EVALUATION OF UNMARKED DELETION MUTANTS AS IMPROVED *BRUCELLA* VACCINE STRAINS IN THE MOUSE AND GOAT MODELS

A Dissertation

by

MELISSA MARIE KAHL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2005

Major Subject: Veterinary Microbiology

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Approved by:

Chair of Committee,	Thomas A. Ficht
Committee Members,	L. Garry Adams
	Donald S. Davis
	Renee Tsolis
Head of Department,	Ann Kier

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ABSTRACT

Evaluation of Unmarked Deletion Mutants as Improved *Brucella* Vaccine Strains in the Mouse and Goat Models. (August 2005)

Melissa Marie Kahl, B.S., University of Connecticut

Chair of Advisory Committee: Dr. Thomas A. Ficht

Historical data suggests that prolonged survival of *Brucella* vaccine organisms in the target host enhances immune protection. Recent research has focused upon the development of rough vaccine strains to avoid interference with standard diagnostic tests. Rough organisms are rapidly cleared from the host, however. In an effort to develop improved vaccine strains, we have screened signature tagged mutagenesis banks to identify mutants with varying survival characteristics. We hypothesize that in order for a vaccine to be efficacious, it must survive in the host. In order to test this, we constructed marked and unmarked deletion mutants of *B. abortus* and *B. melitensis* in genes previously demonstrated by transposon mutagenesis to attenuate *in vivo* and *in vitro* virulence. Survival and efficacy of these novel deletion mutants were then evaluated in the mouse model. The *asp24* mutants, which persist for extended periods *in vivo,* appear superior as a vaccine candidate compared to approved vaccine strains S19 and Rev1 in the mouse model against either homologous or heterologous challenges. Once enhanced protection against infection was demonstrated in the mouse, components of immune function that appeared to be most important were identified to correlate the immune response with the observed protection. We demonstrated that the most

persistent mutant, Δ*asp24*, affords the greatest protection in mice against virulent challenge. In order to evaluate safety of the novel vaccine strains as well as protection against infection and abortion, we tested selected *B. melitensis* unmarked deletion mutants in a natural host, the goat. The Δ*asp24* mutant was shown to be safe in pregnant goats while providing significant protection against infection and abortion.

DEDICATION

To my parents, Paul and Jeanne Kahl, for their unlimited support, encouragement and love, without which I would not have been able to dedicate myself to my education.

To my husband, Jonathan McDonagh, who is a constant source of strength, support and love.

To Murray, Dixie, Ella, Maggie, A.J, Keeper, Dudley, Lola, Scooter, and KiKi, for always making me smile.

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Thomas Ficht, for his support, wisdom, endless ideas, pushing me when I needed it, and accepting my work kinetics. I was lucky to be guided by a fellow New Yorker.

I would also like to thank my committee members for all their encouragement and support. In particular, Dr. Donald Davis for all the trips to Baton Rouge to help with the goats, Dr. L. Garry Adams for making the time to read my histology slides, and Dr. Renee Tsolis for all her practical experience in the lab when I first began graduate school.

Our collaborators at Louisiana State University, including Dr. Philip Elzer, Sue Hagius, and Joel Walker were invaluable with their help in the goat studies.

To all my labmates and friends throughout the years: to Priscilla Hong for teaching me mouse work, Josh Turse for all the endless computer help and discussions, Carol Turse for keeping the lab running smoothly, Dr. Pei for the wealth of immunology help, and to everyone else for the support and conversations.

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INTRODUCTION AND LITERATURE REVIEW

BACKGROUND

The organism. *Brucella* species are small, facultative, Gram negative, intracellular coccobacilli. They are classified phylogenetically within the α-2 subdivision of Proteobacteria. The majority of *Brucella* species are subdivided into various biotypes, or biovars, which are defined depending upon growth characteristics such as necessity for the presence of carbon dioxide, growth on dyes, H_2S production, lysis by phage, and agglutination by monospecific sera (80). The biotypes most commonly utilized in research laboratories are strain 2308 (S2308) which is a *B. abortus* biovar 1, 16M which is a *B. melitensis* biovar 1, and strain 1330, which is a *B. suis* biovar 1 (59). The genomes of most *Brucella* typically consist of two circular chromosomes of approximately 2.1 and 1.5 Mb, without plasmids, distinct from many other bacterial species. One species of *Brucella* that differs from this scheme is *B. suis* biovar 3, which has only one chromosome, sized at 3.3 Mb. *B. suis* biovars 1, 2, and 4 are sized slightly smaller. Overall, chromosome I encodes the majority of genes involved in transcription, translation, and protein synthesis. Chromosome II contains many genes involved in membrane transport, energy metabolism, and regulation (40, 59).

This dissertation will follow the style of the journal, Infection and Immunity.

Animal brucellosis. Most species of *Brucella* are the etiologic agents of brucellosis, a disease that affecting numerous animal species that is also a zoonotic disease for humans (6, 23, 27, 40, 53). There are seven different species of *Brucella* classified primarily by their preferential or normal host. *B. melitensis* infects goats, sheep, cows, camels, dogs and humans, *B. abortus* infects cattle, elk, bison, coyotes, swine, and humans, *B. suis* swine, reindeer, dogs, and humans, *B. ovis* sheep, *B. canis* dogs, *B. neotomae* the desert wood rat, and the seventh is unnamed but infects marine mammals (21, 40, 79, 80). Exposure in animals most commonly occurs through inhalation, or ingestion of infected fetal or contaminated tissues, or in some cases may be sexually transmitted (40). Acute infections in some target ruminant hosts are observed after some species of *Brucella* invade the chorionic trophoblast cells of the placenta, resulting in abortion of the fetus. Persistence is then classified by the organism's ability to reside in reproductive tissues and the mammary gland and lymph nodes, chronically shedding into the milk (28). In animals, brucellosis may cause economic losses due to the abortions and infertility (36, 56).

