10% neutral buffered formalin (NBF;ThermoScientific) for a minimum of 48h. Fetal spleen, liver, lung, heart, kidney, reproductive tract, and umbilicus were collected from each fetus and fixed in 10% NBF. Tissues were then routinely processed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). Histologic changes of the spleen, liver, placenta, and mammary gland were scored for inflammation type and severity in a blinded fashion by a board-certified veterinary pathologist (Table B1).

3.3.8. Immunohistochemistry to detect *Brucella* antigen. Five micrometer tissue sections of placenta and mammary gland were adhered to positively charged glass slides for immunohistochemistry. Slides were routinely processed through a series of xylene and ethanol steps before antigen retrieval was performed using 1:10 EMS Solution A (Electron Microscopy Services, Hatfield, PA) in a 2100 Antigen Retriever (Aptum Biologics Ltd. Southampton, UK), according to manufacturer protocol. Endogenous

peroxidase and alkaline phosphatase were blocked by 10 m incubation with Bloxall Blocking Solution (Vector Laboratories, Burlingame, CA) followed by 20 m blocking with normal goat serum (Vector Laboratories). Primary incubation was performed overnight at 4° C with *Brucella* polyclonal rabbit antibody (Bioss Antibodies, location) at dilution of 1:800. As a negative control, tissue sections were incubated with rabbit nonimmune serum diluted in PBS. A Vectastain Elite® ABC HRP Kit (Vector Laboratories) with an avidin/biotinylated anti-rabbit secondary antibody was used according to the manufacturer's instructions. Antigen was visualized with a Betazoid DAB chromagen kit (Biocare Medical, Pachecho, CA). The slides were counterstained with Meyer's hematoxylin III.

3.3.9. Analysis of humoral immune response to infection by indirect ELISA. Blood samples were collected from pregnant guinea pigs at day 0, 14, and 28 p.i. For the vaccination trial, serum samples were collected at 0, 4-, 12-, 14-, and 16-weeks post-vaccination. iELISA for anti-*Brucella* specific IgG was performed with guinea pig sera as previously described (81). Briefly, Nunc MaxiSorp 96 well plates (ThermoFischer) were coated with 25 ng/well *B. abortus* 2308 heat-killed lysate and held overnight at 4°C. Plates were washed three times and blocked with 3% skim milk for 2 h at room temperature. Guinea pig sera were diluted to a concentration of 1:1000, and 100 µl was added to plates and incubated at 37°C for 1h. Plates were washed and goat anti-guinea pig IgG (H+L) (KPL, Seracare, Milford, MA) was added at a concentration of 1:2000 and incubated at 37°C for 1h. To detect peroxidase activity, SigmaFast OPD peroxidase substrate (Sigma-Aldrich, St. Louis, MO) was incubated for 30 m at 37°C, and

absorbance was measured at 450nm. Assays were performed in triplicate and results are presented as the mean value of three replicates.

3.3.10. Statistical analysis. Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons to evaluate the kinetics of infection. To determine vaccine efficacy, multiple comparisons between vaccinated and unvaccinated pregnant guinea pigs was performed using 2-way ANOVA followed by Tukey's multiple comparisons. All tests were performed by using GraphPad Prism v6 (GraphPad Software, San Diego, CA).

3.4. Results

3.4.1. Intratracheal inoculation results in systemic infection. A previous study demonstrated that non-pregnant guinea pigs will develop fever, splenomegaly, and systemic colonization following IT inoculation with $1x10^7$ CFU *B. melitensis* (81). However, the response of the pregnant guinea pig to infection with *Brucella* spp. has not been well established. To determine if IT inoculation of pregnant guinea pigs would result in similar clinical signs and systemic colonization, guinea pigs at mid-gestation (~30-35 days) were inoculated with 50 µl of $1x10^7$ CFU *B. melitensis* 16M or 50 µl PBS. Mid-gestation was selected as the optimal time point to inoculate similar to natural hosts infected during this period of infection induces the highest rate of abortions and stillbirths (58). Following inoculation, guinea pigs were euthanized at weekly intervals to determine the kinetics of infection during pregnancy.

An important clinical marker of brucellosis in humans is fever. Non-pregnant female guinea pigs develop fever, defined as temperature ≥39.5° C, approximately 14-18

days following IT inoculation with $1x10^7$ CFU (81, 83) . As expected, the mean body temperature was increased in pregnant guinea pigs inoculated with *B. melitensis* compared to those receiving PBS (Fig. 3.1 Panel A) The mean temperature of the guinea pigs inoculated with *B. melitensis* was statistically increased at 8-weeks of gestation compared to PBS controls and to guinea pigs at 5- and 7-weeks of gestation (Fig. 3.1 Panel B). Development of fever in the pregnant guinea pig correlates with the time frame of fever development in non-pregnant guinea pigs and in human cases (6, 81).

Splenomegaly or spleen enlargement is another common finding in human and animal cases of brucellosis. Furthermore, non-pregnant guinea pigs develop splenic enlargement following IT inoculation. The spleens of pregnant guinea pigs were weighed at the time of euthanasia to determine if infection affected weight. Spleen weight was not affected at 5- or 6-w.g. However, infection with *B. melitensis* significantly increased spleen weight at 7 to 8-w.g., as seen in non-pregnant guinea pigs ($P<0.001$, Fig. 3.1 Panel C).

Brucella spp. have a well-defined tropism for organs of the reticuloendothelial organs such as the spleen, liver, and lung in experimental animal models and natural hosts. To determine if IT inoculation resulted in colonization of systemic organs, spleen, liver, and lung were evaluated by culture on Farrell's media. By 1-week p.i./5-w.g., 3 of 4 pregnant guinea pigs (75%) had colonization of at least one of tissue (Fig. 3.1 Panels D-F). By 2-weeks p.i./6-w.g., colonization of the spleen, liver, and lung was detected in 100% of the pregnant guinea pigs (n=4), and mean colonization was statistically increased ($P<0.0001$) from 2 to 4-weeks p.i./6-8-w.g. (Fig. 3.1 Panels D-F). This pattern

was similar to that seen in non-pregnant female guinea pigs in which bacteria colonized systemic organs by 2-weeks p.i. and persisted for 4-weeks p.i. (81).

To corroborate colonization findings, spleen, liver, and lung were collected for histopathology. Slides were graded in a blinded fashion for inflammation type and severity as previously described (Appendix Table B1)(81). By 1-week p.i., histopathologic changes in the lung were confined to mild pulmonary edema (Figure B2). Lung lesions from week 6 of gestation to week 8 consisted of an embolic pattern of aggregates of neutrophils and macrophages in the alveoli and bronchioles, which suggests the guinea pigs developed bacteremia. Histopathologic findings in the spleen were similar to those seen in non-pregnant female guinea pigs and included lymphoid hyperplasia during the early stages of infection that progressed to foci of macrophages by 3 and 4-weeks p.i./7-8-w.g. (Figure B2). The most significant pathology in the liver occurred in the 3 and 4-weeks p.i./7-8-w.g. groups and included lesions commonly associated with brucellosis in people such as microgranulomas, hepatic necrosis, and lymphocytic periportal inflammation (Figure B2). Taken together these results indicate that pregnant guinea pigs develop histopathological changes typically observed in natural cases of *Brucella* infection.

3.4.2. Infection of pregnant guinea pigs with *B. melitensis* results in spontaneous abortion, placental colonization and inflammation, and vertical transmission. Reproductive disease is a common sequela of infection with *B. melitensis* during pregnancy in natural hosts, but adverse pregnancy events may also occur in pregnant women (11). In order to study the pathogenesis of reproductive disease, an

animal model is needed that has similar placentation and replicates key features of the disease such as abortion and fever. To determine if pregnant guinea pigs would offer an improved animal model for reproductive brucellosis, guinea pigs (n=4) at mid-gestation (~4 weeks) were infected with 50 µl of $1x10^7$ CFU *B. melitensis* 16M or 50 µl PBS via intratracheal inoculation. To determine if placental and uterine colonization correlated with the development of clinical signs, placenta and uterus were cultured on Farrell's media. No colonization was noted in the PBS group or at 5-w.g., and only 1 pregnant guinea pig in the 6-w.g. group had a low level of colonization (2.6 logs) of the placenta (Fig. 3.2 Panel A), which correlated with the lack of clinical symptoms seen in these groups. However, by 6-w.g., 3 of 4 (75%) pregnant guinea pigs had colonization of the placenta, but the level of colonization was highly variable with a range of 1 to 9.5 logs. By 8-w.g., placenta of 4 of 4 (100%) pregnant guinea pigs were colonized. This data suggests that it takes approximately 3-weeks in the pregnant guinea pig to establish an infection severe enough to result in spontaneous abortion and stillborn offspring.

Guinea pigs were then monitored twice daily for adverse pregnancy events defined as hemorrhagic vaginal discharge or abortions. Since infection can also result in stillborn offspring, crown to rump length was measured to estimate the stage of gestation for each fetus to determine if *in utero* fetal death had occurred. No abortions or stillborn offspring were noted in the negative controls or at 5- or 6-w.g. (Table 3.1). However, by 7-w.g., 1 of 4 pregnant guinea pigs (25%) had bloody vaginal discharge on day 21 p.i., and 3 of 17 (17.6%) offspring were stillborn (Fig. 3.2 Panel C). Within the 8-w.g. cohort, 1 of 5 (20%) guinea pigs spontaneously aborted at 24-days p.i. and in total, 6 of

20 (30%) offspring were stillborn (Table 1). These results indicate that IT inoculation of pregnant guinea pigs with *B. melitensis* generates adverse obstetric outcomes in guinea pigs.

In the natural host, *Brucella* spp. infection results in colonization of the placenta and also generates a severe necrotizing lesion that precedes reproductive failure. To further characterize the effect of infection on the placenta in the pregnant guinea pig, samples were graded on a histologic scale (0-4) for type and degree of inflammation, necrosis, and edema (Appendix Table 1) and were stained with a polyclonal *Brucella*-specific antibody to confirm *Brucella* antigen within areas of inflammation. Beginning at 6-w.g., small foci of neutrophils and histiocytes were scattered throughout the labyrinth layer of the placenta, which is the site of fetal-maternal blood exchange (84). IHC revealed intracellular antigen within the chorioallantoic epithelium (Fig. 3.2 Panel D) suggesting the earliest infection may be of the cytotrophoblasts, which are derived from the maternal side of the placenta (85). Inflammation became more severe and widely distributed by 7 and 8-w.g., and the character of the lesion shifted from small infiltrates to multifocal aggregates of histiocytes and foci of necrosis in the labyrinth. IHC with a *Brucella*-specific antibody confirmed the etiology of these lesions, which demonstrated abundant intracellular antigen within inflammatory foci centered on the site of fetal/maternal blood exchange (Fig. 3.2 Panel D). Since necrotizing placentitis is the most common histologic placental lesion in ruminant cases of brucellosis, these results indicate that the pregnant guinea pig replicates a key feature of disease.

