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Aerosol Infection of BALB/c Mice with *Brucella melitensis* and *Brucella abortus* and Protective Efficacy against Aerosol Challenge▽

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Brucellosis is a zoonotic disease with a worldwide distribution that can be transmitted via intentional or accidental aerosol exposure. In order to engineer superior vaccine strains against *Brucella* species for use in animals as well as in humans, the possibility of challenge infection via aerosol needs to be considered to properly evaluate vaccine efficacy. In this study, we assessed the use of an aerosol chamber to infect deep lung tissue of mice to elicit systemic infections with either *Brucella abortus* or *B. melitensis* at various doses. The results reveal that *B. abortus* causes a chronic infection of lung tissue in BALB/c mice and peripheral organs at low doses. In contrast, *B. melitensis* infection diminishes more rapidly, and higher infectious doses are required to obtain infection rates in animals similar to those of *B. abortus*. Whether this difference translates to severity of human infection remains to be elucidated. Despite these differences, unmarked deletion mutants BAΔ*asp*24 and BMΔ*asp*24 consistently confer superior protection to mice against homologous and heterologous aerosol challenge infection and should be considered viable candidates as vaccine strains against brucellosis.

Brucellosis is a zoonosis affecting numerous species of domestic animals, wildlife, and humans (13–15). Humans are commonly infected as a result of contact with infected animals, ingestion of contaminated animal products such as milk, milk products, or meat, or laboratory exposure. Abattoir workers may also acquire the disease via aerosol exposure (2, 3, 13–15, 18, 31). The *Brucella* species most pathogenic to humans include *Brucella melitensis*, *B. suis*, *B. abortus*, and *B. canis*, all of which are distributed worldwide but are most common in Mediterranean countries, the Middle East, India, Mexico, Central Asia, and Central and South America (3, 10, 13, 17, 22, 23). Brucellosis has been reported to be the most common zoonotic infection worldwide, with over 500,000 new infections reported annually (22, 23). Human disease manifests as prolonged febrile illness (undulant fever), flu-like symptoms, night sweats, headache, depression, and arthritis, and infection can lead to chronic illness, such as meningitis and endocarditis (2, 3, 8, 14, 22, 31).

Documented evidence of aerosol transmission of these organisms has emphasized the recent focus on the use of *Brucella* as a potential bioterrorism agent (5, 20). In the 1950s, *B. suis* was the first agent weaponized in the United States, and *Brucella* has been evaluated for this purpose by several other countries as well (3, 22). It has been estimated that as few as 10 to 100 organisms comprise an infectious aerosol dose in humans, and *Brucella* is therefore considered highly infectious when it is delivered in this manner (3). As a result, *B. melitensis*, *B. abortus*, and *B. suis* have been classified as category B agents by the Centers for Disease Control and Prevention and the National Institute of Allergy and Infectious Diseases (1, 22). The absence of a safe and efficacious vaccine for use in humans underscores the concern.

In order to engineer superior vaccine strains against *Brucella* species for use in animals as well as humans, the potential for challenge infection via aerosol exposure needs to be considered to properly evaluate vaccine efficacy. Previous studies have considered intranasal infection of mice and guinea pigs as models for aerosol exposure (1, 2, 10, 12, 13, 18, 29). In this study, we evaluated the kinetics of systemic infection in the mouse model after aerosol exposure of deep lung tissue to both *B. abortus* and *B. melitensis* in order to establish a novel exposure route in the mouse model. This route of infection was utilized to evaluate the protective efficacy of selected *Brucella* mutants previously shown by our lab to elicit significant protection in mouse and goat models (14, 15).

MATERIALS AND METHODS

Bacteria and bacterial culture. The *B. abortus* 2308 and *B. melitensis* 16M wild-type strains used for challenge, as well as unmarked deletion strains used for vaccination studies, were routinely grown on tryptic soy agar (TSA) (Difco Laboratories) at 37°C in an atmosphere containing 5% (vol/vol) CO_2. Virulent *B. abortus* strain S2308 was obtained from Billy Deyoe at the National Animal Disease Center in Ames, IA. *B. melitensis* biovar 1 (16M) was originally obtained from ATCC and was reisolated from an aborted goat fetus (14). Unmarked deletion strains used in this study (BAΔ*asp*24, BMΔ*asp*24, BAΔ*virB2*, BMΔ*virB2*, BAΔ*man*BA, and BMΔ*man*BA) were engineered previously and tested for survival and protective efficacy in BALB/c mice (15). All bacterial strains were stored frozen at −80°C in medium supplemented with 50% (vol/vol) glycerol and grown on TSA for immediate use in each experiment. 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 retrospectively to accurately determine the number of organisms in the inoculum. Lung, liver, and spleen samples from mice were plated onto Farrell's medium to select for *Brucella.* Farrell's medium is TSA supplemented with 5 mg/liter nalidixic acid, 25,000 IU/liter bacitracin, 100 mg/liter cycloheximide, 5000 IU/liter polymyxin B sulfate, 20 mg/liter vancomycin, 100,000 IU/liter nystatin (*Brucella* selective supplement; Oxoid), 10% (vol/vol) horse serum, and 2% (wt/vol) dextrose).

Kinetics of aerosol infection of *Brucella* in mice. The survival or persistence of wild-type strain *B. abortus* 2308 or *B. melitensis* 16M was evaluated using groups of 6- to 8-week-old female BALB/c mice (Jackson Laboratories) following aero-

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sol exposure via a Madison aerosol chamber (College of Engineering Shops, University of Wisconsin, Madison) (30) to three different doses of *Brucella* added to the chamber nebulizer: 5×10^7, 5×10^8, and 5×10^9 CFU/ml. The actual numbers of infectious organisms inhaled by the mice at each dose were determined directly by euthanizing a group of four or five mice immediately after removal from the chamber via carbon dioxide asphyxiation, homogenizing the lungs in 1 ml PBS, and plating the preparations onto Farrell's medium to determine the number of CFU in the combined lung tissue for each mouse. For kinetic studies, mice were euthanized at 1, 2, 4, 6, or 8 weeks postinfection. At each time point, the lungs, liver, and spleen were collected and weighed, homogenized in 1 ml PBS, and serially diluted, and 200 μl of each dilution was plated onto Farrell's medium. Recovered bacteria were enumerated to evaluate the persistence of each strain.

Efficacy studies. The mouse model was used to evaluate the efficacies of unmarked deletion mutants (previously shown to protect mice against virulent *Brucella* by intraperitoneal [i.p.] challenge) against subsequent aerosol challenge. Groups of four or five female 6- to 8-week-old BALB/c mice were vaccinated via i.p. injection of 1×10^6 CFU/ml of unmarked deletion mutant or PBS for naïve controls. Mice were subsequently challenged with an aerosol chamber dose of 5×10^9 CFU/ml of the homologous wild-type strain at 20 weeks postvaccination. Four weeks after the virulent challenge (corresponding to 24 weeks postvaccination), the mice were euthanized, and the lungs, liver, and spleen were extracted, weighed, homogenized in 1 ml PBS, serially diluted, and plated onto Farrell's medium to measure recovery of the challenge organism.