Human brucellosis. Humans may become infected, commonly by *B. abortus, B. melitensis*, and *B. suis*, after contact with infected animals, inhalation of aerosolized organism, or ingestion of contaminated animal products including meat, milk products and milk (7, 56, 69). In humans, *B. melitensis, B. abortus,* and *B. suis* infections cause a reoccurring, or undulant, fever, arthritis, osteomyelitis, spondylitis, dementia, and rarely meningitis or endocarditis (21, 32, 36). Human to human transmission is very rare,

though it has been reported from tissue transplantation or sexual contact (32). Even with proper antibiotic treatment for humans, chronic infections with recurrent periods of disease may occur. The actual incidence of brucellosis in humans is unknown due to poor surveillance and documentation of the disease, but is estimated to be between <0.01 to >200 per 100,000 people in highly endemic areas (32). Areas with high prevalence of brucellosis include the Middle East, southwest Asia, South and Central America, South-Eastern Europe, Africa, the Caribbean and the Mediterranean Basin countries (32, 36).

Due to the highly infectious nature of the organism from aerosol and oral modes of transmission, where only a few organisms would be sufficient to cause infection, *Brucella* are considered a biowarfare agent. Model systems for biological attacks with aerosol *Brucella* estimate a cost of $477.7 million per 100,000 people exposed to the attack, with 413 deaths and 82,500 cases of clinical disease (32). The events of 9/11 broadened the scope of *Brucella* research and interest to include the demand for better protection and reaction of this country against potential bioterrorist threats. There is no effective or approved *Brucella* vaccine for use in humans (7, 32, 40, 56).

Transmission. The normal portal for entry for *Brucella* in animals occurs through nasal, oral, and pharyngeal cavities, where the bacteria penetrate the mucosal epithelium. Depending upon the particular *Brucella* species, the primary route of exposure is varied. *B. abortus* and *B. melitensis* predominately infect the oropharnyx, whereas *B. suis*, *B. canis*, and *B. ovis* naturally transmit through the genital tract in animals (1). They are transported in the host to regional lymph nodes in phagocytic cells

or alone. The bacteria then spread and multiply inside macrophages in lymph nodes, spleen, liver, bone marrow, mammary glands, and reproductive organs (40).

Pathogenesis. *Brucella* species have the ability to resist killing by professional phagocytes, such as neutrophils and macrophages, and are allowed to multiply in the same cells for maintenance of chronic infections (40, 66). It is hypothesized that *Brucella* are able to replicate intracellularly by induction of virulence genes, resulting in persistence of the organism in the host and subsequent disease (41).

There are two documented ways *Brucella* manipulate this intracellular niche. The first is accomplished through a recently identified virulence factor, the Type IV secretion system, encoded by the *virB* operon. It has been demonstrated that *Brucella* evade macrophage killing through VirB-dependent sustained interactions with the endoplasmic reticulum, preventing fusion of *Brucella*-containing vacuoles with lysosomes, which is necessary for intracellular survival and multiplication. *Brucella virB* mutants exhibit reduced survival *in vitro* and in mice, due to the inability of the organism to prevent this fusion and subsequently replicate (8, 10, 11, 14, 41).

O antigen is also involved in the avoidance of the endosome pathway that normally kills pathogens. It has been shown that smooth strains of *B. suis* contained within phagosomes do not fuse with lysosomes, whereas rough versions fuse rapidly (41, 63). Rough mutants have also been shown to be taken up by macrophages in greater numbers than smooth organisms and become cytopathic for the macrophages, potentially recruiting immune factors and/or aiding the spread of organism to neighboring cells (60).

The defined role of O antigen in *Brucella* pathogenesis is unclear, but it does hold an important role in virulence of the organism.

Current prophylaxis. Overall, prevention and control of the disease in target animal species relies mainly upon detection of infected animals and their elimination from herds. Vaccination is used to provide some level of immunity for protection against infection and reduction of transmission by reducing abortions (36). Vaccination with live vaccines has proven much more successful than vaccination with heat-killed organisms or subunits (42, 49, 54, 55, 69, 78, 81). In the majority of the United States, *B. abortus* infection in cattle has been successfully eradicated by the combined use of vaccination, testing for infection, and slaughter of seropositive animals. Commonly, strain 19 (S19) and more recently, rough *Brucella* strain 51 (RB51), have been used for vaccination in cattle herds. S19 has several disadvantages, including inducing humoral responses that interfere with standard diagnostic tests, leading to confusion distinguishing reactor animals that were vaccinated from naturally infected ones. S19 protects only 65 to 75 percent of vaccinated cattle, can cause abortions if given to pregnant animals, and it is pathogenic to humans as well (36, 58, 76). RB51 was created as an improved vaccine strain that would not interfere with currently available diagnostic tests, since it lacks O antigen. It was derived by repeated passage of 2308 on solid media containing various concentrations of rifampin and penicillin, and is less virulent than S19 in mice (36, 68, 76). Accidental exposure of humans to RB51 through needle sticks or eye splashes can result in local adverse events, and it is possible that systemic

brucellosis could develop (4). Rev. 1 vaccine was created to prevent *B. melitensis* infections in goats and sheep. It is resistant to streptomycin, induces positive serology, and though attenuated is still virulent in pregnant animals and humans (69).

Disease prevention in target wildlife species. The vaccine strains S19 and RB51 vaccines are not ideal for all ruminant species. In particular, S19 may cause abortion when administered to pregnant bison and elk and RB51 fails to protect most bison and elk from challenge infection (17, 22). RB51 vaccination in caribou and reindeer is not safe and can cause abortions from the vaccination itself (17). As a result, improved vaccines need to be created that will combine safety and efficacy to target species, including livestock and relevant wildlife. The optimal vaccine candidate should be unable to colonize the reproductive tissues in the pregnant ruminant, yet would persist long enough to stimulate a protective immune response for protection against abortion and infection. This is particularly important in large free-ranging herds of bison and elk, where determining the pregnancy status of animals is virtually impossible. In bison and elk, outbreaks of brucellosis are devastating to the state and domestic herds of surrounding states. States may lose *Brucella*-free status if the disease is detected in domestic herds. After the loss of *Brucella*-free status cattle, bison, elk and deer are not easily transported from these states, which is a huge economic loss for the livestock industry. There is a demand for safe *Brucella* vaccines with significant levels of protection for both animals and in humans.