Finally, to characterize the effect of maternal infection on fetal colonization (vertical transmission), tissues (spleen, liver, lung, and stomach contents) were collected from each fetus. Fetuses in the negative control groups and at 5-w.g. had appropriate C-R length indicating that early fetal death did not occur, and no bacteria were recovered from any tissue (Table 3.1). Even though all of the fetuses had appropriate C-R length in the 6-w.g. group, colonization of the liver and lung was detected in 4 of 17 (23.5%) indicating that bacteria are capable of crossing the placental barrier and infecting the fetuses by 2-weeks p.i./5w.g. (Table 3.1). Interestingly, although only 17.6% of fetuses appeared stillborn by 3-weeks p.i., colonization occurred in 11 of 17 fetuses (64.7%) suggesting that infection of the fetus does not necessarily result in fetal death. Vertical transmission occurred in 6 of 20 (30%) fetuses from pregnant guinea pigs at 8-w.g. Tissue culture revealed that lung is the best tissue for confirming colonization of the fetus and further indicate that pregnant guinea pigs develop disease with similar clinical manifestations as natural hosts and humans.

3.4.3. Intratracheal inoculation results in colonization and inflammation of the mammary gland. Colonization of the mammary gland is an important means of transmission of disease from infected ruminants to humans because *Brucella* spp. are shed in the milk (58, 59). It is unknown if this aspect of infection would be replicated in a pregnant guinea pig model, so left and right mammary gland were collected for culture on Farrell's media following IT inoculation with 50 µl of $1x10^7$ CFU *B. melitensis* 16M. Histopathology and IHC were performed to correlate colonization with microscopic evidence of infection.

A low level of colonization (1 log) of the mammary gland was detected 1-week p.i. in 1 of 4 pregnant guinea pigs (25%)(Fig. 3.3 Panels A and B), which was associated with a minimal infiltrate of neutrophils (Fig. 3.3 Panel C). No antigen was detected by IHC 5-w.g. By 6-w.g. colonization was detected in 4 of 4 (100%) pregnant guinea pigs in at least one half of the mammary gland. Histologically, this was accompanied by a mild to moderate interstitial infiltrate of neutrophils and macrophages at 6-w.g., and *Brucella* antigen was detected by IHC within the cytoplasm of macrophages in the interstitium and in glandular epithelial cells (Fig. 3.3 Panel C). By 3-weeks p.i., the inflammatory population had shifted towards one reflective of chronic inflammation including macrophages, lymphocytes, and plasma cells. Again, *Brucella* antigen was detected within both macrophages and glandular epithelium (Fig. 3.3 Panel C). At 8-w.g., aggregates of macrophages were multifocally scattered throughout the interstitium and replaced mammary acini (Fig. 3.3 Panel C). These results indicate that intratracheal inoculation results in disseminated infection and could lead to shedding in the milk. This data further supports that the pregnant guinea pig is an appropriate animal model to study all aspects of *Brucella*-induced disease.

3.4.4. Infection with *B. melitensis* stimulates a *Brucella*-specific IgG humoral response. Humans and animals infected with *Brucella* spp. will develop a *Brucella*-specific antibody response. Previous data indicates that non-pregnant guinea pigs develop an IgG response to challenge with *B. melitensis.* iELISA was used to evaluate the kinetics of *Brucella*-specific IgG with guinea pig sera at 0, 7, 14, 21, and 28-days p.i. Infection with *Brucella melitensis* resulted in a statistically significant increase in IgG

level beginning 6-w.g. ($P<0.0001$) and continuing through 8-w.g. ($P<0.0001$) (Figure B3).

3.4.5. Vaccination with S19 and 16MΔ*vjbR* + Quil-A prevented fever and adverse pregnancy events. After demonstrating that pregnant guinea pigs develop clinical disease when infected with *B. melitensis* via IT inoculation, the pregnant guinea pig was next evaluated as an animal model for vaccine safety and efficacy. To do so, the protocol depicted in Figure B1 and described in the methods section was developed. Non-pregnant guinea pigs were vaccinated with $1x10^9$ CFU/100 µl S19 to simulate the vaccination dose and schedule used in domestic animals in which non-pregnant female cattle are vaccinated with $3x10^8$ to $5x10^9$ S19 organisms subcutaneously (58). *B. abortus* S19 is widely used to vaccinate cattle against brucellosis and is the reference strain by which other vaccines are measured (58). 16MΔ*vjbR* has been extensively evaluated by our laboratory in the mouse model where it has proven safe and efficacious (63, 82). However, the vaccine has not been evaluated in pregnant mice, and therefore efficacy in the pregnant guinea pig was evaluated to further demonstrate the utility of the pregnant guinea pig as a model.

Pregnancy was confirmed by abdominal palpation, and pregnant guinea pigs at approximately 35 d/4-weeks of gestation (range: 35-40 d) were challenged via IT inoculation with 50 µl of $1x10^7$ CFU *B. melitensis* 16M (84). The estimated gestation range at the time of challenge was calculated by measuring the C-R length of the offspring, comparing it to an established chart of C-R length for fetal guinea pigs, and subtracting from 62, which is the average length of gestation for the guinea pig (84).

Even though guinea pigs were vaccinated with *B. abortus* S19, animals were challenged with *B. melitensis* because the effects of this strain on pregnancy had been established and previous studies have demonstrated cross-protection amongst strains in guinea pigs and cattle (55, 86). Clinically, protection was defined as absence of fever and adverse pregnancy events including stillbirths or abortions.

As expected, pregnant guinea pigs sham vaccinated with PBS + Quil-A had a higher mean daily temperature than vaccinated guinea pigs (Fig. 3.4 Panel A). Intriguingly, none of the pregnant guinea pigs vaccinated with S19 or the 16MΔ*vjbR* + 10 μg Quil-A groups developed fever and had a lower mean daily temperature compared to 16M inoculated guinea pigs indicating vaccination protected against one of the key clinical features of disease (Fig. 3.4 Panel A).

Vaccine efficacy was evaluated by determining if vaccination prevented or reduced colonization in tissues. Spleen, liver, lung, and left and right mammary gland were collected at the time of parturition or 28-days p.i. to evaluate colonization and histopathologic changes. As the kinetics study indicated pregnant guinea pigs would develop colonization within 28-days p.i., animals in the vaccine efficacy study were euthanized at this time point to evaluate protection against systemic infection. Spleen weight was also assessed because it was expected that vaccination would reduce splenomegaly following challenge with 16M.

Spleen weight was significantly decreased in guinea pigs previously vaccinated with S19 ($P<0.001$) and 16MΔ*vjbR* ($P<0.01$) compared to unvaccinated controls following challenge with WT (Fig. 3.4 Panel B). When colonization of organs was

evaluated following challenge, guinea pigs that were previously vaccinated with S19 had a statistically significant reduction in mean colonization (Fig. 3.4 Panels C-E) of the spleen ($P<0.001$), liver ($P<0.001$), and lung ($P<0.0001$). In 4 of 6 (66.7%) animals, S19 vaccination reduced colonization to below the limit of detection (10 CFU/g tissue) following challenge. Vaccination with 16MΔ*vjbR* did not result in a significant decrease in colonization in the majority of tissues compared to unvaccinated controls, with the exception that vaccination decreased colonization of the lung ($P<0.05$) (Fig. 3.4 Panel E).

When the spleen was examined by light microscopy, S19 vaccinated animals had mild lymphoid hyperplasia indicating immune stimulation but not active inflammation. However, the spleen of 16MΔ*vjbR* vaccinated guinea pigs had microscopic lesions which resembled those seen in unvaccinated controls suggesting vaccination did not prevent a low level of infection from occurring after challenge with WT. When a histologic grading scale was applied, the 16MΔ*vjbR* vaccinated group had an intermediate degree of inflammation (mean=0.86) compared to S19 vaccinated (mean=0.2) and unvaccinated controls (mean=1.39).

After demonstrating that pregnant guinea pigs develop colonization of the mammary tissues following IT inoculation with *B. melitensis*, efficacy was evaluated by determining if vaccination would prevent or decrease colonization following challenge. S19 resulted in a significant decrease in mean colonization of the left mammary gland ($P<0.01$) and right mammary gland ($P<0.001$). Guinea pigs vaccinated with 16MΔ*vjbR*

had a more modest decrease in colonization of the left mammary gland ($P<0.05$) and no significant difference in the right (Fig. 3.4 Panels F-G).

Adverse pregnancy events such as abortion and stillbirths secondary to infection with *B. melitensis* are a common disease manifestation, and a primary goal of vaccination is to prevent the development of reproductive disease (4, 6). As expected, spontaneous abortion and/or stillbirths were noted in three of the unvaccinated guinea pigs (60%), but neither vaccinated group had abortions or stillbirths. Adverse pregnancy events were associated with a mean colonization of 5.99 logs in the unvaccinated guinea pigs (Fig. 3.5 Panel A). Even though the vaccines protected against the development of clinical signs, neither vaccine was 100% effective at preventing colonization of the uterus and placenta. However, S19 resulted in statistically significant reduction in mean colonization of the uterus ($P<0.01$) and placenta ($P<0.05$) compared to unvaccinated guinea pigs following challenge (Fig. 3.5 Panels A-B). As noted previously, colonization of the placenta predisposes the animal to pregnancy loss. Thus, vaccination with S19 and 16MΔ*vjbR* likely prevented the development of placental insufficiency that leads to pregnancy loss. When the placenta was evaluated by light microscopy and a histologic grading scale was applied, S19 had a lower mean histologic score (0.24) than 16MΔ*vjbR* (0.55) or unvaccinated (1.82) (Fig. 3.5 Panel C). S19 vaccinated guinea pigs had rare infiltrates of macrophages within the labyrinth, but 16MΔ*vjbR* and unvaccinated guinea pigs had more extensive areas of inflammation following challenge with WT (Fig. 3.5 Panel E).

To monitor the immune response to vaccination, *Brucella*-specific IgG antibody response was measured by analyzing serum samples from pre-vaccination to 16-weeks post-vaccination. Both S19 and 16MΔ*vjbR* had a statistically significant ($P<0.001$) IgG response as early as 4-weeks post-vaccination (Figure B4). In response to challenge with $1x10^7$ CFU *B. melitensis*, the memory response in both vaccines was rapid and statistically increased compared to unvaccinated animals that were challenged. Interestingly, even though S19 resulted in protection below the limit of detection in 66.7% of pregnant guinea pigs, the IgG response was significantly less than that provided by 16MΔ*vjbR* indicating IgG response is inadequate to predict protection.

Table 3.1. Crown to rump length, number of offspring, fetal viability, and colonization of fetuses from pregnant guinea pigs infected with $1x10^7$ CFU *B. melitensis* IT.