To evaluate cross-species protection, groups of four or five female 6- to 8-week-old BALB/c mice were vaccinated with either the BAΔ*asp*24 or BMΔ*asp*24 unmarked mutant strain and challenged as described above with the heterologous wild-type strain 20 weeks postvaccination. Four weeks after the virulent challenge, the mice were euthanized, and the challenge organisms recovered were enumerated as described above.

For both experiments, vaccine efficacy is described in the text as a measure of protective immunity or units of protection (U), representing differences in bacterial burden in the spleen of challenge organism for naïve and vaccinated mice ($\log_{10}$ wild type recovered from unvaccinated mice $-$ $\log_{10}$ wild type recovered from vaccinated mice).

Statistical analysis. Data from aerosol infection kinetics and efficacy studies were expressed as mean CFU ± standard error and are presented below in graphs as the $\log_{10}$ *Brucella* CFU recovered per organ. Culture-negative organs were assigned a value of 4 CFU/organ, which is below the limit of detection of 5 CFU/organ. Spleen weight data from aerosol kinetics studies were plotted as the mean spleen weight (in mg) ± standard error. The statistical significance of differences between vaccinated animals was evaluated by analysis of variance (ANOVA) by comparing the spleen weights for a group of five naïve, unvaccinated BALB/c control mice (data not shown) to the spleen weights for all mice receiving the same chamber dose inoculum, followed by Dunnett's multiple-comparison test.

In efficacy studies vaccinated and subsequently challenged mice were compared to mice receiving PBS as a vaccine control that were challenged with the wild-type organism. The statistical significance of differences between vaccinated animals was analyzed by ANOVA followed by Tukey's honestly significant difference posttest comparing all groups to one another. For all ANOVAs, P values less than 0.05 were considered statistically significant.

RESULTS

Kinetics of aerosol infection with *B. abortus*. Mice were infected with three different doses of *B. abortus* 2308 via aerosol challenge to evaluate the kinetics of infection in the lungs, livers, and spleens. Mice receiving a dose of 5×10^7 CFU/ml added to the chamber nebulizer actually inhaled much less challenge organism into the total lung tissue; the average was determined to be 415 organisms per mouse (2.62 logs) (Fig. 1A). At this dose, lung colonization with 2308 gradually increased over the first 4 weeks postchallenge and then gradually decreased over the following 4 weeks to 90% of the maximum value. Despite this slight decrease, colonization by the organism in the other tissues was consistent with a chronic infection. Colonization of the liver, although barely detectable at 1 week postchallenge, steadily increased over the first 4 weeks postchallenge and then declined negligibly between weeks 4 and 8. The spleens of infected mice displayed a colonization pattern similar to that of the livers, although the total number of CFU recovered was consistently higher. Spleen colonization increased between weeks 4 and 8, consistent with a persistent infection.

Mice receiving doses of 5×10^8 and 5×10^9 CFU/ml added to the chamber nebulizer on average inhaled 1,266 and 34,560 organisms per mouse (3.1 and 4.54 logs), respectively (Fig. 1B and 1C). Other than the difference in the numbers of organisms inhaled and a 0.5- to 1.0-log increase in lung colonization, the difference in the persistence of the organism in each of the tissues evaluated was negligible.

Kinetics of aerosol infection with *B. melitensis*. As described above for *B. abortus*, mice were infected with three different doses of *B. melitensis* 16M via the aerosol route to evaluate the kinetics of infection in the lungs, livers, and spleens. Mice receiving a dose of 5×10^7 CFU/ml added to the chamber nebulizer inhaled an average of 805 organisms per mouse (2.91 logs) (Fig. 2A). Mice receiving a dose of 5×10^8 CFU/ml added to the chamber nebulizer inhaled an average of 7,988 organisms per mouse (3.9 logs) (Fig. 2B). Mice receiving a dose of 5×10^9 CFU/ml added to the chamber nebulizer inhaled an average of 12,520 organisms per mouse (4.10 logs) (Fig. 2C). It is not clear whether the failure to record a 10-fold increase in inhaled organisms at the highest dose was the result of the viscosity of the inoculum (i.e., the more concentrated culture did not pass through the nebulizer as well as lower doses) or the breathing patterns of the mice in the last chamber run.

At the lowest dose, lung, liver, and spleen colonization with 16M displayed an erratic colonization pattern with large variations between mice, despite the fact that these mice received on average a higher dose than animals infected with *B. abortus.* Overall, the increased levels of colonization in all tissues (aside from the initial lung colonization immediately after exposure) peaked by 4 weeks after exposure, although the levels were lower than the challenge dose. Tissue colonization followed the same profile over the first 4 weeks, with liver and spleen colonization subsequent to lung colonization, and reached a peak by 4 weeks postexposure. However, in contrast to colonization by *B. abortus*, systemic colonization of the liver and spleen waned over the next 4 weeks, and there was a 90 to 99% reduction in the number of CFU.

Splenomegaly induced by aerosol exposure. The colonization profiles described above parallel those observed for i.p. inoculations and, except for lung involvement, confirm that introduction via aerosol exposure results in a similar course of systemic infection. As an alternative measure of disease in the mice, spleen weights were also recorded to observe splenomegaly patterns. Mice infected with *B. abortus* did not have significant splenomegaly until 6 weeks postinfection with the 5×10^8 and 5×10^9 CFU/ml chamber doses or until 8 weeks postinfection with the lower dose (5×10^7 CFU/ml) (Fig. 3A). This inflammation correlated with the levels of bacteria in the spleen, which began to peak or plateau at 4 to 6 weeks postinfection depending upon the dose. This pattern is analogous to that observed following i.p. inoculation, where splenomegaly mirrored spleen colonization but was delayed.