CREATION OF DELETION MUTANTS IN *B. abortus* AND *B. melitensis*

INTRODUCTION

The drawback of using transposon mutants as vaccine strains in their present state is the very element that allowed identification in the first place: the kanamycin resistance. Since it is not desirable to release an antibiotic resistant *Brucella* strain into the field, particularly one possessing the ability of a movable element (transposon), removal of this resistance while retaining attenuation of the mutant is ideal. There are several methods commonly employed to successfully create unmarked gene deletions in various bacterial species. Targeting specific genes by removing them, leaving behind no antibiotic resistance, has several advantages over insertion of a transposon or antibiotic resistance gene. Insertional mutagenesis risks the accidental creation of polar effects, particularly when the element is inserted within an operon. It is also possible to unintentionally create secondary mutations elsewhere in the chromosome, rather than only in the gene of interest (46). By utilizing homologous recombination between the bacterial chromosome and a plasmid carrying cloned chromosomal sequences and a selectable marker, unmarked, in-frame deletions can be created. Mutants generated in this fashion were used to demonstrate a correlate between persistence of the mutant and efficacy as a vaccine.

Numerous attenuated mutants have been constructed in *Brucella* species via transposon mutagenesis (2, 27, 38). The signature tagged mini Tn5 has proven to be extremely efficient for identifying several *Brucella* genes encoding factors or products

vital for survival and virulence within the host simultaneously in a single mouse (38, 44). Mutants defective for genes required early in infection were rapidly cleared from the mouse model and from macrophages in culture. Mutants defective for genes required later in infection were retained longer in the mouse model, and depending on the defect may or may not show any difference in the relatively short time span of the macrophage assay (<72 hours) (dissertation by Priscilla Hong). Since mice infected via intraperitoneal injection with wild-type *B. abortus* may retain bacteria within the spleen for up to 24 weeks post-infection, the degree of persistence can therefore be compared to wild-type infection (38). Mutants may be assigned to tentative groups based upon their rate of clearance from the mouse. Mutants that clear within two weeks of infection are considered highly attenuated, and are designated as class I or II mutants depending on whether they recover to normal levels (class II) or do not recover (class I) later. Class II mutants are of little use as vaccine strains. Class III mutants demonstrate reduced survival only after 8 weeks of infection, such as S19 in the mouse (74).

In order to evaluate protection as a function of persistence of the vaccine strain mutants representing two of the different classes described above were selected for further study. Rough mutants were selected as one representative of class I mutants due to a lack of interference with current diagnostic tests from an interrupted O-antigen portion of the lipopolysaccharide (LPS). Organisms with intact LPS are considered smooth, whereas organisms with interruptions or deletions in LPS components are referred to as rough. Lipopolysaccharide consists of three domains: lipid A, the core oligosaccharide, and O antigen (48, 63, 77). The perosamine O-side chain is an

immunodominant antigen, evoking the majority of the antibody response of animals and humans infected or vaccinated with smooth *Brucella* species (68). O-antigen is the best studied virulence factor in *Brucella*, yet the basis for the attenuated phenotype of rough mutants remains contentious (2, 30, 48, 63, 77). Reliance on O-antigen for serological diagnosis of brucellosis is based on potent antibody responses directed against this cell surface antigen and interest in the use of rough organisms as vaccines stems from the ease of detection of anti-O-antigen antibodies (48, 69). However, the rapid clearance of rough organisms is often considered a weakness of rough vaccine strains preventing a long-term protective immune response (48). Furthermore, the immunity induced by RB51 appears to vary from host to host, and is ineffective in several wildlife species (17, 22).

The gene chosen for a rough vaccine candidate was *manBA*. Phosphomannomutase, encoded by *manB*, is an enzyme that converts mannose-6-phosphate to mannose-1-phosphate, which is necessary for perosamine synthesis and in turn O-antigen. In the mouse model as well as in tissue culture, mutants of this locus exhibit reduced survival, and were chosen to evaluate efficacy as a vaccine strain (2, 30, 48, 63, 77).

Because of the controversy surrounding the use of rough vaccines we chose to also evaluate a second organism from the first class of mutants (group I). Mutants defective in the *vir*B operon were also unable to establish persistent infections past two weeks in the mouse STM screen. This region is homologous to the *virB* operon in *Agrobacterium tumefaciens* in which it encodes a Type IV secretion system. This

system is described as a family of proteins forming a complex serving to secrete or export macromolecules (8, 10). Various mutants in this operon are avirulent in the mouse model, suggesting that a completely functional VirB system is necessary for the establishment and maintenance of infection (10, 18), and reduced survival in cultured macrophages (8-11, 18, 41).

In conjunction with Dr. Renée Tsolis's lab, marked and unmarked deletion mutants of *virB*2 were made. In *A. tumefaciens*, this gene encodes a bacterial cell surface pilus necessary for protein export (8, 30). It is hypothesized that without the formation of the pilus, factors or proteins that may be secreted by the VirB system of *Brucella*, which have yet to be identified, do not reach their destination, thus reducing virulence of the strain. It was a logical step to construct a knockout of this gene, since the pilus is thought to be directly responsible for the transport of effector molecules that may be involved in the virulence of *Brucella.*

Another mutant identified by Tn5 mutagenesis that would fall into the Class I mutants is *cyd*B of the *cyd*DCBA operon of *B. abortus*. In *E. coli*, the genes *cyd*A and *cyd*B encode the two subunits of the membrane-bound alternate terminal oxidase for microaerobic respiration, cytochrome bd, which may enhance survival at low oxygen concentrations or by limiting production of oxygen radicals (15, 23). Without cytochrome bd oxidase in *E. coli*, oxygen levels can increase in the cytoplasm to detrimental quantities. The Tn5 mutant demonstrated a reduction in survival in the mouse model due to the loss of oxidase activity. This locus was chosen due to its rapid

attenuation in vivo, and because it contains both known virulence determinants, the virB system and LPS.

Mutants that are not rapidly cleared in the mouse model have exhibited superior protective immunity (Hong, et al, unpublished). It was desirable to choose a class III mutant to compare survival with vaccine efficacy. The *asp24* gene was selected, as mutants defective in the 24 kDa acid shock protein persist for extended periods without causing the typical chronic infection. Originally, this protein was identified by its increased expression within macrophages, and under low pH conditions *in vitro (45)*. Adaptation to low pH occurs for *Brucella* within the first few hours of growth during a period in which intracellular numbers of bacteria declines to 1-10% of bacterial uptake (62). It is hypothesized that a protein such as Asp24 is therefore important for replication and dissemination of *B. abortus* in the host cells during later stages (45). In the mouse (G. Song, data not published), *asp24*::kan mutants exhibit approximately one and a half logs reduction in survival by 8 weeks infection and no reduction was detected in macrophages *in vitro*. Mutants of this gene will be used to correlate survival with protection.