CR length was measured from the base of the skull to the point of the hip in cm and is reported as average (avg.) length in centimeters (cm). Fetal viability reported as number (no.) and percentage born stillborn. Fetal spleen, liver, lung, and stomach contents were cultured on Farrell's media and results are reported as avg. $\log_{10}$/g. Maternal infection variably resulted in vertical transmission to the fetus. Results of culture of stomach contents reported as number and percentage with growth.

Week of gestation	Avg. C-R length (cm)	No. of offspring	Stillborn No. (%)	Spleen (Avg. $\log_{10}$/g)	Liver (Avg. $\log_{10}$/g)	Lung (Avg. $\log_{10}$/g)	Stomach contents No. (%)
Week 5	40.8	24	0/24 (0)	0	0	0	0/24 (0)
Week 6	46.8	21	0/21 (0)	0.02	0.14	0.13	0/21 (0)
Week 7	59.9	17	3/17 (17.6)	0.93	1.13	0.93	6/17 (35.3)
Week 8	50.8	20	6/20 (30)	0.57	0.86	0.40	2/20 (10)

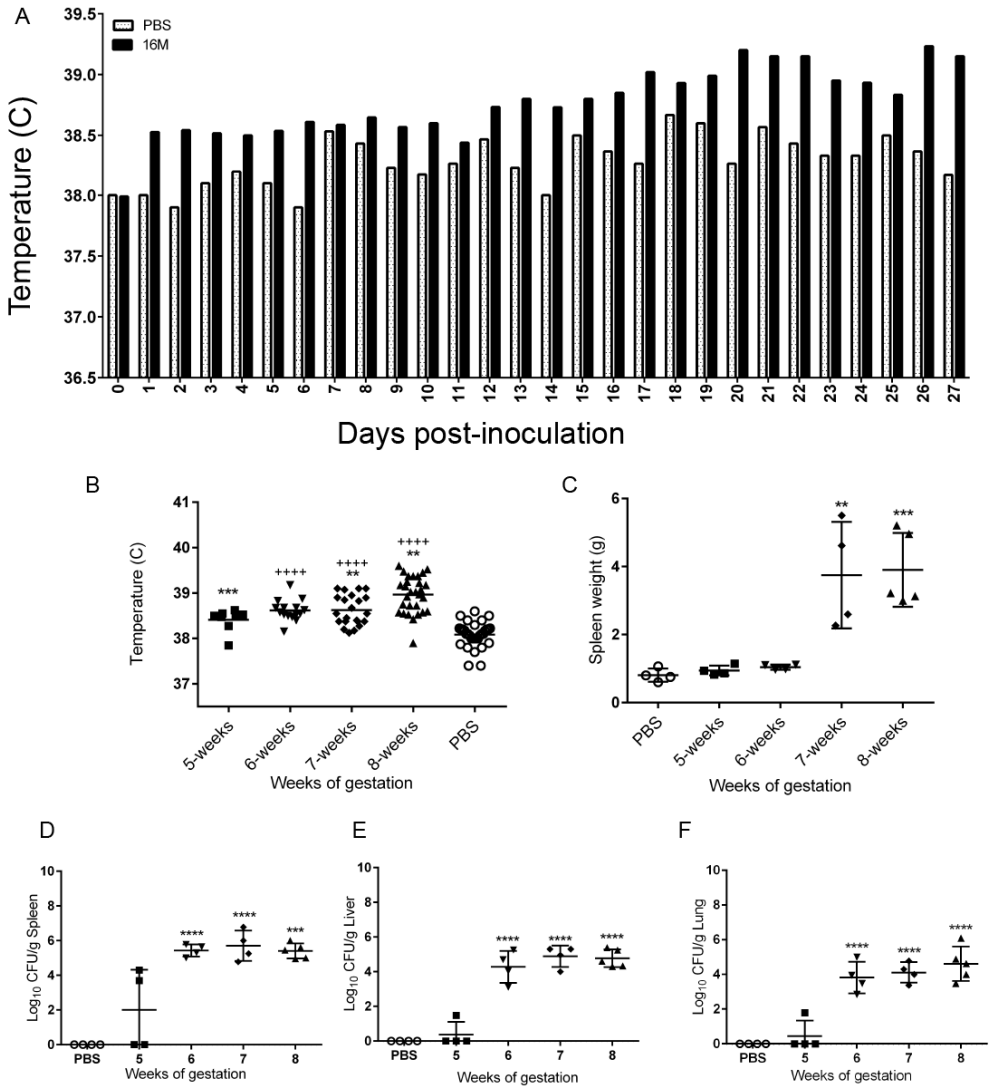
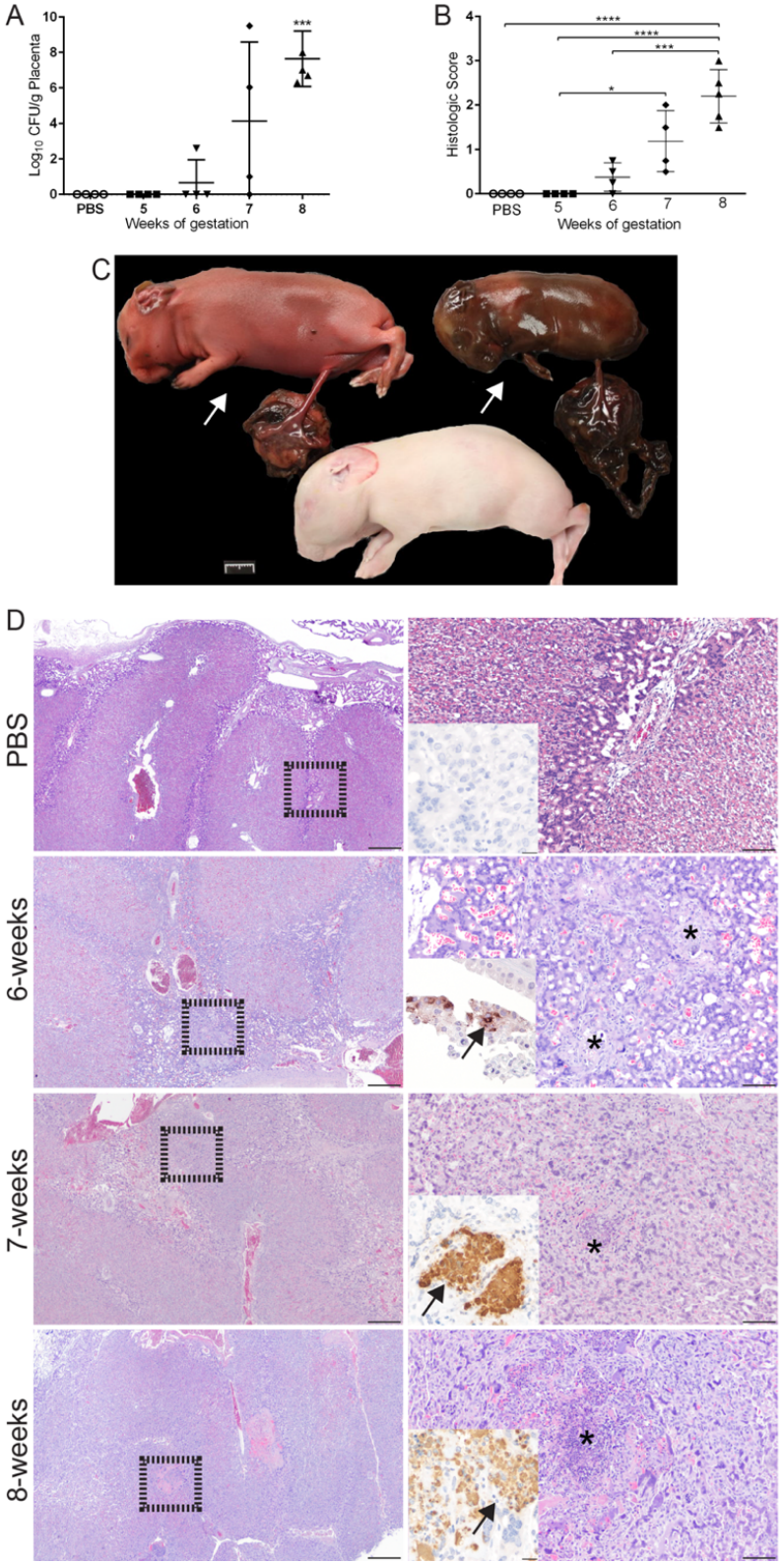


Figure 3.1. *Brucella* infection results in a higher mean temperature, splenomegaly, and systemic colonization.

(A) Kinetics of temperature following intratracheal inoculation with $1x10^7$ CFU *B. melitensis* 16M. Implantable subcutaneous microchips were monitored daily using a handheld DAS-7000 reader. Mean daily temperature was increased in the guinea pigs inoculated with *B. melitensis* compared to PBS controls. (B) Mean temperature was compared between groups using ANOVA followed by Dunnett's multiple comparisons. ++++, P<0.0001 compared to 8-weeks of gestion. **P<0.01, ***P<0.001, compared to PBS controls. (C) Spleen weight was compared between groups using ANOVA. **P<0.01, ***P<0.001 compared to PBS controls. Bacterial colonization from pregnant guinea pigs at 5-,6-,7-, and 8-weeks of gestation following intratracheal inoculation with $1x10^7$ CFU *B. melitensis*. Spleen (D), liver (E), and lung (F) were collected at each time point for bacterial culture on Farrell's media. The recovery of organisms is plotted as the total CFU/g (means ± standard deviation). Mean recovery per gram of tissue was compared between times points and uninfected control guinea pigs. Statistical significance was determined by ANOVA followed by Dunnett's multiple comparisons. Three asterisks, P <0.001. Four asterisks, P <0.0001.

Figure 3.2. Level of placental colonization correlates with clinical signs and severity of histologic lesions.

(A) A representative placenta sample from each pregnant guinea pig (n=4) per time point was cultured on Farrell's media. The recovery of organisms is plotted as the total CFU/g (mean ± s.d.). Mean recovery per gram of tissue was compared between times points and uninfected control guinea pigs. Statistical significance was determined by ANOVA followed by Dunnett's multiple comparisons. * P <0.05. ***, P <0.001. (B) Histologic scores from placenta. H&E stained sections of placenta were graded in a blinded fashion for edema (0-1), mononuclear infiltrate (0-4), necrosis (0-4), and bacteria (0-1). Values represent the means histological score per group (n=4). Values that are significantly different are indicated by bars and asterisks. *** P<0.001, **** P<0.0001 (C) Representative gross image of discolored and desiccated stillborn fetuses (white arrows) with inappropriate C-R length and necrosis of the subplacenta from a pregnant guinea pig 3-weeks post-infection. Note the apparently normal fetus from the same pregnancy. Marker = 1cm. (D) Representative sections of H&E-stained and IHC-labeled placenta at the time of abortion or study endpoints. Note the abundant intracytoplasmic staining in the chorionic epithelium at 6-weeks of gestation (arrow) indicating maternal infection precedes fetal infection. By 7-weeks of gestation, multifocal areas of necrosis (asterisk) with abundant intracytoplasmic *Brucella* antigen scattered throughout the labyrinth of the placenta (inset, arrow). Lesions become more numerous and larger by 8-weeks of gestation (asterisk). Left column H&E, Magnification 2x; Bar= 500 μm. Dashed box indicates field selected for higher magnification in the left column. Right column H&E, Magnification 10x; Bar = 50 μm. Inset, IHC Magnification 40x; Bar = 25 μm.