Mice infected with the lowest chamber dose of 16M did not

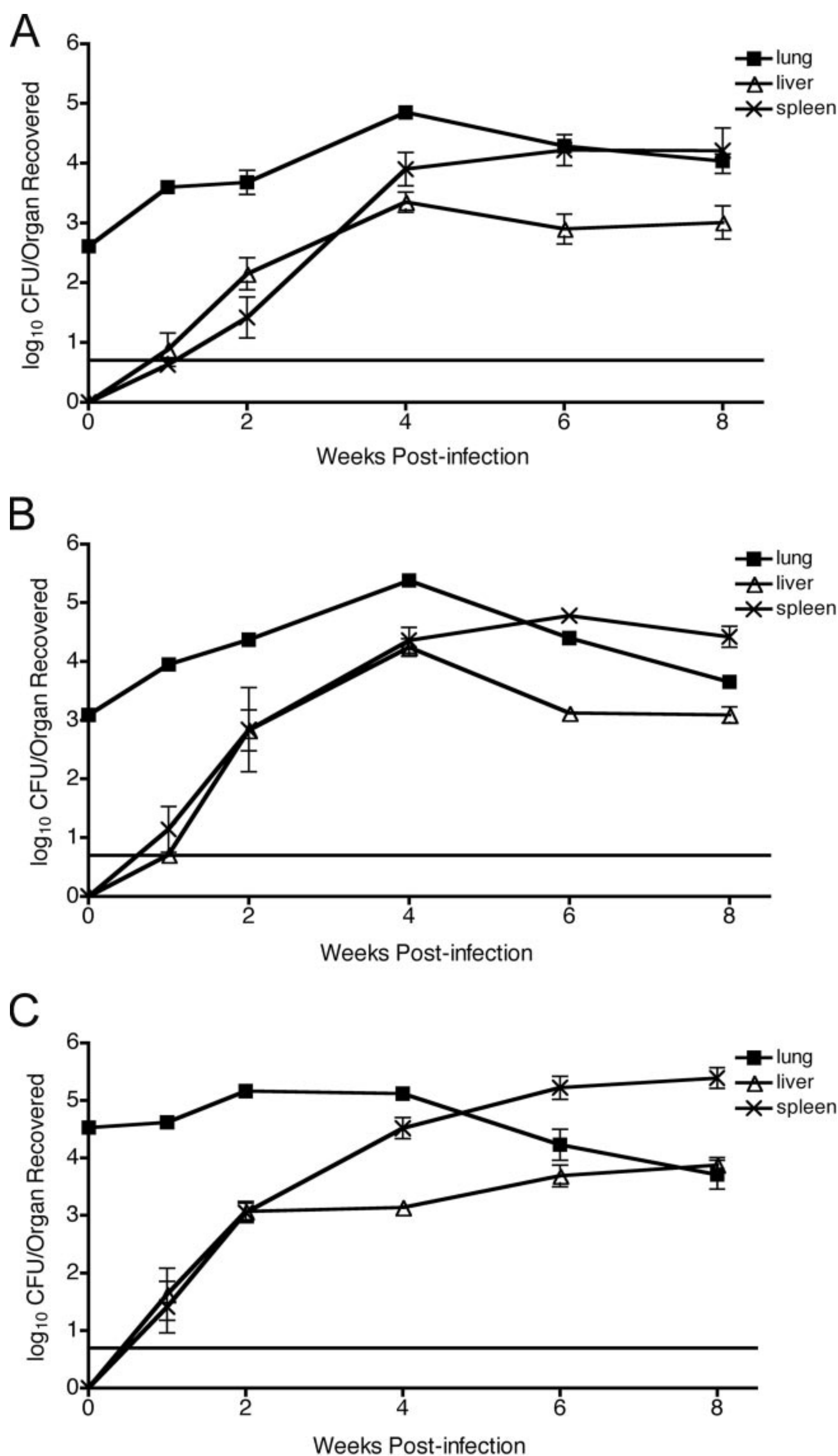

FIG. 1. Kinetics of clearance of *B. abortus* 2308 from BALB/c mice. Four or five female BALB/c mice were infected with aerosolized 2308 in a Madison aerosol chamber using three different chamber doses, 5×10^7 CFU/ml (A), 5×10^8 CFU/ml (B), or 5×10^9 CFU/ml (C). The initial lung colonization was evaluated immediately after challenge to determine the quantity of *Brucella* inhaled for each chamber dose. Mice were euthanized at 1, 2, 4, 6, or 8 weeks postchallenge to determine the numbers of *Brucella* persisting in the lungs, livers, and spleens. The recovery of organisms is plotted as the total CFU/organ (means ± standard errors). The solid line at 0.7 log represents the lower limit of detection, which is ≥5 CFU.

display significant splenomegaly, which correlated with the erratic infection observed (Fig. 3B). Mice infected at the 5×10^8-CFU/ml chamber dose presented with significant splenomegaly 6 weeks postinfection, subsequent to the peak bacterial load observed in the spleen. Mice infected at the highest chamber dose, 5×10^9 CFU/ml, exhibited splenomegaly earlier, at 4 weeks postinfection, paralleling the increased spleen colonization evident at 1 week postchallenge.