MATERIALS AND METHODS

Bacterial strains and plasmids for cloning. *E. coli* cultures were grown on Luria-Bertani (LB, Difco Laboratories) plates overnight at 37°C with or without supplementation of kanamycin (100 mg/l), carbenicillin (100 mg/l) or chloramphenicol (50 mg/l). Frozen stocks were prepared in LB broth supplemented with 50% glycerol

(v/v) and stored at –80°C. *E. coli* cultures used included: DH5α F$^-$ ϕ80*dlac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 r*ec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1(Invitrogen), Top10 (F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G) (Invitrogen), DH10B(F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *nup*G) (Invitrogen).

Various plasmids were utilized for cloning and electroporation purposes: pBluescriptKSII+ from Stratagene (f1+ origin, Ap^R, β-galactosidase α-fragment, pUC origin, *lac* promoter), pKD4 (FLP/FRT, Km^R) (37), pCR2.1 from Invitrogen (TA overhang for cloning, Ap^R, Kan^R), pEX18Ap (*sacB*, Ap^R *)* (70), and pBBR1.mcs (Cm^R).

Recombinant plasmid construction. In order to construct a vector to eliminate a gene of interest, primers were designed to amplify sequences flanking the gene to be deleted. These flanking regions are called the 5' and the 3' fragment, and were joined to one another using overlap extension PCR using specially designed PCR primers. Importantly, the reverse primer of the 5' fragment and the forward primer of the 3' fragment include sequence complimentary to the opposite fragment and a unique restriction site. The 5' and 3' fragments were amplified in separate reactions, removed from a gel and then purified. The two products were then used as template for PCR (52). The ends of the joined product were trimmed by digestion with the restriction enzymes engineered into the primers and the final fragment was gel purified for cloning into pBluescript KSII+. The vector was digested with the same restriction enzyme on the 5'

and 3' ends of the overlap fragment, and subsequently dephosphorylated to prevent re-ligation of the vector. The gel purified overlapping PCR product was then ligated to pBluescript.

The plasmid created by inserting an antibiotic resistance cassette between the upstream and downstream fragments of this backbone vector was used to generate marked deletion mutants. A kanamycin cassette was amplified via PCR from the plasmid pKD4 with primers containing the unique restriction site located within the overlap of the backbone vector, and inserted. pKD4 is a plasmid created for the FLP-FRT system whose Kan cassette is the nptII version from Tn5 (16).

The construction of the plasmid for creation of unmarked deletion mutants entailed cloning of the original overlapping PCR product (without the kanamycin cassette) into a plasmid containing *sacB*, which is pEX18Ap (37). The novel construct was removed and inserted into pEX18Ap using the appropriate restriction enzymes. This construct is referred to as the unmarked plasmid.

Transformation and selection of recombinant plasmids. Following ligations of the PCR product to the vector, potential clones were transformed into DH5α, DH10B, or Top10 competent cells. 40μl of cells were mixed with a 1μl portion of the ligation mixture and kept on ice for 30 minutes. Samples were then heat shocked at 42°C for 30 seconds, then immediately placed on ice for 2 minutes. SOC (6% trypticase soy broth (w/v), 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$ and 20mM glucose was added to the samples, which were then incubated at 37°C with agitation for one hour. After the

incubation period, portions of the culture were plated onto solid media supplemented with appropriate antibiotics and incubated overnight. Colonies were picked and grown overnight in 5 ml cultures with appropriate antibiotics. Recombinant plasmids were purified (Sigma Mini Prep kit) and analyzed using restriction enzymes.

Bacterial strains for electroporation. Virulent *B. abortus* strain S2308 was obtained from Billy Deyoe at the National Animal Disease Center in Ames, Iowa, from an aborted calf passaged once in culture, and frozen as glycerol stocks at –80°C. *B. melitensis* biovar 1 (16M) was originally acquired from Dr. Philip Elzer at Louisiana State University in Baton Rouge, Louisiana, from an aborted goat fetus. After usage of this strain in the 2002 goat safety trial, another virulent isolate was obtained from a Texas A&M aborted goat fetus. This strain was cultured, kept as frozen stocks, and used as the parental strain used to generate deletion mutants, as well as the virulent challenge strains for controls and in efficacy studies. To prepare cells for electroporation, 16M and S2308 were grown from the frozen stocks on tryptic soy agar (TSA; Difco Laboratories) or *Brucella* agar (Difco Laboratories) to confluence for 3 days at 37°C. Bacteria were harvested by carefully scraping bacteria off the plate with a metal spreader into 6 ml of PBS (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.4mM KH_2PO_4, pH 7.3). Bacterial suspensions were removed from the plate using a pipette and transferred into a 50ml conical tube. Typically, two plates of each species were prepared and harvested. The final suspension of 10 ml contains approximately $4x10^{11}$ CFU/ml as estimated turbidometrically using a Klett meter. Alternatively, isolated colonies were

selected following 72 hours of growth and used to inoculate 5 ml of fresh tryptic soy broth (TSB; Difco Laboratories). These cultures were grown for 48 hours to achieve saturation. Fifty μl of the saturated culture was used to inoculate 50 ml of fresh TSB, and cultures are incubated overnight with vigorous shaking at 37°C for 16 (early log) to 24 (late log) hours, depending upon the growth phase desired. Liquid cultures grown 24 hours contained approximately $4x10^9$ CFU/ml.