A
Log10 CFU/g Placenta
PBS
5
6
7
8
Weeks of gestation
***
B
Histologic Score
****
****
***
*
PBS
5
6
7
8
Weeks of gestation
C
D
PBS
6-weeks
7-weeks
8-weeks

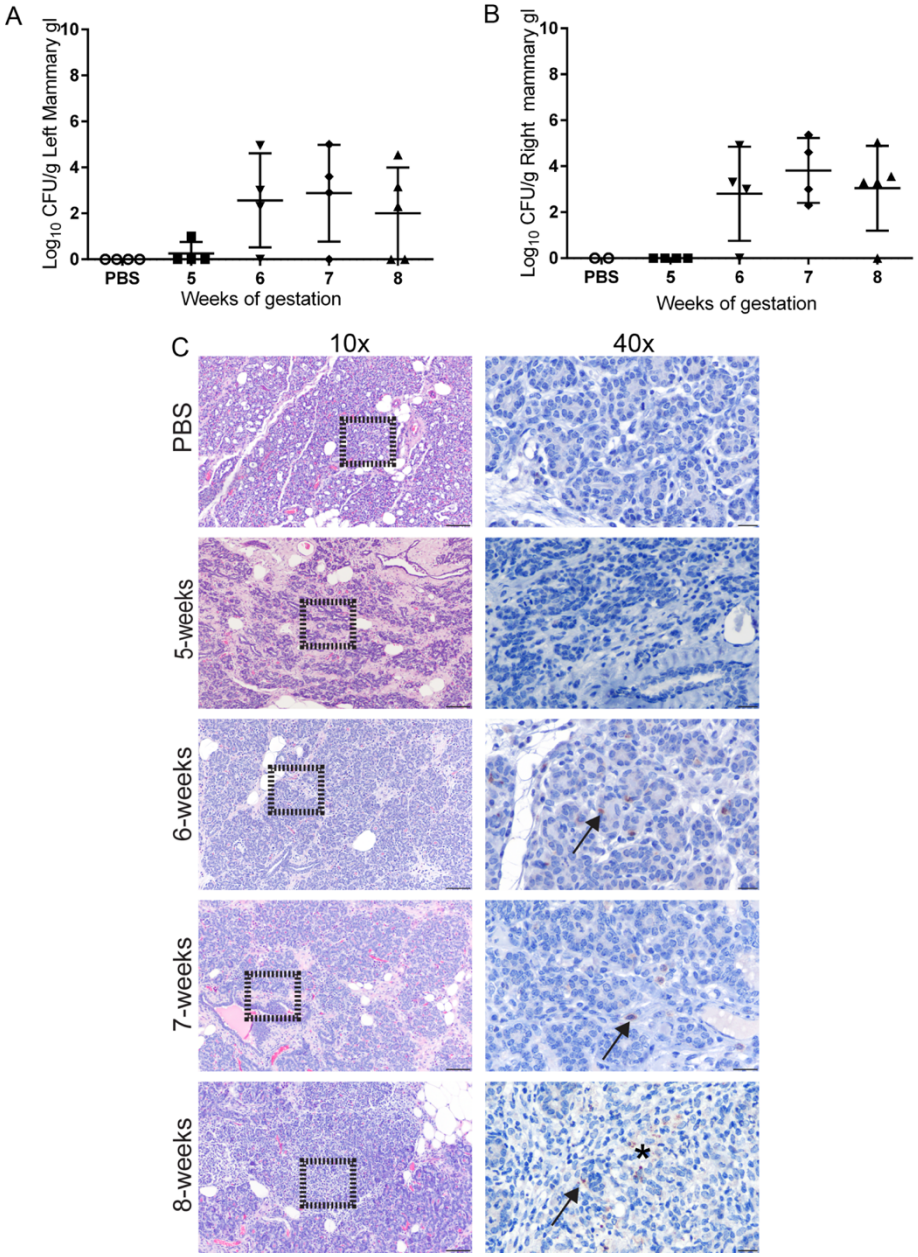


Figure 3.3. Colonization of the mammary gland results in inflammation.
Colonization of the left (A) and right (B) mammary gland following IT inoculation with $1x10^7$ CFU *B. melitensis*. The recovery of organisms is plotted as the total CFU/g (mean ± s.d.). Mean recovery per gram of tissue was compared between times points and uninfected control guinea pigs. Differences between time points and negative controls are not statistically significant. Representative H&E and IHC images of mammary gland (C) at each time-point post-inoculation. Left column, H&E, Magnification 10x; Bar = 50 µm. Right column, Higher magnification of area delineated by black outline at 10x. Multifocal macrophages and epithelial cells contain granular intracellular (arrows) and extracellular (*) *Brucella* antigen. IHC with polyclonal anti-*Brucella* antibody, Magnification 40x; Bar = 25 µm.

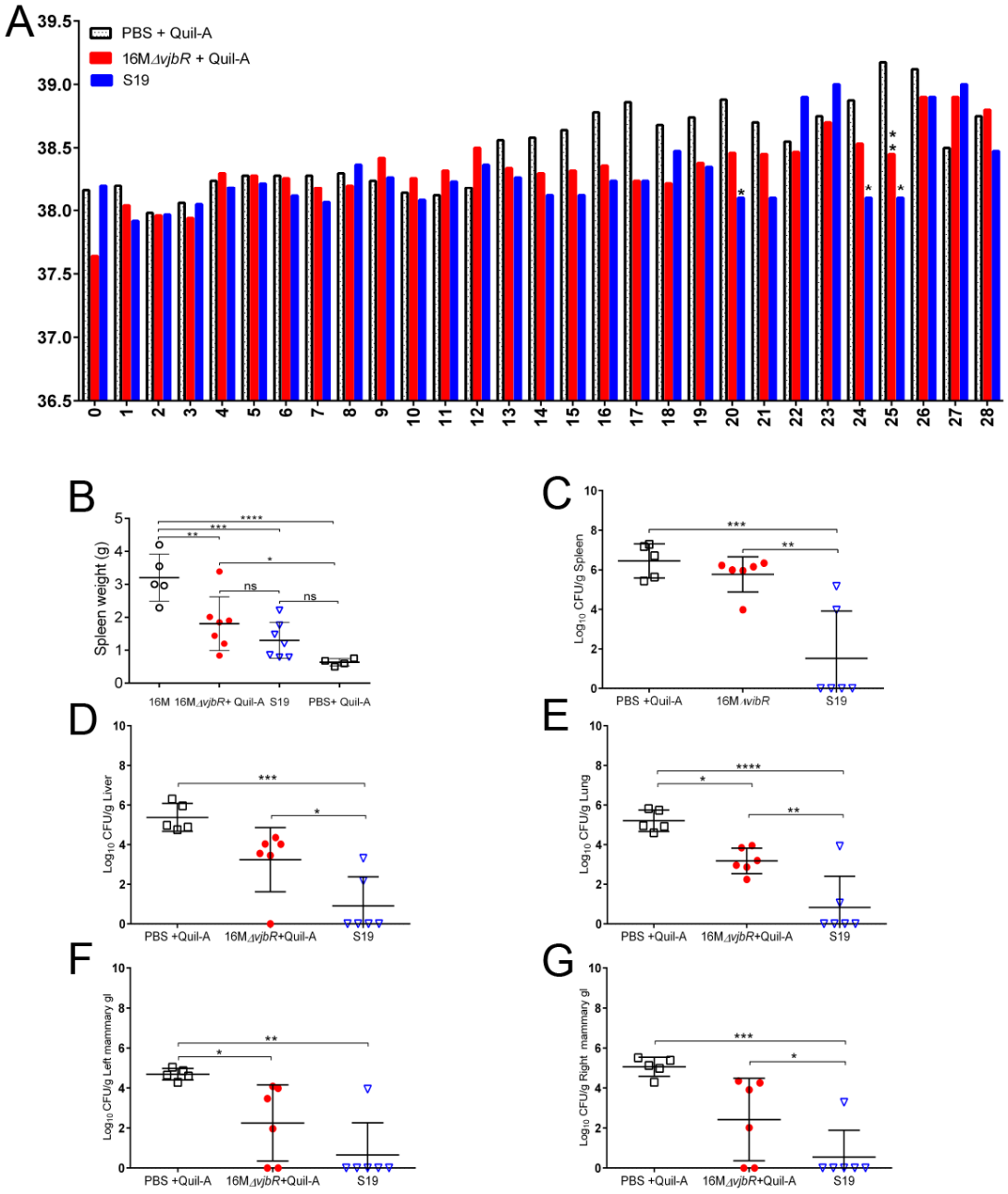
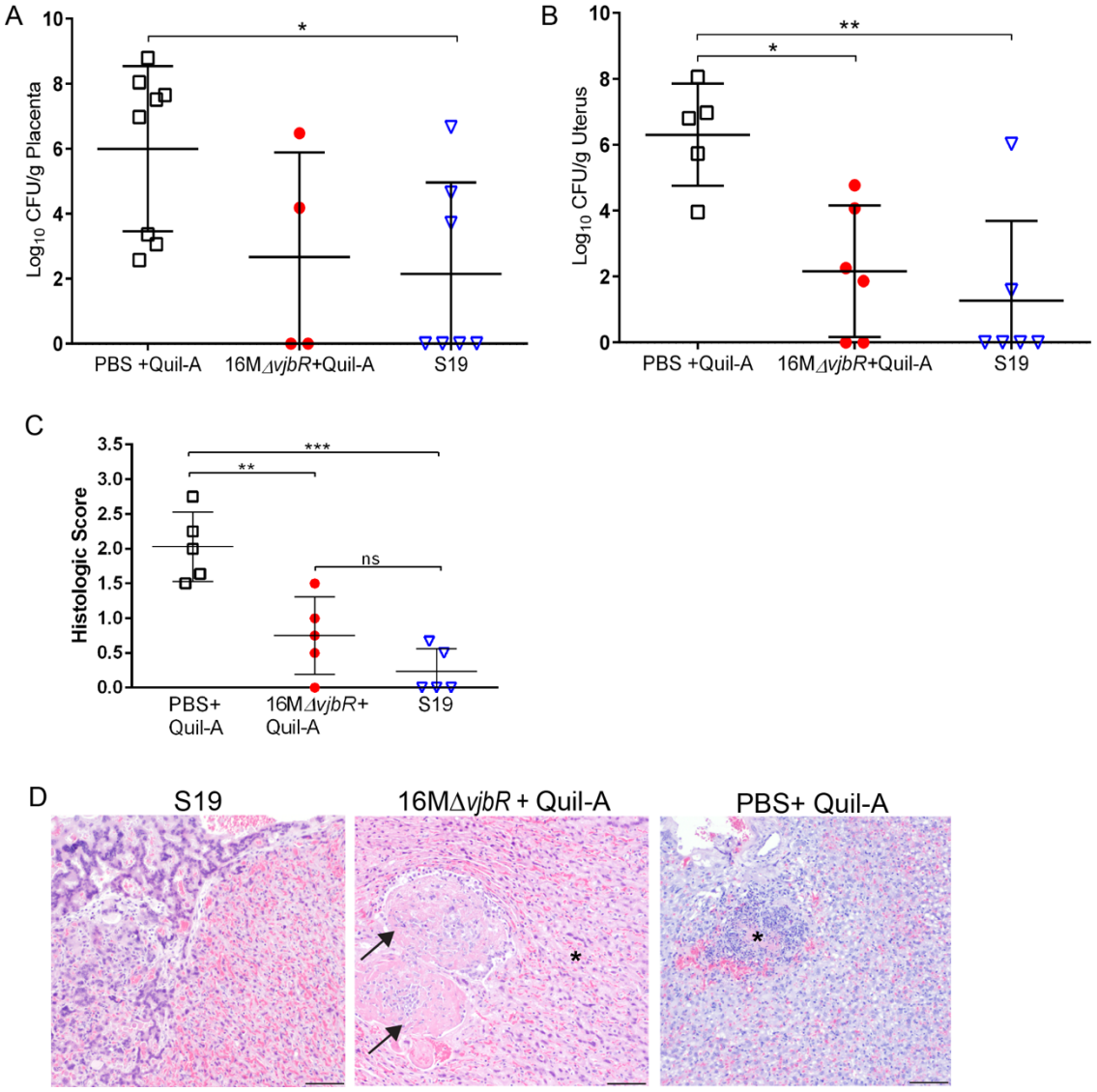