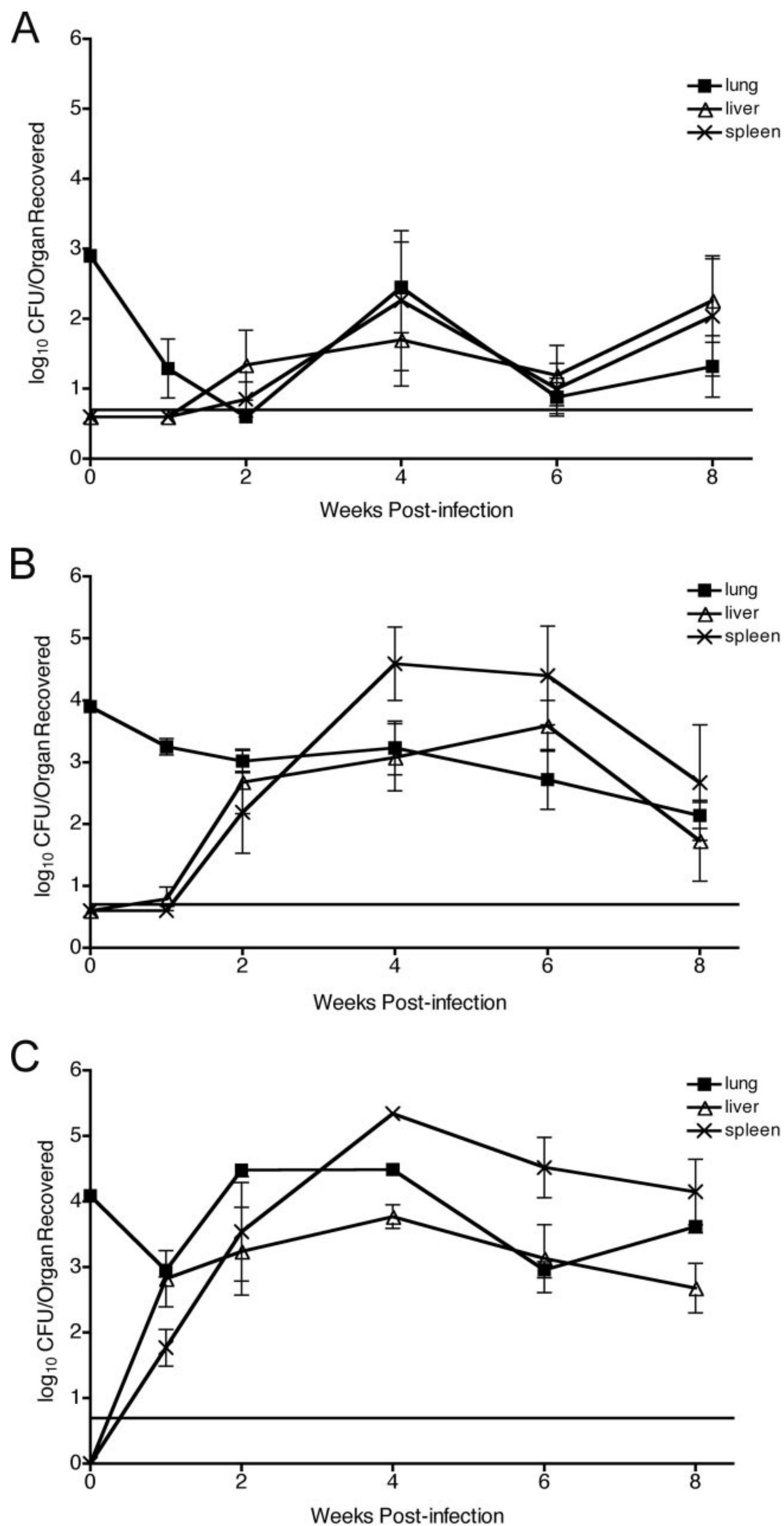

FIG. 2. Kinetics of clearance of *B. melitensis* 16M from BALB/c mice. Four or five female BALB/c mice were infected with aerosolized 16M in a Madison aerosol chamber using three different chamber doses, 5×10^7 CFU/ml (A), 5×10^8 CFU/ml (B), or 5×10^9 CFU/ml (C). The initial lung colonization was determined immediately after challenge to determine the quantity of *Brucella* inhaled for each chamber dose. Mice were euthanized at 1, 2, 4, 6, or 8 weeks postchallenge to determine the numbers of *Brucella* persisting in the lungs, livers, and spleens. The recovery of organisms is plotted as the total CFU/organ (means ± standard errors). The solid line at 0.7 log represents the lower limit of detection, which is ≥5 CFU.

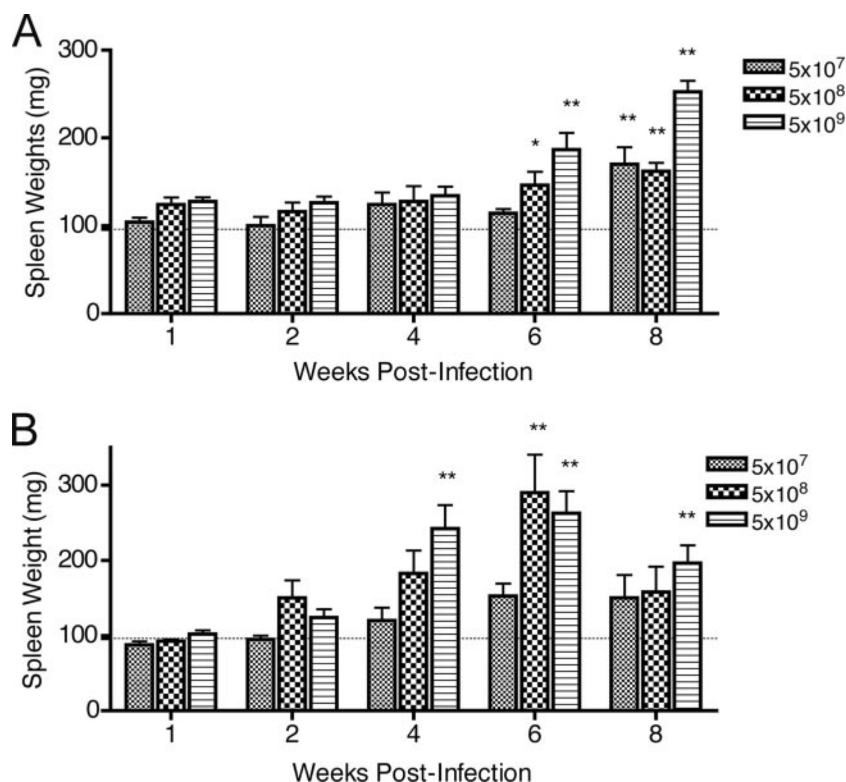

FIG. 3. Evaluation of splenomegaly induced by *Brucella* aerosol challenge. Spleen weights of mice infected in *B. abortus* (A) and *B. melitensis* (B) aerosol kinetics studies were recorded as a measure of splenomegaly induced by the infection. The data are the averages and standard errors for all mice in a treatment group. Statistical significance was determined by ANOVA, followed by Dunnett's multiple-comparison test comparing the spleen weights of a group of five unchallenged BALB/c mice to the spleen weights of all mice receiving the same chamber inoculum dose. The dotted line represents the average spleen weight of uninfected mice (96 mg). One asterisk, $P < 0.05$; two asterisks, $P < 0.01$.

Protective efficacy against homologous *B. abortus* aerosol challenge. To evaluate the vaccine potential of selected *B. abortus* unmarked mutants BAΔ*asp*24, BAΔ*virB2*, and BAΔ*man*BA against virulent aerosol infection, the level of protection provided against wild-type colonization was assessed. Protective efficacy was expressed as a value relative to the nonvaccinated controls challenged with the wild type at each time point, which was obtained by subtracting the mean number of CFU/organ recovered from vaccinated mice from the mean number of CFU/organ recovered from age-matched nonvaccinated aerosol-challenged controls. The highest chamber dose, 5×10^9 CFU/ml, was chosen for the challenge inoculum, and mice were challenged with the wild type (S2308) 20 weeks after i.p. vaccination with each mutant. Colonization by the wild type was determined 4 weeks after challenge (corresponding to 24 weeks postvaccination) in the spleen, liver, and lungs.

As described previously for i.p. challenge and as described above, the spleens of mice typically have the highest bacterial burden and spleen tissue is the most useful tissue for monitoring *Brucella* infection. In these experiments the bacterial burden in the spleen following aerosol challenge was reduced 2.02 U by vaccination with BAΔ*asp*24 and by 0.86 U by vaccination with BAΔ*virB2* relative to the burden in naïve mice ($P < 0.001$ and $P < 0.05$, respectively) (Fig. 4C). In contrast, the rough BAΔ*man*BA mutant was unable to elicit significant protective immunity relative to the immunity of naïve mice (−0.06 U; $P > 0.05$).

At 24 weeks postvaccination, less protection was observed in the lungs of mice vaccinated with BAΔ*asp*24 (0.83 U) and BAΔ*virB2* (0.43 U), although the protection was significantly greater than that observed for naïve controls ($P < 0.001$ and $P < 0.05$, respectively) (Fig. 4A). The rough mutant, BAΔ*man*BA, protected mouse lungs to a lesser degree (0.35 U, which was not significant).

Despite the large size of the liver, the colonization was 2 logs less than that observed in either the spleen or lung, and although the total recovery of wild-type organisms from livers was reduced by an average of 1.04 U, there were too few animals to provide statistical significance (Fig. 4B). Furthermore, the variation between mice in the BAΔ*asp*24-vaccinated group was substantial, possibly masking the degree of protection afforded in the liver, since for two of five mice no challenge organisms were recovered from the liver. No protection was evident in BAΔ*virB2*- or BAΔ*man*BA-vaccinated animals (−0.03 and −0.44 U of protection, respectively).