Creation of marked mutants in *B. abortus* and *B. melitensis*. Marked deletion mutants were created in *Brucella* though electroporation of the marked plasmid into either 16M or S2308. Depending upon whether cultures are grown in liquid or on plates, $2x10^{10}$ to $4x10^{11}$ CFU of bacteria, respectively, were pelleted via centrifugation. The supernatant was removed and the cell pellet washed three times with ice-cold water. All centrifugation steps were performed at 1700 x g and 4°C for 15 minutes. After the final wash, the supernatant was decanted, leaving approximately 1 ml in the bottom of the conical to resuspend the cells. Seventy μl of these cell suspensions were used per electroporation with varying amounts of plasmid (1-5 μl total, approximating 1 μg DNA) in a pre-chilled 1mm gap cuvette (BioRad). The cells were shocked in a BTX electroporation apparatus set at 2.2-2.5kV and 246Ω. One ml of SOC-B [6% trypticase soy broth (w/v), 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, and 20mM glucose] medium was immediately added to cuvette, and the suspension was transferred to a fresh tube (43). The tubes were left at room temperature for 10 minutes, then incubated overnight at 37°C with agitation to prevent settling in the tube.

Approximately 12-18 hours later, 100 μl portions of the cultures were plated onto TSA containing kanamycin @100 μg/ml. When colonies arose, they were then replica-plated onto TSA containing kanamycin @100 μg/ml and carbenicillin @100μg/ml. Marked deletion mutants were kanamycin resistant (Km^R) and carbenicillin sensitive (Cb^S). Verification of mutant genotype was obtained via PCR and Southern blot analysis, to ensure that the gene of interest was deleted and the kanamycin cassette is retained.

Creation of unmarked mutants in *B. abortus* and *B. melitensis*. The unmarked plasmid, containing the *sacB* gene, insert, and *bla* gene (same function as carbenicillin) was used for electroporation into marked deletion strains. Utilizing the newly created marked strain enhanced selection, since loss of kanamycin resistance identified unmarked mutants formed via allelic exchange. Electroporation conditions were identical to those described in the construction of marked mutants. After electroporation, cells were plated onto TSA containing carbenicillin @100μg/ml instead of kanamycin to select for the first homologous recombination, i.e. a co-integration. As colonies appeared on the surface of these plates, they were replica-plated onto (a) sucrose plates (TSA without salt, containing 6% (w/v) sucrose, without antibiotic), and (b) TSA containing carbenicillin @100μg/ml. Colonies that grew on carbenicillin (Cb^R) but not sucrose ($sucrose^S$) were co-integrates with functional *sacB* gene, which was imperative for counter-selection to create the unmarked mutant. Resolution of co-integration occurred spontaneously and was selected for by inoculating 5 ml of sucrose broth (TSB, without salt or antibiotics, and supplemented with 6% (w/v) sucrose) and

incubating for 24 hours with agitation at 37°C. After 24 hours of growth, the cultures did not look saturated because the sucrose was toxic to the majority of the cells, but there were enough cells for plating. Aliquots of 100 μl from undiluted, 10 fold, and 100 fold serial dilutions were spread onto sucrose containing media, and incubated at 37°C until growth was evident. Colonies were replica-plated onto (a) sucrose media and (b) TSA containing kanamycin @100μg/ml. Unmarked deletion mutants were sucrose tolerant, resulting from the loss of the integrating plasmid containing *sacB*, and kanamycin sensitive, since the original kanamycin cassette was replaced during plasmid integration.

Validation of gene deletions. Genomic DNA extracts were prepared for deletion mutants using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol. These genomic preps were used as template for PCR, using the primers created for the overlapping PCR to detect the deleted region.

For Southern blots, genomic extracts were digested with restriction enzymes that either flank the deleted gene or cut one time within the region. Samples were electrophoresed overnight on a 0.8% (w/v) agarose gel using constant voltage. The gel was soaked in 0.25N HCl for approximately 15 minutes with agitation. The gel was rinsed with dd$H_2$0, and then placed in 0.4N NaOH for 10 minutes. DNA was transferred to nylon (ny+) membrane overnight in 0.4N NaOH. The membrane is then prehybridized 4-16 hours at 68°C in prehybridization buffer (1.5X SSPE, 0.1% SDS, 0.1% NaPPi, 0.5% Blotto, 10% PEG, 0.1 mg/ml ssDNA).

Probes were prepared by a hot PCR method, incorporating a P^{32}-labeled dATP into the PCR product. PCR was performed normally except for the substitution of this radioactive dATP for unlabeled dATP, and the inclusion of unlabeled dTTP, dGTP, and dCTP to the reaction. After PCR, the product was run through G-50 resin to remove unincorporated radiation. The purified product was then counted on a scintillation counter to determine the specific activity (cpm/μg DNA); amount of labeled probe was adjusted based upon size of membrane, using 100,000 cpm/cm^2 of blot. The probe was then heat denatured for 10 minutes at 95°C, then quenched on ice. Five to ten ml of hybridization buffer (same as prehybridization buffer) was added to the probe and the blot was hybridized for at least 4 hours to overnight. Blots were washed 2 times in 2X SSPE/0.1% SDS at room temperature for 10 minutes, 2 times in 1X SSPE/0.1% SDS at room temperature for 10 minutes, 1 time in 0.1X SSPE/0.1% SDS at room temperature for 10 minutes, then 1 time in 0.1X SSPE/0.1% SDS at 50°for 10 minutes. The blots were exposed to either a phosphoimager or x-ray film for the desired period to give the best signal before development.

RESULTS

Construct development for mutant creation. Primers were designed using MacVector™ to create gene knockouts in *Brucella* species. The primers are listed in Table 1. The conditions for the various primer pairs are listed in Table 2. In total, nine vectors were constructed by a two-round PCR technique to create marked and unmarked