Figure 3.4. Vaccination prevents fever and reduces colonization of the systemic tissues.

(A)Vaccination with 16MΔ*vjbR* and S19 decreased the mean daily temperature following IT challenge with 1x10^7 CFU *B. melitensis*. (B) To evaluate splenomegaly, spleen was collected at necropsy and weighed. Mean spleen weight was decreased in vaccinated groups compared to 16M challenged guinea pigs. Spleen (C), liver (D), lung (E), left mammary gland (F), and right mammary gland (G) were assessed for colonization by culturing on Farrell's media and is plotted as the total CFU/g (mean ± s.d.). Statistical significance was determined by ANOVA followed by Tukey's multiple comparisons. * P <0.05. **, P <0.01. ***, P <0.001. ****, P <0.0001.

Figure 3.5. Vaccination reduces colonization and inflammation of the placenta following challenge with *B. melitensis*.

Colonization of the placenta (A) and uterus (B) in pregnant guinea pigs previously vaccinated with either $1x10^9$ CFU/100 µl *Brucella abortus* S19 (n=6), *Brucella melitensis* 16M Δ*vjbR* + 10 µg Quil-A (n=6), or sham vaccinated with 100 µl sterile PBS + 10 µg Quil-A (n=5). Twelve-weeks post-vaccination and at mid-gestation (~35-40 days of gestation), pregnant guinea pigs were challenged by IT inoculation with $1x10^7$ CFU *B. melitensis*. The recovery of organisms is plotted as the total CFU/g (mean ± s.d.). Mean recovery per gram of tissue was compared between vaccinated and unvaccinated guinea pigs using ANOVA followed by Dunnett's multiple comparisons. * $P < 0.05$. **, $P < 0.01$. (C) H&E stained sections of placenta were graded in a blinded fashion for edema (0-1), mononuclear infiltrate (0-4), necrosis (0-4), and bacteria (0-1). Values represent the means histological score per group (n=5). Values that are significantly different are indicated by bars and asterisks. ** P<0.01, *** P<0.001, ns= not significant (D) Representative images of H&E stained placenta from each group. No lesions were noted in the S19 vaccinated guinea pigs. Areas of coagulative necrosis (asterisk) were noted adjacent to thrombi (arrow) in 16M Δ*vjbR* + Quil-A vaccinated guinea pigs. PBS + Quil-A inoculated guinea pigs developed foci of necrosis (asterisk) following challenge with 16M. Magnification 10x; Bar = 50 µm.


A
Log10 CFU/g Placenta
PBS +Quil-A
16MΔvjbR+Quil-A
S19
B
Log10 CFU/g Uterus
C
Histologic Score
PBS+ Quil-A
16MΔvjbR+ Quil-A
ns
D
S19
16MΔvjbR + Quil-A
PBS+ Quil-A

3.5. Discussion

Reproductive disease is an important consequence of infection with *Brucella* spp., but the pathogenesis is not fully understood. Infection in pregnant guinea pigs was evaluated because guinea pigs have similar placentation to humans and have been used successfully to model other bacterial reproductive pathogens such as *Treponema pallidum* (syphilis), *Chlamydia trachomatis*, and *Listeria monocytogenes* (87). Despite the apparent relevance of the pregnant guinea pig, the pregnant mouse model is more commonly utilized to investigate events underlying reproductive failure. When pregnant mice are inoculated at day 4.5 of gestation with WT or live attenuated strains (LAV), fetal death and resorption will occur (20, 88, 89). However, the mouse model does have some limitations including differences in placentation, degree of trophoblast invasion, and a short gestation that may make it less suitable for fully exploring the reproductive pathogenesis of *Brucella* spp. (35). This study demonstrates that pregnant guinea pigs are a more physiologically relevant model because they develop fever, abortions, and stillbirths when infected with *B. melitensis,* as seen in humans and natural hosts. Importantly, because 75-80% of pregnant guinea pigs develop fever in response to infection, body temperature can be used as an indicator of vaccine safety and efficacy in future studies.

Infectious aerosols are a known transmission route for brucellosis in both humans and animals, so IT inoculation was evaluated in the pregnant guinea pig to determine if reproductive disease would occur (2). IT inoculation is a more physiologically relevant route of infection compared to i.p., and a previous study demonstrated that it reliably

results in clinical signs and systemic disease in guinea pigs (81). Pregnant guinea pigs have rarely been used in brucellosis research with only one previous study evaluating the pregnant guinea pig as a model for *Brucella* spp. infection. In the 1970s, Bosseray and Diaz inoculated pregnant guinea pigs via intramuscular inoculation (IM) with $5x10^4$ *B. abortus* 544, which resulted in a 50% abortion rate (37). This study was exciting because it demonstrated that pregnant guinea pigs would develop reproductive disease when inoculated with a virulent *Brucella* spp.; however, the authors failed to specify the stage of gestation at the time of inoculation and did not culture the placenta. Additionally, IM is an artificial route of challenge and is less physiologically relevant for exploring the pathogenesis of reproductive disease. The current study confirms that abortions/stillbirths occur from *Brucella melitensis* secondary to aerosol inoculation, which is a means of transmission in naturally-acquired brucellosis. Additionally, pregnant guinea pigs developed fever secondary to IT inoculation further supporting the ability of this model to replicate natural disease. Therefore, the pregnant guinea pig model shows promise as a model to evaluate the pathogenesis of *Brucella*-associated adverse pregnancy outcomes in women (6, 37).

While the pregnant guinea pig is a good model for brucellosis, research with guinea pigs does have limitations. With far fewer studies conducted in guinea pigs compared to mice, limited reagents are commercially available to fully characterize the guinea pig immune response. However, the guinea pig genome has been sequenced and new annotations are continually added, indicating that a lack of reagents may become less of an impediment to using the guinea pig model. Another obstacle to completing

these studies is the additional space required to house animals under BSL3 conditions. During pregnancy, guinea pigs weigh up to 1.5 kg and require larger cages and cage racks. These limitations may be why pregnant mice have been used more extensively to evaluate the tropism and effects of *Brucella* on the gravid uterus (20, 77, 90, 91). The mouse model also has the advantage of short generation time and a large number of commercially available reagents to evaluate the immunological response to infection (35).

An important goal of developing the pregnant guinea pig as a model for brucellosis is not only to have a better model for investigating the pathogenesis of reproductive disease but to also have an animal model to evaluate vaccine candidates. A recent meta-analysis, which investigated the mouse model for *Brucella* vaccine development found that the protection index has remained stable despite the development of novel subunit vaccines, DNA vaccines, and LAV mutants over the past thirty years (8). This suggests that while mice are an important animal model for investigating the pathogenesis of brucellosis, they may not be the best model for evaluating vaccines. As an example, *Brucella* Δ*cydBA* was attenuated in a BALB/c mouse model (92). When the same mutant was evaluated in pregnant goats, a natural host for *B. melitensis*, the LAV mutant colonized organs at the same level as the WT strain (68).

The guinea pig could prove a useful alternative animal model for developing and evaluating novel vaccines against brucellosis. Historically, guinea pigs were instrumental for developing and testing the safety and efficacy of commonly used

Brucella vaccines such as the *B. melitensis* mutant Rev. 1 and *B. abortus* S19 vaccines (52, 53). Guinea pigs were also used to compare the protection provided by the various vaccine strains against field isolates and were used to determine if vaccination against one strain offered cross protection (27, 54-57). Due to the similarities in disease manifestation, it was expected that vaccines or antigens which generate an active immune response in the guinea pig would be suitable for testing in humans and large animals (15, 52, 93, 94).

Previous studies have demonstrated that S19 is protective in a non-pregnant guinea pig challenge model, but efficacy in pregnant guinea pigs has not been evaluated (95). S19 was used because it is the reference strain against which new vaccines are compared, and it is classified as a BSL2 agent allowing the breeding portion of the experiment to be conducted in group-housed open-bank caging available under BSL2 conditions (58, 96). Typically, Rev. 1 is used to protect small ruminants from infection with *B. melitensis*; however, Rev. 1 is classified as a select agent and vaccinated animals must be housed in a BSL3 level facility. This places a significant impediment on breeding experiments due to the strict caging regulations at BSL3 and associated increase in the animal numbers required. As the use Rev. 1 in this context was not feasible, vaccine candidate, *B. melitensis* 16MΔ*vjbR,* which can be used under BSL2 conditions and has been extensively evaluated in a mouse model (63, 82). Quil-A was added as an adjuvant because previous studies in mouse models using 16MΔ*vjbR* suggested the need for additional immune stimulation to generate a robust response to vaccination (82). Quil-A promotes a cellular and humoral immune response and is on the

list of approved adjuvants for use in human vaccines (97). The results of this study confirm the protective capacity of S19 and suggests that 16MΔ*vjbR* requires additional modifications such as adding a booster dose or a different adjuvant to achieve a similar level of protection as S19. Even though guinea pigs were vaccinated with *B. abortus* LAV S19, vaccination was capable of providing protection against challenge with wild-type *B. melitensis*. Cross-protection has previously been demonstrated in the guinea pig and mouse models as well as in dairy cattle (55, 56, 86, 98).