Protective efficacy against homologous *B. melitensis* aerosol challenge. Mice were vaccinated i.p. with *B. melitensis* unmarked deletion strains BMΔ*asp*24, BMΔ*virB*2, and BMΔ*man*BA and allowed to rest for 20 weeks prior to aerosol challenge with 16M. The highest chamber dose, 5×10^9 CFU/ml 16M, was chosen for

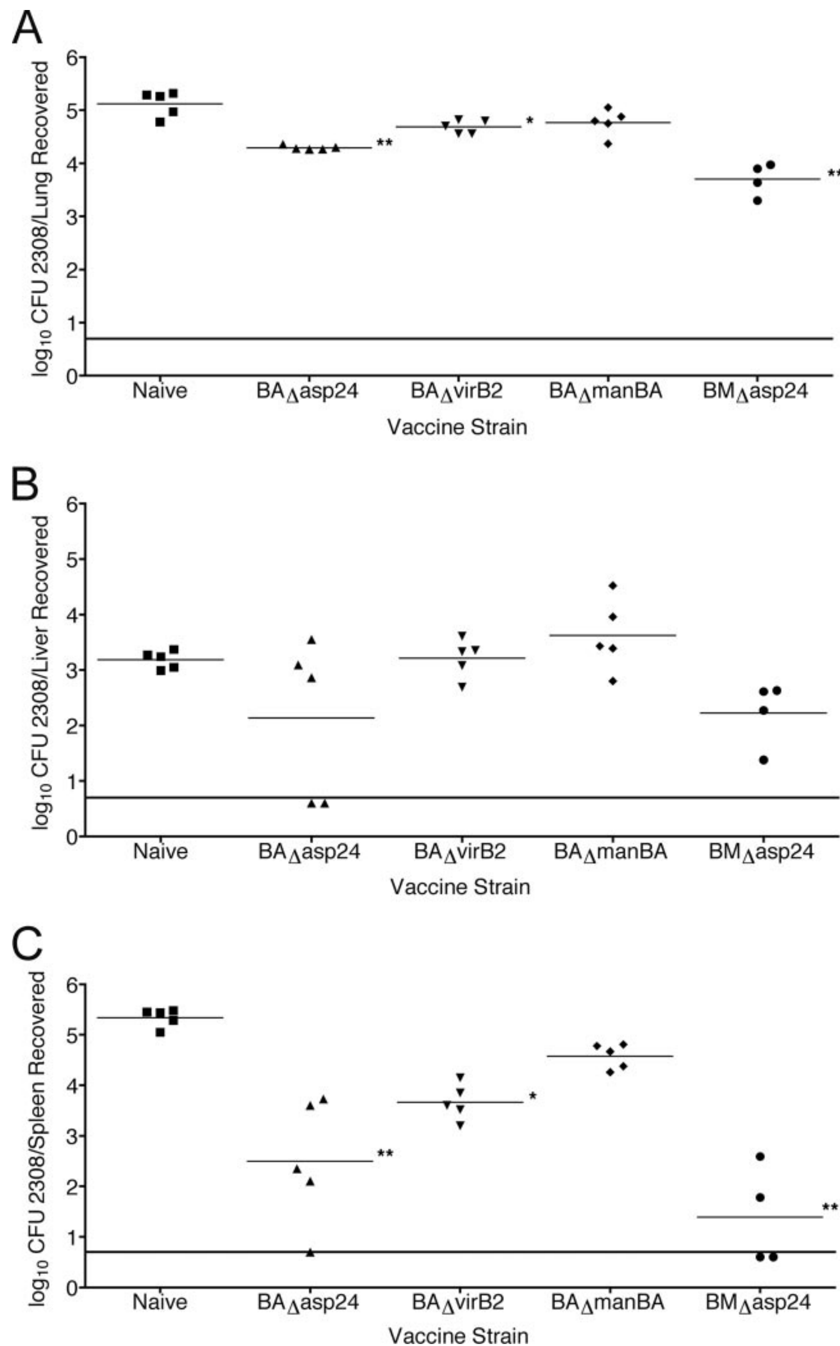

FIG. 4. Evaluation of the efficacy of vaccination against *B. abortus* aerosol challenge. Groups of four or five female 6- to 8-week-old BALB/c mice were vaccinated via i.p. injection of 1×10^6 CFU/ml of unmarked deletion mutant BAΔ*asp*24, BA*virB2*, BAΔ*man*BA, or BMΔ*asp*24 or PBS for naïve controls. Mice were subsequently challenged with an aerosol chamber dose of 5×10^9 CFU/ml *B. abortus* 2308 at 20 weeks postvaccination (heterologous challenge for BMΔ*asp*24). Four weeks after the virulent challenge, the mice were euthanized, and lungs (A), livers (B), and spleens (C) were extracted to measure the recovery of the challenge organisms. Data are expressed as the $\log_{10}$ recovery of 2308 from the entire organ, and the results for individual mice in each treatment group are shown. The solid line at 0.7 log represents the lower limit of detection, which is ≥5 CFU. For comparisons with naïve control animals using ANOVA with Tukey's honestly significant difference posttest, one asterisk indicates that the *P* value is <0.05 and two asterisks indicates that the *P* value is <0.01.

the challenge inoculum. The mice were euthanized 4 weeks postchallenge (corresponding to 24 weeks postvaccination).

Spleens of mice were highly protected against aerosol 16M challenge by BMΔ*asp*24 (3.99 U), and for three of five mice no recoverable 16M was detected in the spleen ($P < 0.001$) (Fig. 5C). BMΔ*man*BA-vaccinated mice were also significantly protected compared to naïve controls (1.59 U) ($P < 0.05$). BMΔ*virB2* was unable to elicit significant protective immunity in the spleens (0.92 U; $P > 0.05$).