TABLE 1. Primer design for mutant construction and validation

Primer name	Sequence (restriction enzyme engineered)	Fragment
TAF101	5'-GGAATTCGGCAAAGCGAGTGGGTGATTAG-3' (EcoRI)	*asp24* upstream
TAF102	5'-CGGGATCCTGAGCAAGTGCGGGAATAGC-3' (BamHI)	*asp24* upstream
TAF103	5'-CGGGATCCTGGGAATGGAGCGGCTTTAG-3' (BamHI)	*asp24* downstream
TAF104	5'-GCTCTAGATTTGAACACTTGGCGATAGCG-3' (XbaI)	*asp24* downstream
TAF105	5'-CGACTGAAGGCTGGACAGAC-3'	5'of *asp24* deletion
TAF106	5'-CAAACTGGCTGGAGGAAGC-3'	3' of *asp24* deletion
TAF300	5'-CGGGATCCCGCACGTCTTGAGCGATTGTGTAGG-3' (BamHI)	Kan cassette
TAF301	5'-CGGGATCCCGGGACAACAAGCCAGGGATGTAAC-3' (BamHI)	Kan cassette
TAF356	5'-CGGGATCCCTGGAGGAAAACAATCTGGG-3' (BamHI)	*manBA* upstream
TAF357	5'-AAGACGGCGCGCCCGAACCTGTATCTGCCTG-3' (AscI)	*manBA* upstream
TAF358	5'-GTTCGGGCGCGCCGTCTTAACCCAAAACCGCTTCGTA-3' (AscI)	*manBA* downstream
TAF359	5'-GCTCTAGAGGGTTTTCTGATCGATCTGGTAGC-3' (XbaI)	*manBA* downstream
TAF204	5'-GGCGCGCCACGTCTTGAGCGATTGTGTAGG-3' (AscI)	Kan cassette
TAF205	5'-GGCGCGCCGGACAACAAGCCAGGGATGTAAC-3' (AscI)	Kan cassette
TAF160X	5'-GCTCTAGAGCGTGCTCGGCATTCTCATTATCC-3' (XbaI)	*cydBA* upstream
TAF297	5'-TACCGGATCCGGAAAGACCCAAAGTCAGAGGC-3' (BamHI)	*cydBA* upstream
TAF298	5'-TTCCGGATCCGGTATTTTTCCTGGCTTTTGGG-3' (BamHI)	*cydBA* downstream
TAF299	5'-GCTCTAGAGCAAGGCTCACTTTATGCGGAG-3' (XbaI)	*cydBA* downstream

TABLE 2. PCR conditions for the different sets of primers used to generate knockout mutants

PCR Conditions for Primer Pairs

Primer Pair	Hot Start	Denature	Anneal	Elongation	# cycles	Final elongation
101/102	95°C 5 min	95°C 30 sec	58°C 30 sec	72°C 45 sec	30	72°C 10 min
103/104	95°C 5 min	95°C 30 sec	58°C 30 sec	72°C 45 sec	30	72°C 10 min
101/104	95°C 5 min	95°C 30 sec	58°C 30 sec	72°C 45 sec	30	72°C 10 min
105/106	95°C 5 min	95°C 30 sec	58°C 30 sec	72°C 1.5 min	30	72°C 10 min
160X/297	95°C 5 min	95°C 30 sec	62°C 30 sec	72°C 45 sec	30	72°C 10 min
289/299	95°C 5 min	95°C 30 sec	62°C 30 sec	72°C 45 sec	30	72°C 10 min
160X/299	95°C 5 min	95°C 30 sec	65°C 30 sec	72°C 1.5 min	30	72°C 10 min
356/357	95°C 5 min	95°C 30 sec	63°C 30 sec	72°C 1 min	35	72°C 10 min
358/359	95°C 5 min	95°C 30 sec	63°C 30 sec	72°C 45 sec	35	72°C 10 min
356/359	95°C 5 min	95°C 30 sec	63°C 30 sec	72°C 1.2 min	35	72°C 10 min
300/301	95°C 5 min	95°C 30 sec	65°C 30 sec	72°C 1.5 min	30	72°C 10 min
204/205	95°C 5 min	95°C 30 sec	65°C 30 sec	72°C 1.5 min	30	72°C 10 min

gene deletions into *B. abortus* and *B. melitensis* (Fig. 1). Nomenclature and details for engineered plasmids are listed in Table 3.

Constructs to delete *manBA*. The constructs pMMK33 and pMMK31 were engineered to delete *manBA (*Fig. 2). Primers TAF 356 and TAF 357 were used to amplify the region 5' of the asp24 gene. Primers TAF 358 and TAF 359 were used to amplify the area 3' of the deletion (Tables 1 and 2). After purification of these products, they were used as templates in a second round of PCR with TAF 356 and TAF 359 to ligate the products together. This product was digested with BamHI and XbaI and cloned directionally into pBluescript to create pMMK29. Cloning of the same PCR product into pEX18Ap elicited pMMK31. Insertion of the kanamycin resistance gene from pKD4 via amplification with primers TAF204 and TAF205 into the center of the overlap product of pMMK29 by the engineered AscI site created pMMK33 (Table 3).

Constructs to delete *cydBA*. The constructs pMMK14 and pMMK11 were engineered to delete *cydBA* (Fig. 3). Primers TAF 160-X and TAF 297 were used to amplify the region 5' of the asp24 gene. Primers TAF 298 and TAF 299 were used to amplify the area 3' of the deletion (Tables 1 and 2). After purification of these products, they were used as templates in a second round of PCR with TAF 160-X and TAF 299 to ligate the products together. This product was digested with XbaI and cloned non-directionally into pBluescript to create pMMK14. Cloning of the same PCR product into pEX18Ap elicited pMMK11. Insertion of the kanamycin resistance gene from