Vaccination with both S19 and 16MΔ*vjbR* generated a robust *Brucella*-specific IgG response. Interestingly, the IgG response to challenge in guinea pigs vaccinated with 16MΔ*vjbR* + Quil-A was stronger than that of S19; however, this increase in IgG level did not correlate with superior protection against colonization or inflammation. While the humoral immune response is often used to evaluate the response to vaccination, the immune response to infection with *Brucella* spp. is largely dependent on the T cell response (99). In particular, several studies have demonstrated that vaccine protection relies on stimulating a strong Th1 response with evidence that Th2 cells are less effective in controlling infection (100-102). 16MΔ*vjbR* induced a robust total IgG response but did not enhance protection, which suggests that vaccination with 16MΔ*vjbR* may skew the humoral immune response towards a less effective IgG1/Th2 phenotype (102). However, we were unable to confirm this speculation due to a lack of a commercially available reagent for anti-guinea pig IgG1.

It is interesting to note that vaccination with S19 prevented fever following challenge with 16M, but the response of the guinea pig to vaccination with S19 was not

evaluated. Previous reports of accidental exposure and a single trial using prisoner volunteers indicate that vaccination with BA-19 or Rev. 1 results in fever and symptoms of brucellosis in people (6, 96). As we did not monitor temperature during the period of vaccination, we are unable to conclude if vaccination with S19 or 16MΔ*vjbR* produce fever in guinea pigs. Additional studies could illuminate this point and better define the guinea pig as a model for vaccine safety trials.

3.6. Conclusion

Understanding the pathogenesis of brucellosis associated reproductive disease is a crucial aspect to prevent disease in both humans and animals. Herein we present compelling data that the pregnant guinea pig is an excellent model for evaluating the events that underlie reproductive failure. The pregnant guinea pig also offers improvements upon the mouse model for vaccine studies because the two clinical endpoints that are of importance for human health, fever and adverse pregnancy events, could be evaluated. Future studies using the pregnant guinea pig could be performed to develop novel therapeutics or vaccines. This study indicates that pregnant guinea pigs should be considered appropriate models to evaluate vaccine candidates in the pipeline because they can provide valuable information about clinical signs of infection as well as the appropriate tissue targets to assess protection.

4. CONCLUSIONS

Brucellosis research has largely depended on intraperitoneal inoculation (ip) of the mouse to characterize disease and investigate the immune response to infection (17). However, ip is an artificial route of inoculation and thus does not recapitulate the events of natural disease transmission. The most common routes of inoculation in humans are ingestion of contaminated milk or inhalation of infectious aerosols (4). Thus, aerosol models offer a more physiologically appropriate inoculation route to study disease. Several aerosol inoculation routes have been developed including a breathing apparatus, intranasal, or whole-body exposure (9, 40, 50). Intratracheal inoculation provides a targeted means of generating infectious aerosols and removes the dose variability associated with other aerosol models that depend on the animal's respiration rate and limits cross infectivity through non-target routes such as mucous membranes or ingestion. This work characterized a novel aerosol route of inoculation in the non-pregnant and pregnant guinea pig and demonstrates that this inoculation method reliably results in disease in both. The combination of the guinea pig and IT could offer a novel challenge model that mimics natural disease transmission.

This work also demonstrates that inoculation via infectious aerosols rapidly leads to systemic colonization. Importantly, since the lung is a portal of entry for *Brucella*, IT inoculation of the guinea pig could be used to determine if vaccination would provide protection against an aerosol challenge. Intranasal inoculation in the mouse model indicates that aerosol infection modulates the resident immune cell phenotype and

affects the protective immune response at the level of the lung (50, 103). As a targeted approach to generating pulmonary infection, IT could be used to investigate factors that drive the inflammatory response and how vaccination modulates the immune response to infection.

Brucellosis remains a threat to both human and animal health in endemic regions, and vaccination is a key control measure used to limit disease in animals. In order to better design and develop vaccine candidates for human use, it may be necessary to return to the roots of vaccine development and again utilize the guinea pig. The mouse model has been used extensively to evaluate the immune response to infection and to interrogate vaccine candidates, but mice do not develop clinical signs of brucellosis such as fever that are a hallmark of disease in humans (17). This limits the utility of the model because fever cannot be evaluated as an endpoint in vaccine or therapeutic studies. In contrast, our research demonstrates that guinea pig develop fever when inoculated with *B. melitensis*. Guinea pigs could be used to screen vaccine candidates for their ability to reduce or prevent infection as well as to mitigate the clinical aspects of disease. This could streamline the development of promising vaccine candidates by early removal of those that fail to provide protection in the guinea pig.

An unexpected but intriguing finding from the infection of non-pregnant guinea pigs revealed that *B. melitensis* is capable of colonizing the uterus independent of pregnancy. This finding is particularly relevant because it had not been described before, but reports from endemic countries suggests a connection between infection and reduced fertility (11). It is possible that infection of the non-gravid uterus could lead to infertility

in both humans and animals. Utilizing IT inoculation, the effect of infection on the estrous cycle could be evaluated to determine if infection inhibits the progression of estrus. As such, the guinea pig could be used to determine a link between infection and diminished fertility.

A secondary application of the inoculation route and guinea pig could be to investigate the pathogenesis of brucellosis associated reproductive disease. Chapter 3 provides compelling data that the pregnant guinea pig is an excellent model for evaluating the events that underlie reproductive failure. Despite being recognized as a cause of adverse pregnancy events and infertility for over 130 years in both animals and humans, it is unknown what factors lead to preferential colonization of reproductive tissues (11). The pathogenesis of placental colonization and tropism is an active area of research. Recent attention has focused on the type 4 secretion system (T4SS), which is an important virulence mechanism whose function is to secrete effector proteins across the bacterial cell membrane (104-106). Structural components of the T4SS are encoded by the *virB* operon (*virB1-virB12*), and it is well-established that *virB* mutants display an attenuated phenotype *in vitro* and *in vivo* (104, 107).

The pregnant guinea pig offers an exciting animal model for exploring the events that precede reproductive failure. Studies using microarray analysis have already identified several factors that may contribute to placental tropism including BtpB and VceC (88, 107-110). RNAseq is a more sensitive methodology for detecting gene expression and could offer further insight into genes that are differentially expressed at the level of the placenta. Our data indicates differences in colonization in systemic

organs such as the spleen and liver compared to the reproductive tissues. Intriguingly, colonization of the reproductive organs lags behind the spleen, but the CFU/g is higher in reproductive tissues. We hypothesize that gene expression of T4SS effectors differs between the spleen and placenta leading to enhanced virulence and tropism of *Brucella* spp. for the placenta.

Overall, the research presented herein makes a strong argument that intratracheal inoculation of the guinea pig is an improved model for evaluating vaccine candidates and has untapped potential for investigating the pathogenesis of reproductive disease.

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APPENDIX A

Table A1. Histologic scoring system for lesions of brucellosis in the guinea pig.

Splenic Lesions	Score	Description
Histiocytic inflammation	0	None
	1	Minimal—One focus per 4x objective
	2	Mild—Two to four foci per 4x objective
	3	Moderate—Five to 10 foci per 4x objective
	4	Marked—>10 per 4x objective
Neutrophilic accumulation	0	None
	1	Minimal— few cells identified
	2	Mild—multiple small foci <10 cells
	3	Moderate—1-2 foci of >10 cells
	4	Marked—Multiple foci of >10 cells
Necrosis	0	None
	1	Minimal—one focus per 10x objective
	2	Mild—Two to four foci per 10x objective
	3	Moderate—Five to ten foci per 10x objective
	4	Marked— >10 foci per 10x objective

Hepatic Lesions	Score	Description
Periportal inflammation	0	None
	1	Minimal— <25% affected
	2	Mild—25%
	3	Moderate—50%-75% affected
	4	Marked—100% affected
Microgranulomas	0	None
	1	Minimal—One focus per 10x objective
	2	Mild—Two to four foci per 10x objective
	3	Moderate—Five to 10 foci per 10x objective
	4	Marked—Fills the 10x objective
Random necrosis	0	None
	1	Minimal—one focus per 10x objective
	2	Mild—Two to four foci per 10x objective
	3	Moderate—Five to ten foci per 10x objective
	4	Marked— >10 foci per 10x objective

Uterine Lesions	Score	Description
Myometrial inflammation	0	None
	1	Minimal—One focus per 4x objective
	2	Mild—Two to four foci per 4x objective
	3	Moderate—Five to 10 foci per 4x objective
	4	Marked—>10 per 4x objective
Endometrial neutrophilic inflammation	0	None
	1	Minimal— few cells identified
	2	Mild—multiple small foci <10 cells
	3	Moderate—1-2 foci of >10 cells
	4	Marked—Multiple foci of >10 cells
Edema	0	None
	1	Present

Pulmonary Lesions	Score	Description
Granulomas	0	None
	1	Minimal—One focus per 4x objective
	2	Mild—Two to four foci per 4x objective
	3	Moderate—Five to 10 foci per 4x objective
	4	Marked—>10 per 4x objective
Neutrophilic accumulation	0	None
	1	Minimal— few cells identified
	2	Mild—multiple small foci <10 cells
	3	Moderate—1-2 foci of >10 cells
	4	Marked—Multiple foci of >10 cells
Necrosis	0	None
	1	Minimal—one focus per 10x objective
	2	Mild—Two to four foci per 10x objective
	3	Moderate—Five to ten foci per 10x objective
	4	Marked— >10 foci per 10x objective
BALT hyperplasia	0	None
	1	Present

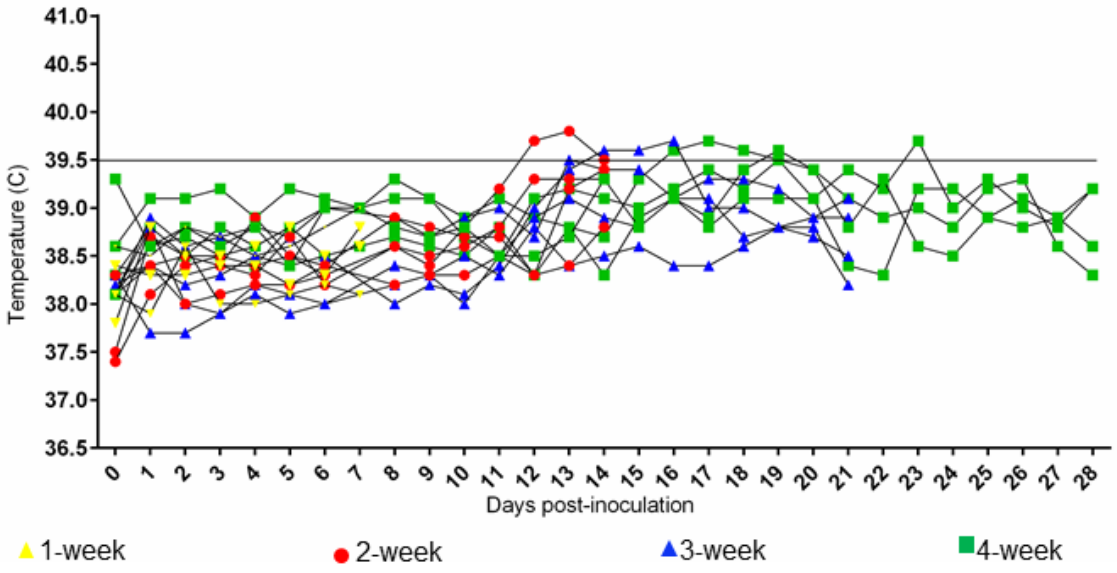

Figure A1. Kinetics of body temperature in guinea pigs.
Body temperature changes in guinea pigs (n=4) after intratracheal inoculation $1x10^7$ CFU *B. melitensis* 16M. The solid line at 39.5° C indicates the threshold for fever. Guinea pigs developed fever beginning at day 12 post-infection.