Lung colonization in mice indicated that there were much

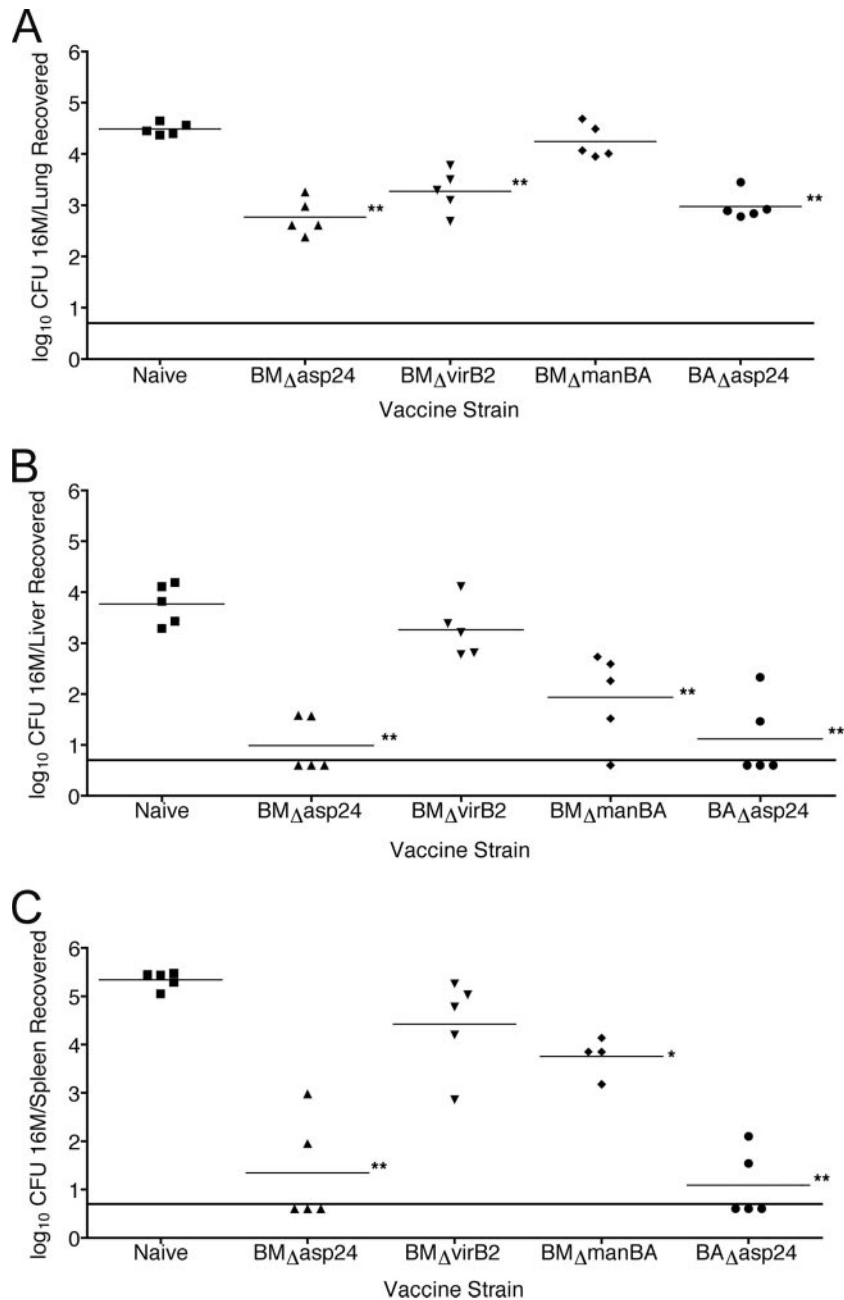

FIG. 5. Evaluation of the efficacy of vaccination against *B. melitensis* aerosol challenge. Groups of four or five female 6- to 8-week-old BALB/c mice were vaccinated via i.p. injection of 1×10^6 CFU/ml of unmarked deletion mutant BMΔ*asp*24, BM*virB2*, BMΔ*man*BA, or BAΔ*asp*24 or PBS in naïve controls. Mice were subsequently challenged with an aerosol chamber dose of 5×10^9 CFU/ml *B. melitensis* 16M at 20 weeks postvaccination (heterologous challenge for BAΔ*asp*24). Four weeks after the virulent challenge, the mice were euthanized, and lungs (A), livers (B), and spleens (C) were extracted to measure recovery of the challenge organism. Data are expressed as the $\log_{10}$ recovery of 16M from the entire organ, and the results for individual mice in each treatment group are shown. The solid line at 0.7 log represents the lower limit of detection, which is ≥5 CFU. For comparisons with naïve control animals using ANOVA with Tukey's honestly significant difference posttest, one asterisk indicates that the *P* value is <0.05 and two asterisks indicates that the *P* value is <0.01.

lower levels of protection against virulent 16M challenge by vaccination with BMΔ*asp*24 (1.72 U) and BMΔ*virB*2 (1.22 U), but the levels were significantly greater than those for naïve controls ($P < 0.001$ for both) (Fig. 5A). The rough mutant, BMΔ*man*BA, protected mouse lungs to a lesser degree (0.25 U, which was not significant).

In contrast to *B. abortus* colonization, *B. melitensis* reached higher levels in the tissues, providing statistically significant results. As a result, significant levels of protection against 16M aerosol infection were observed after vaccination with BMΔ*asp*24 (2.78 U), and three of five mice had no recoverable 16M in their livers ($P < 0.001$) (Fig. 5B). The livers of mice vaccinated with

BMΔ*man*BA also exhibited significant protection compared to naïve controls (1.83U) ($P < 0.001$). BMΔ*virB2* also protected the livers (0.51 U, which was not significant).

Protective efficacy against heterologous aerosol challenge with *B. abortus* and *B. melitensis*. Heterologous challenges of vaccinated mice were performed to evaluate if the highly protective mutants, BAΔ*asp*24 and BMΔ*asp*24, elicit cross-species protection. When mice were challenged with 2308, the BMΔ*asp*24 mutant significantly protected mouse lungs (1.42 U) ($P < 0.001$) and spleens (3.13 U) ($P < 0.01$) (Fig. 4A and 4C). In this case, organisms were cultured from only two of four mouse spleens. The livers were not significantly protected, although there was an evident reduction (0.96 U) (Fig. 4B). When mice were challenged with 16M, the BAΔ*asp*24 mutant significantly protected the lungs (1.51 U) (Fig. 5A) ($P < 0.001$). In the liver, mice were protected (2.65 U), and the challenge organism was not recovered from three of five mice (Fig. 5B) ($P < 0.001$). The spleens of mice were significantly protected (4.25 U), and the challenge organism was not recovered from three of five mice (Fig. 5C) ($P < 0.001$).

DISCUSSION

Human brucellosis is often associated with aerosol transmission and is therefore considered a biowarfare threat. Protection afforded by novel *Brucella* vaccine strains should therefore consider challenge via inhalation of infectious organism to evaluate vaccine efficacy. Aerosol chambers have been successfully used to study infections with several different organisms, including *Mycobacterium tuberculosis*, *Bacillus anthracis*, and *Coxiella burnetti* in mice and guinea pigs (11, 16, 25–27). Aerosol exposure has also been utilized previously to infect rhesus macaques with *B. melitensis*, but this is the first evaluation of aerosol infection in mice, which have been widely used to model human infections (17).

In this study, a Madison aerosol chamber was utilized to infect groups of mice with *B. abortus* or *B. melitensis*. Use of the Madison chamber leads to infection of deep lung tissue, specifically the delivery of droplet nuclei to alveolar spaces, as might be experienced as a result of inhalation during aerosol exposure (26, 30). Ranges of inoculum doses were evaluated to determine differences in the levels of infectivity between the two bacterial species, as well as to establish the kinetics of systemic infection. Under these controlled conditions in an enclosed environment, it was found that as little as a 5-min exposure to 5×10^7 CFU/ml is sufficient to cause infection in the lungs and systemically in mice. However, consistent results for *B. melitensis* required exposure to doses that were 10 to 100 times greater. Although organisms were detected on the fur of animals using cotton swabs, the recovery data suggested that the levels were in the range from 100 to 1,000 organisms per mouse, which is well below the oral dose necessary for infection (12).