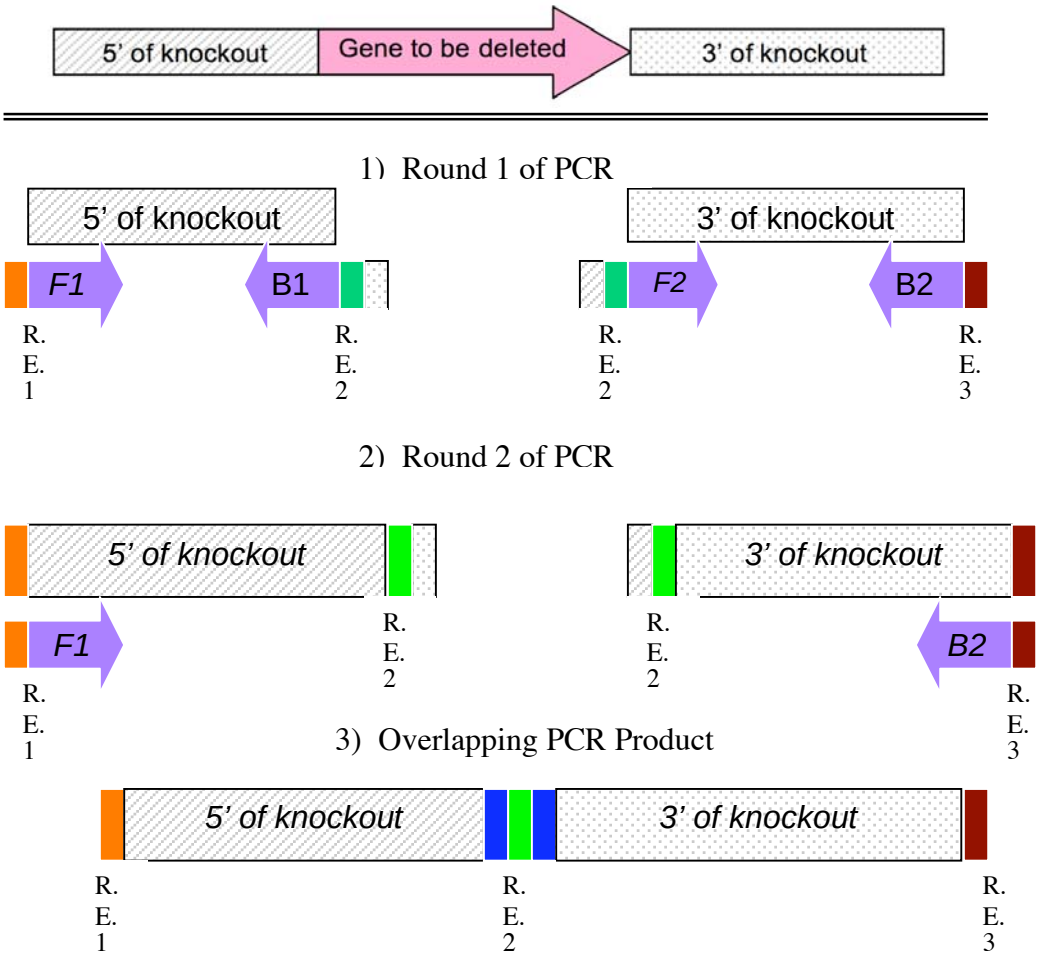

FIG. 1. Overlapping PCR to generate knockout mutant constructs. The first round of PCR amplifies areas 5' and 3' of the gene to be deleted. The forward primer of the 5' product (F1) contains a unique restriction site (R.E. 1). The reverse primer of the 5' knockout (B1) contains another unique restriction site (R.E. 2), as well as an area of homology to the 3' fragment. The forward primer of the 3' product (F2) contains the same restriction site as the reverse primer of the 5' fragment (R.E. 2) as well as an area of homology to the 5' fragment. The reverse primer of the 3' fragment (B2) contains a unique restriction site (R.E. 3). The second round of PCR utilizes the PCR products from the first round as templates for a reaction involving (F1) and (B2) as the primers. From this PCR, one product is produced, the overlapping PCR product, which joins the two products by the areas of overlap and the unique center restriction site (R.E. 2). The overlapping PCR product can be cloned into a plasmid via R.E.1 and R.E. 3, and antibiotic resistance markers can be inserted through R.E. 2.

TABLE 3. Plasmid nomenclature

Plasmid Nomenclature:	Plasmid Details:	Plasmid Purpose:
pMMKB	TAF101/TAF104 cloned into pEX18Ap	To make unmarked *asp24* deletion
pMMK8	TAF101/TAF104 cloned into pBluescript	Intermediate plasmid
pMMK16	pMMK8 separated by TAF300/TAF301 (kanamycin resistance)	To make marked *asp24* deletion
pMMK29	TAF356/TAF359 cloned into pBluescript	Intermediate plasmid
pMMK31	TAF356/TAF359 cloned into pEX18Ap	To make unmarked *manBA* deletion
pMMK33	pMMK29 separated by TAF204/205 kanamycin resistance gene	To make marked *manBA* deletion
pMMK14	TAF 160X/TAF299 cloned into pBluescript	Intermediate plasmid
pMMK15	pMMK14 separated by TAF 330/TAF301 kanamycin resistance gene	To make marked *cydBA* deletion
pMMK11	TAF 160X/TAF 299 cloned into pEX18Ap	To make unmarked *cydBA* deletion
pAV2.2	(18)	Plasmid to make marked *virB2* deletion
pAS1.1	(18)	Plasmid to make unmarked *virB2* deletion