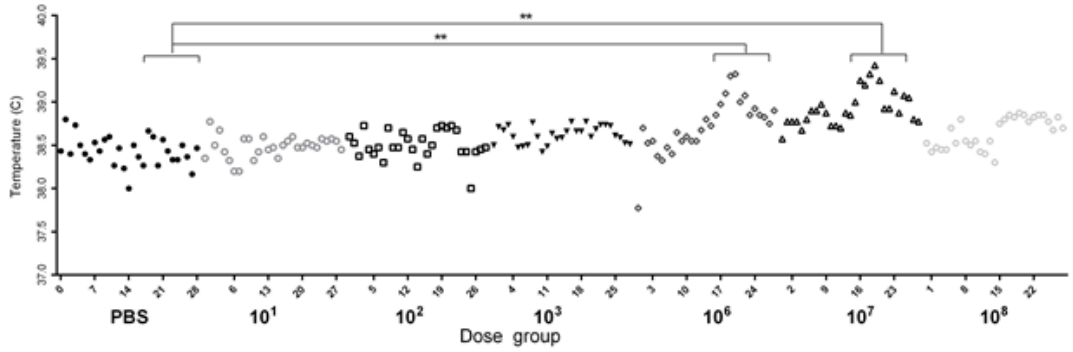

Figure A2. *Brucella melitensis* induces fever in guinea pigs.
Comparison of body temperature differences between uninfected controls (PBS) and dose groups. Statistical significance by ANOVA followed by Dunnett's multiple-comparisons. The mean daily temperature was compared between the uninfected controls and the dose groups. Two asterisks, P <0.01.

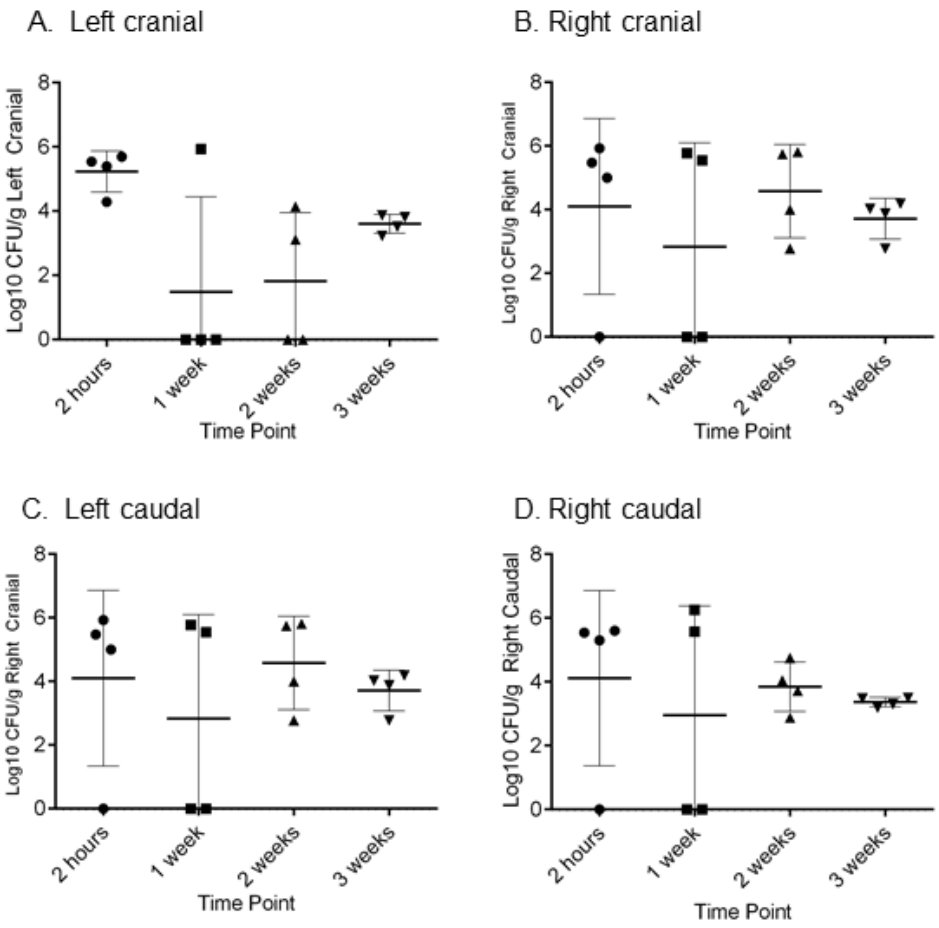

Figure A3. Intratracheal inoculation results in even distribution of aerosolized particles throughout all lung fields.

The distribution of aerosolized *B. melitensis* 16M in the lung lobes of guinea pigs inoculated with $1x10^7$ CFU/50 µl was evaluated at 2-hours and 1,2, and 3-weeks post-inoculation. The lung was divided into four regions defined as left, right, cranial, and caudal, and tissue colonization was determined by region. The horizontal bar is the mean per group with standard error.

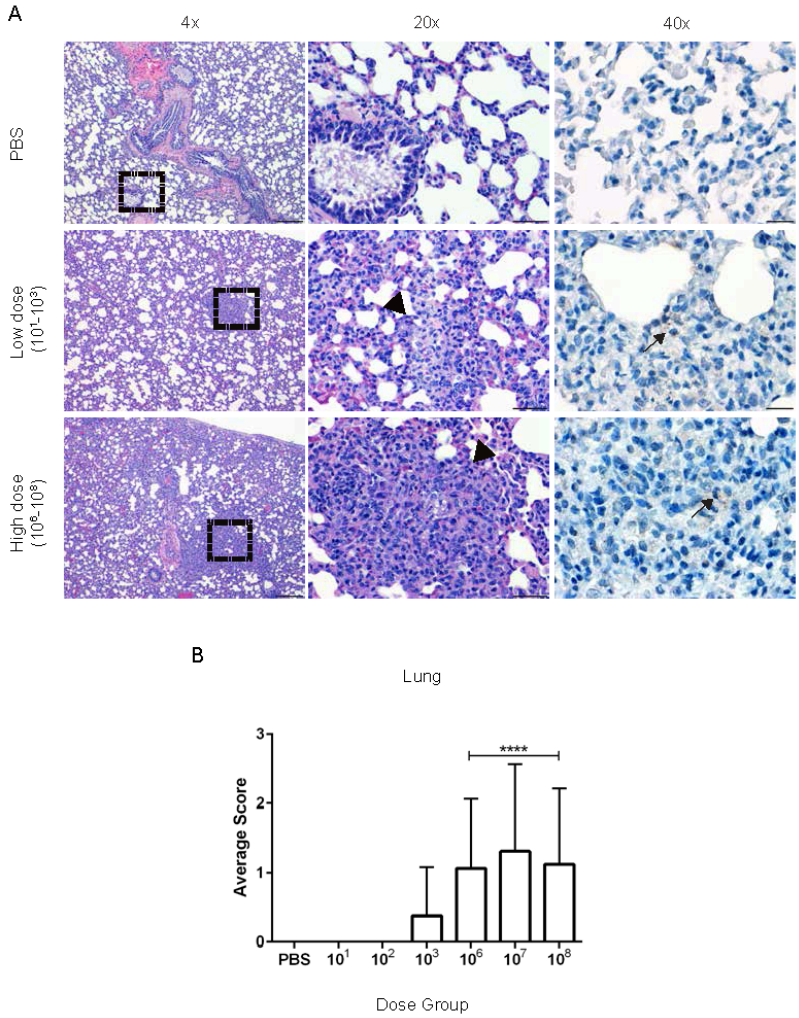

Figure A4. Histopathology of the lung following *Brucella* infection.
Representative images of histopathology and immunohistochemistry of the lung following intratracheal inoculation with PBS (top), *B. melitensis* 16M at low dose (middle), high dose (bottom) at 30-days post-inoculation. Sections were scored for severity from 1-4 (Table Appendix 1) based neutrophilic inflammation, number and size of microgranulomas and necrosis, and bronchoalveolar hyperplasia. The white dashed box in the left panel indicates the section highlighted for higher magnification in the middle and right panels. Foci of histiocytic inflammation were seen in the low and high dose groups (arrowheads), but the lesions were larger in the high dose group. *Brucella* antigen was detected within alveolar macrophages in areas of inflammation by IHC (arrows). Magnification 4x (left, H&E, bar= 200 μm), 20x (middle, H&E, bar= 50 μm), 40x (right, Anti-*Brucella* IHC, bar=20 μm).

APPENDIX B

Table B1. Histologic scoring system for lesions of the placenta in the guinea pig.