It has been previously reported that variation in the *Brucella* exposure dose in primates alters the kinetics of trafficking to peripheral organs (18). This effect was also evident here for the murine model after aerosol inoculation. *B. abortus* infection of mice via the aerosol route results in immediate colonization of lung tissue that is sustained or increases over time, indicating a capacity to replicate within the lung. Bacterial colonization of livers and spleens was delayed as long as 2 weeks before noticeable colonization in these organs became evident, and this colonization occurred without diminution within the lungs, consistent with replication of the organism. *B. melitensis* infections of mice (including lung tissue) appeared to be variable early in infection at lower doses compared to *B. abortus* infections. Significant systemic colonization of spleens and livers was evident as early as 1 week postinfection only in the group receiving the highest dose and occurred 2 weeks postinfection with lower challenge doses.

Comparison of the durations of *Brucella* infections in mice exposed by the i.p. and intravenous routes also revealed a more persistent and chronic infection with *B. abortus* than with *B. melitensis* (6, 7, 9, 15, 21). Although the present studies considered time points up to only 8 weeks postinfection, a similar trend was observed with the aerosol exposure described here. It may be concluded that *B. abortus* infection via the aerosol route elicits a persistent and relatively constant bacteremia in BALB/c mice, requiring a much lower inoculum dose than that required for *B. melitensis*. Persistence in the lung and the chronic nature of the infection appear to extend the period of optimum systemic colonization for both organisms.

Splenomegaly is a well-known clinical manifestation associated with *Brucella* infection that correlates with increased numbers of mononuclear cells and appears to be dependent on the bacterial burden (9, 17–19, 28). Splenomegaly was evident after aerosol exposure with both *B. abortus*, with which splenomegaly was delayed until approximately 6 weeks postexposure, and *B. melitensis*, with which splenomegaly was evident earlier at 4 weeks postexposure. Overall, the gross size of the spleens was not as great as the gross sizes observed when other routes of infection were used, although splenomegaly remained a significant marker of disease in these mice (4, 9, 28).

The highest chamber dose, 5×10^9 CFU/ml, was chosen as the challenge inoculum in efficacy trials. This dose closely mimics the doses used in previous efficacy studies for the same vaccine strains when mice were challenged with 1×10^4 CFU/mouse via the i.p. route (15). The mice in the present experiments received 3.3×10^4 CFU 2308/mouse or 1.3×10^4 CFU 16M/mouse via the aerosol route, and therefore the levels of protection afforded, particularly in the spleens, could be compared for the two studies. It is important to note, however, that in previous studies mice were euthanized 1 week postchallenge, whereas here they were euthanized 4 weeks postchallenge. The 4-week time point was chosen to allow adequate infection of the peripheral organs in order to properly evaluate efficacy.

It is interesting to observe that, particularly for *B. abortus* challenges, the protection afforded to the lungs against infection is not as marked (although for three vaccine strains it is significant) as the protection afforded to the spleen. There are several possible explanations for this observation. The first and most obvious possible explanation is the use of the i.p. route of vaccination. Although not known with certainty, organisms are not expected to traffic to lung tissue, and therefore the lungs may not elicit the same immune response upon challenge infection that would be expected if they were primed by vaccination. Alternatively, it is possible that a lack or diminishment of an inflammatory response in the lungs masks the efficacy of vaccination. It has been previously demonstrated that lungs of mice inoculated intranasally with 16M, although colonized, do

not show substantial histologic changes associated with the infection or an indication of inflammatory responses (18). In addition, rhesus macaques infected with 16M via the aerosol route did not develop significant pathological lesions in lung tissues (17). Epithelial cells of the lungs have been shown to control inflammation and immune responses in the airways and alveoli (24). Protection against aerosol challenge may therefore be enhanced if the vaccine strain itself is delivered via mucosal routes, including oral or nasal delivery.

Another possible explanation for the persistence of *B. abortus* in the lung tissue, even in vaccinated mice, is that *B. abortus* challenge was performed with a dose that overwhelmed the immune response. Particularly for *B. abortus*, we demonstrated that persistent infection also resulted from aerosol exposure at doses that were 10- to 100-fold reduced, and therefore lower doses could be used for challenge.

In this study, we established a novel infection model of *Brucella* infection in BALB/c mice that parallels natural exposure. We showed that consistent *B. abortus* aerosol infection of mice is possible using as few as 4×10^2 organisms per animal, whereas infection with *B. melitensis* requires between 8×10^3 and 1×10^4 organisms per mouse to be consistent. It was also demonstrated that the BAΔ*asp*24 and BMΔ*asp*24 unmarked deletion mutants, which have been previously shown to elicit superior protection against i.p. challenge, also evoke significant protection against *B. abortus* and *B. melitensis* homologous and heterologous aerosol challenges in lungs, livers, and spleens. The BMΔ*asp*24 mutant was also previously tested for safety in pregnant goats and did not cause abortion (14). As such, the Δ*asp*24 deletion mutants remain excellent candidates for further evaluation due to their protective ability, while they remain safe for pregnant animals. Future studies should include vaccination of mice intranasally to enhance mucosal immunity for improvement of memory responses against aerosol infection with *Brucella*.

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REFERENCES

1. **Bhattacharjee, A. K., M. J. Izadjoo, W. D. Zollinger, M. P. Nikolich, and D. L. Hoover.** 2006. Comparison of protective efficacy of subcutaneous versus intranasal immunization of mice with a *Brucella melitensis* lipopolysaccharide subunit vaccine. Infect. Immun. **74:**5820–5825.
2. **Bhattacharjee, A. K., L. Van de Verg, M. J. Izadjoo, L. Yuan, T. L. Hadfield, W. D. Zollinger, and D. L. Hoover.** 2002. Protection of mice against brucellosis by intranasal immunization with *Brucella melitensis* lipopolysaccharide as a noncovalent complex with *Neisseria meningitidis* group B outer membrane protein. Infect. Immun. **70:**3324–3329.
3. **Bossi, P., A. Tegnell, A. Baka, F. Van Loock, J. Hendriks, A. Werner, H. Maidhof, and G. Gouvras.** 2004. Bichat guidelines for the clinical management of brucellosis and bioterrorism-related brucellosis. Eur. Surveill. **9:**E15–E16.
4. **Campos, E., S. L. Cravero, L. Delgui, I. Mora, N. Kahn, A. I. Arese, and O. L. Rossetti.** 2002. *Brucella abortus* INTA2, a novel strain 19 (Delta)bp26::luc (Delta)bmp18 double mutant lacking drug resistance markers. Vet. Microbiol. **87:**1–13.
5. **Davos, D. E., C. F. Cargill, M. R. Kyrkou, J. A. Jamieson, and G. E. Rich.** 1981. Outbreak of brucellosis at a South-Australian abattoir. 2. Epidemiological investigations. Med. J. Aust. **2:**657–660.
6. **Edmonds, M. D., A. Cloeckaert, and P. H. Elzer.** 2002. *Brucella* species lacking the major outer membrane protein Omp25 are attenuated in mice and protect against *Brucella melitensis* and *Brucella ovis*. Vet. Microbiol. **88:**205–221.
7. **Enright, F. M., L. N. Araya, P. H. Elzer, G. E. Rowe, and A. J. Winter.** 1990. Comparative histopathology in BALB/c mice infected with virulent and attenuated strains of *Brucella abortus*. Vet. Immunol. Immunopathol. **26:** 171–182.
8. **Godfroid, J., A. Cloeckaert, J. P. Liautard, S. Kohler, D. Fretin, K. Walravens, B. Garin-Bastuji, and J. J. Letesson.** 2005. From the discovery of the Malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. Vet. Res. **36:**313–326.
9. **Guilloteau, L. A., J. Dornand, A. Gross, M. Olivier, F. Cortade, Y. L. Vern, and D. Kerboeuf.** 2003. Nramp1 is not a major determinant in the control of *Brucella melitensis* infection in mice. Infect. Immun. **71:**621–628.
10. **Hoover, D. L., R. M. Crawford, L. L. Van De Verg, M. J. Izadjoo, A. K. Bhattacharjee, C. M. Paranavitana, R. L. Warren, M. P. Nikolich, and T. L. Hadfield.** 1999. Protection of mice against brucellosis by vaccination with *Brucella melitensis* WR201(16MΔ*purEK*). Infect. Immun. **67:**5877–5884.
11. **Hughes, M. A., C. S. Green, L. Lowchyj, G. M. Lee, V. K. Grippe, M. F. Smith, Jr., L. Y. Huang, E. T. Harvill, and T. J. Merkel.** 2005. MyD88-dependent signaling contributes to protection following *Bacillus anthracis* spore challenge of mice: implications for Toll-like receptor signaling. Infect. Immun. **73:**7535–7540.
12. **Izadjoo, M. J., A. K. Bhattacharjee, C. M. Paranavitana, T. L. Hadfield, and D. L. Hoover.** 2004. Oral vaccination with *Brucella melitensis* WR201 protects mice against intranasal challenge with virulent *Brucella melitensis* 16M. Infect. Immun. **72:**4031–4039.
13. **Izadjoo, M. J., Y. Polotsky, M. G. Mense, A. K. Bhattacharjee, C. M. Paranavitana, T. L. Hadfield, and D. L. Hoover.** 2000. Impaired control of *Brucella melitensis* infection in Rag1-deficient mice. Infect. Immun. **68:**5314–5320.
14. **Kahl-McDonagh, M. M., P. H. Elzer, S. D. Hagius, J. V. Walker, Q. L. Perry, C. M. Seabury, A. B. den Hartigh, R. M. Tsolis, L. G. Adams, D. S. Davis, and T. A. Ficht.** 2006. Evaluation of novel *Brucella melitensis* unmarked deletion mutants for safety and efficacy in the goat model of brucellosis. Vaccine **24:**5169–5177.
15. **Kahl-McDonagh, M. M., and T. A. Ficht.** 2006. Evaluation of protection afforded by *Brucella abortus* and *Brucella melitensis* unmarked deletion mutants exhibiting different rates of clearance in BALB/c mice. Infect. Immun. **74:**4048–4057.
16. **McMurray, D. N., S. S. Allen, A. Jeevan, T. Lasco, H. Cho, T. Skwor, T. Yamamoto, C. McFarland, and T. Yoshimura.** 2005. Vaccine-induced cytokine responses in a guinea pig model of pulmonary tuberculosis. Tuberculosis (Edinburgh) **85:**295–301.
17. **Mense, M. G., R. H. Borschel, C. L. Wilhelmsen, M. L. Pitt, and D. L. Hoover.** 2004. Pathologic changes associated with brucellosis experimentally induced by aerosol exposure in rhesus macaques (Macaca mulatta). Am. J. Vet. Res. **65:**644–652.
18. **Mense, M. G., L. L. Van De Verg, A. K. Bhattacharjee, J. L. Garrett, J. A. Hart, L. E. Lindler, T. L. Hadfield, and D. L. Hoover.** 2001. Bacteriologic and histologic features in mice after intranasal inoculation of *Brucella melitensis*. Am. J. Vet. Res. **62:**398–405.
19. **Mielke, M. E.** 1991. T cell subsets in granulomatous inflammation and immunity to *L. monocytogenes* and *B. abortus*. Behring Inst. Mitt **88:**99–111.
20. **Miller, C. D., J. R. Songer, and J. F. Sullivan.** 1987. A twenty-five year review of laboratory-acquired human infections at the National Animal Disease Center. Am. Ind. Hyg. Assoc. J. **48:**271–275.
21. **Montaraz, J. A., and A. J. Winter.** 1986. Comparison of living and nonliving vaccines for *Brucella abortus* in BALB/c mice. Infect. Immun. **53:** 245–251.
22. **Pappas, G., P. Panagopoulou, L. Christou, and N. Akritidis.** 2006. Biological weapons: *Brucella* as a biological weapon. Cell. Mol. Life Sci. **63:**2229–2236.
23. **Pappas, G., P. Papadimitriou, N. Akritidis, L. Christou, and E. V. Tsianos.** 2006. The new global map of human brucellosis. Lancet Infect. Dis. **6:**91–99.
24. **Pilette, C., Y. Ouadrhiri, V. Godding, J. P. Vaerman, and Y. Sibille.** 2001. Lung mucosal immunity: immunoglobulin-A revisited. Eur. Respir. J. **18:** 571–588.
25. **Russell-Lodrigue, K. E., M. W. Poels, G. Q. Zhang, D. N. McMurray, and J. E. Samuel.** 2005. Hepatitis associated with *C. burnetii* isolates. Ann. N. Y. Acad. Sci. **1063:**176–180.
26. **Russell-Lodrigue, K. E., G. Q. Zhang, D. N. McMurray, and J. E. Samuel.** 2006. Clinical and pathologic changes in a guinea pig aerosol challenge model of acute Q fever. Infect. Immun. **74:**6085–6091.

27. **Smith, D., E. Wiegeshaus, and V. Balasubramanian.** 2000. Animal models for experimental tuberculosis. Clin. Infect. Dis. **31**(Suppl. 3)**:**S68–S70.
28. **Tobias, L., D. O. Cordes, and G. G. Schurig.** 1993. Placental pathology of the pregnant mouse inoculated with *Brucella abortus* strain 2308. Vet. Pathol. **30:**119–129.
29. **Van De Verg, L. L., A. B. Hartman, A. K. Bhattacharjee, B. D. Tall, L. Yuan, K. Sasala, T. L. Hadfield, W. D. Zollinger, D. L. Hoover, and R. L. Warren.** 1996. Outer membrane protein of *Neisseria meningitidis* as a mucosal adjuvant for lipopolysaccharide of *Brucella melitensis* in mouse and guinea pig intranasal immunization models. Infect. Immun. **64:**5263–5268.
30. **Wiegeshaus, E. H., D. N. McMurray, A. A. Grover, G. E. Harding, and D. W. Smith.** 1970. Host-parasite relationships in experimental airborne tuberculosis. 3. Relevance of microbial enumeration to acquired resistance in guinea pigs. Am. Rev. Respir. Dis. **102:**422–429.
31. **Yagupsky, P., and E. J. Baron.** 2005. Laboratory exposures to brucellae and implications for bioterrorism. Emerg. Infect. Dis. **11:**1180–1185.

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