Placenta	Score	Description
Edema	0	Not present
	1	Present
Mononuclear infiltrate	0	Not present
	1	Minimal—One focus per 5 10x objective
	2	Mild—Two to four foci per 5 10x objective
	3	Moderate—Five to 10 foci per 5 10x objective
	4	Marked—>8 per 5 10x objective
Fibrosis	0	Not present
	1	Minimal—One focus per 5 10x objective
	2	Mild—Two to four foci per 5 10x objective
	3	Moderate—Five to 7 foci per 5 10x objective
	4	Marked—>8 per 4x objective
Necrosis	0	Not present
	1	Minimal—one focus per 5 10x objective
	2	Mild—Two to four foci per 5 10x objective
	3	Moderate—Five to 7 foci per 5 10x objective
	4	Marked— >8 foci per 5 10x objective
Bacteria	0	Not present
	1	Present

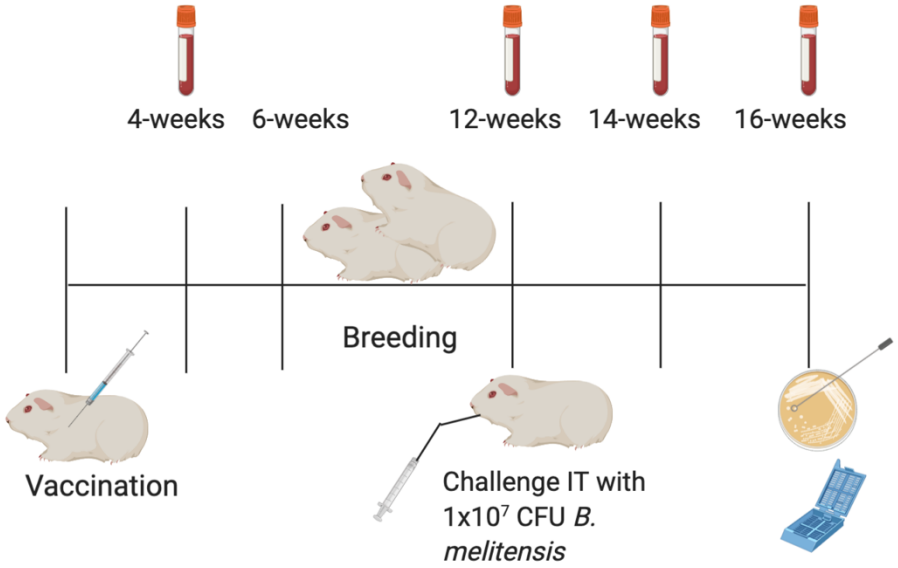

Figure B1. Experimental design.
200-250 g female Hartley guinea pigs were randomly separated into three treatment groups and were vaccinated with $1x10^9$ CFU/100 µl *Brucella abortus* S19 (n=6) or *Brucella melitensis* 16M Δ*vjbR* + 10 µg Quil-A (n=6) by subcutaneous injection in the right inguinal region. Control animals were sham vaccinated with 100 µl sterile PBS + 10 µg Quil-A (n=5). At 6-weeks post-vaccination, female guinea pigs were co-housed with unvaccinated male Hartley guinea pigs for breeding at a ratio of 3:1. After 2-weeks, females were evaluated for pregnancy by abdominal palpation. When guinea pigs were at approximately 30-35 days of gestation, they were transferred to the BSL-3 and challenged with $1x10^7$ *B. melitensis* IT. Blood was collected from the saphenous vein on the day of vaccination (week 0), and at 4-, 12-, 14-, and 16-weeks post-vaccination. All pregnant guinea pigs were euthanized at the time of parturition or 4-weeks post-challenge. Tissue samples were collected for bacteriological culture and histology. Figured generated with www.Biorender.com.

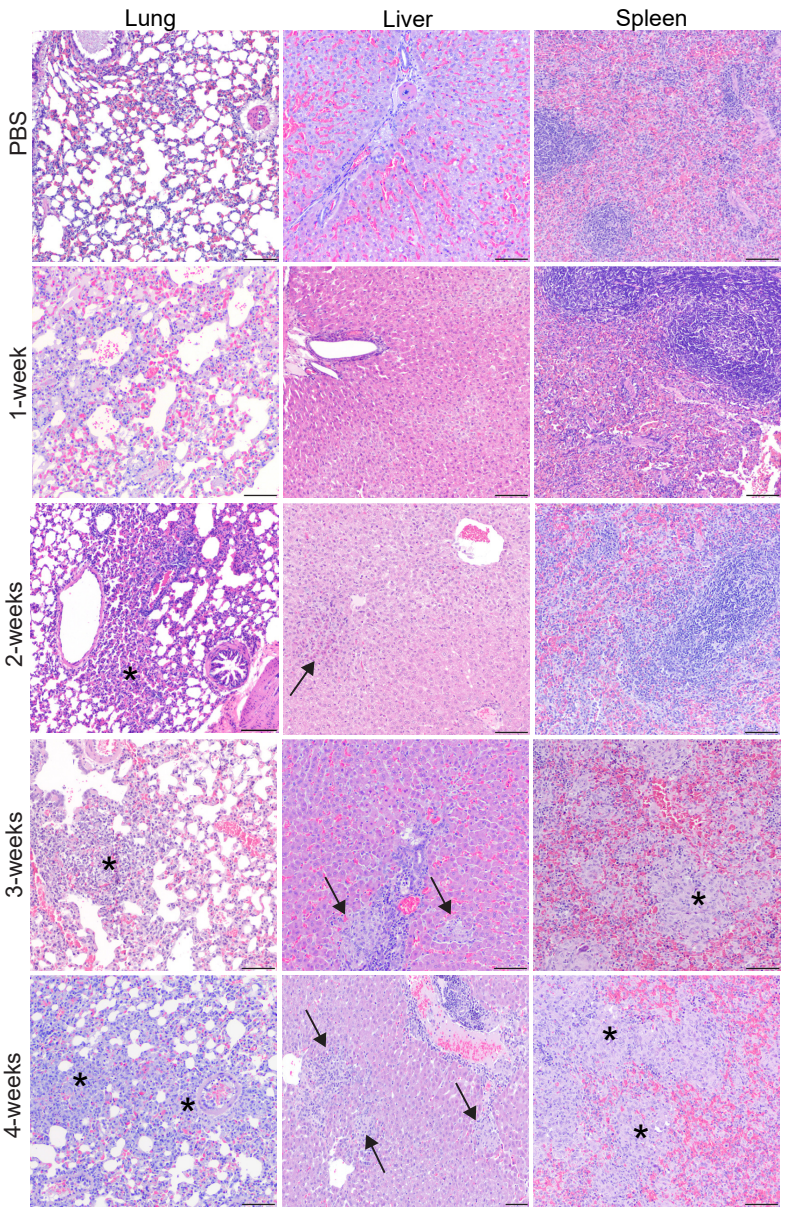

Figure B2. Intratracheal inoculation of pregnant guinea pigs results in systemic inflammatory lesions.

Representative H&E images of lung (left column), liver (middle column), and spleen (right column) from pregnant guinea pigs at 5-,6-,7-, and 8-weeks of gestation following IT inoculation with $1x10^7$ CFU *B. melitensis*. Negative controls received sterile PBS IT, which did not incite an inflammatory reaction in any tissue. The earliest lesion in the lung at 1-week p.i. was mild pulmonary edema that progressed to neutrophilic and histiocytic embolic pneumonia (asterisks). Random foci of lymphohistiocytic inflammation in the liver (arrows) and foci of histiocytic inflammation in the spleen (asterisks) became larger and more numerous from 2 to 4-weeks p.i. H&E, Magnification, 10x; Bar = 100 µm.

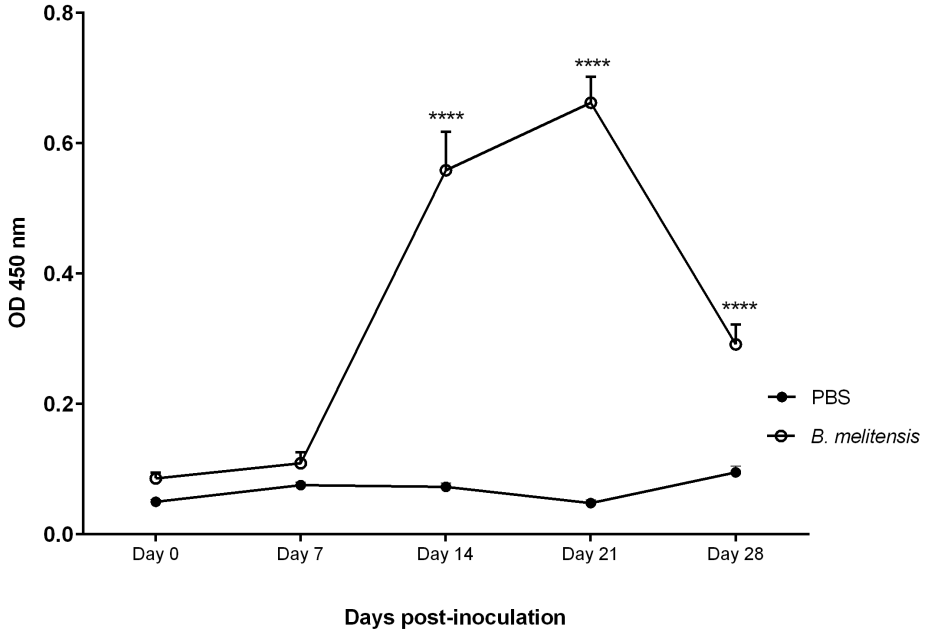

Figure B3. IT inoculation results in a *Brucella*-specific IgG response to infection.
Guinea pigs at mid-gestation (n=4) were challenged with 1x10^7 CFU *B. melitensis* IT or sterile PBS and were euthanized at 7-day intervals for 28-days. Blood was obtained from the lateral saphenous at days 0 and 14 or from cardiac blood at study end-points and was analyzed for Brucella-specific IgG via iELISA. Data are represented as the mean per group per time point. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparisons.

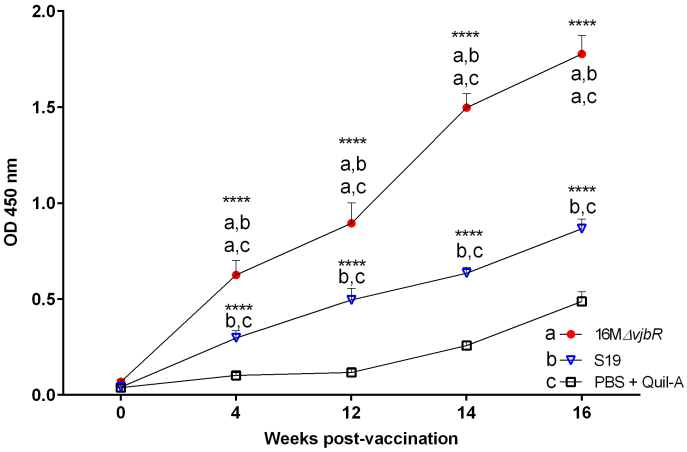

Figure B4. Vaccination promotes a robust *Brucella*-specific IgG response.
Blood samples were obtained from the lateral saphenous vein at 0,4-,12-,14-, and 16-weeks post-vaccination. At mid-gestation and 12-weeks post-vaccination, pregnant guinea pigs were challenged at 12-weeks post-vaccination with 1x10^7 CFU *B. melitensis* IT. iELISA was used to evaluate the IgG response to vaccination. Statistical significance was determined by ANOVA followed by Tukey's multiple comparisons. a- 16MΔ*vjbR*; b- S19; c- PBS+ Quil-A. ****, P <0.0001.