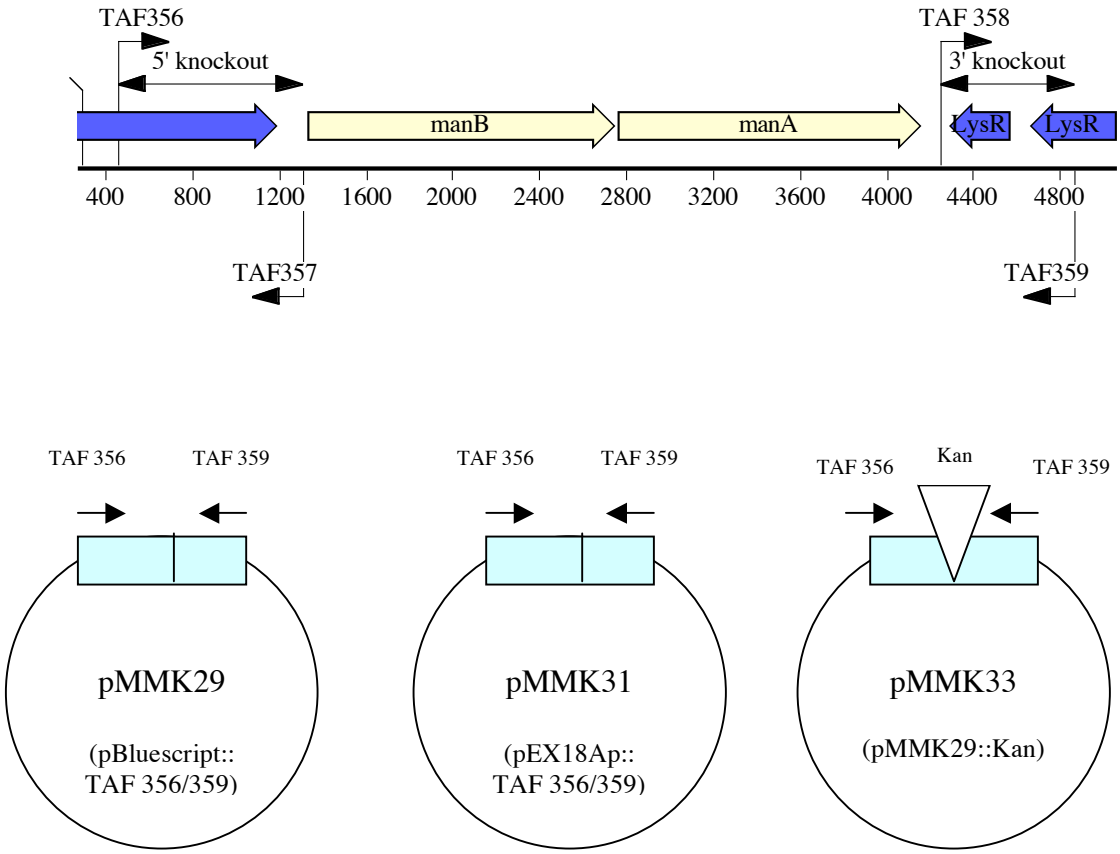

FIG. 2. Creation of plasmids pMMK29, pMMK33, and pMMK31 for *manBA* deletion mutants. The region 5' of *manBA* was amplified using primers TAF 356/ TAF 357 and the region 3' of the gene was amplified using primers TAF 358/TAF 359 as described in the text. The two products were joined together in a second round of PCR using primers TAF 356/ TAF 359. The product was cloned into pBluescript with BamHI and XbaI to elicit pMMK29, and into pEX18Ap with BamHI and XbaI to elicit pMMK31. A kanamycin cassette was amplified from pKD4 with TAF204/205 and cloned into pMMK29 with AscI, which was engineered at the overlapping region that joined the 5' and 3' products together, to elicit pMMK33.

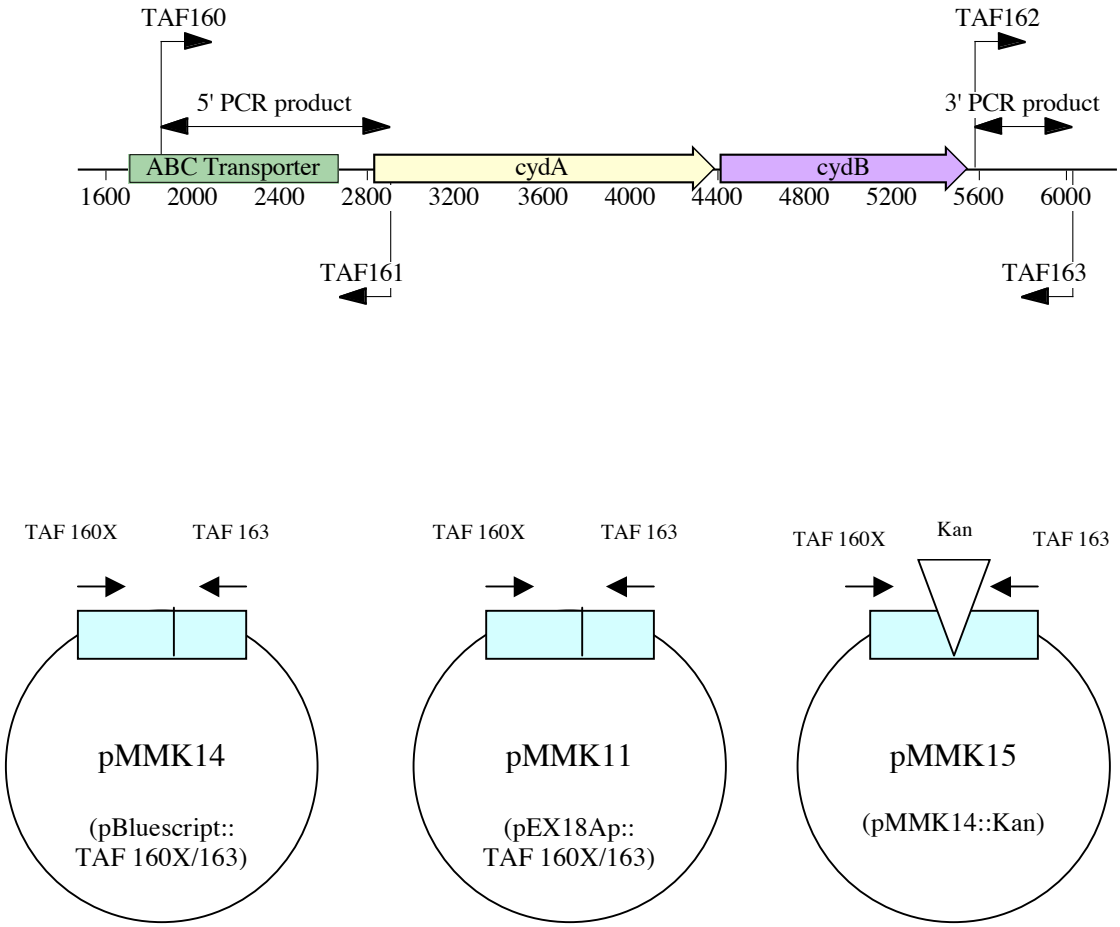

FIG. 3. Creation of plasmids pMMK14, pMMK15, and pMMK11 for *cydBA* deletion mutants. The region 5' of *asp24* was amplified using primers TAF 160X/ TAF 161 and the region 3' of the gene was amplified using primers TAF 162/TAF 163 as described in the text. The two products were joined together in a second round of PCR using primers TAF 160X/ TAF 163. The product was cloned into pBluescript with XbaI to elicit pMMK14, and into pEX18Ap with XbaI to elicit pMMK11. A kanamycin cassette was amplified from pKD4 with TAF300/301 and cloned into pMMK8 with BamHI, which was engineered at the overlapping region that joined the 5' and 3' products together, to elicit pMMK15.

pKD4 via amplification with primers TAF 300 and TAF 301 into the center of the overlap product of pMMK14 by the engineered BamHI site created pMMK15 (Table 3).

Constructs to delete *asp24*. The constructs pMMK16 and pMMKB were engineered to delete *asp24* (Fig. 4). Primers TAF 101 and TAF 102 were used to amplify the region 5' of the asp24 gene Primers TAF 103 and TAF 104 were used to amplify the area 3' of the deletion (Tables 1 and 2). After purification of these products, they were used as templates in a second round of PCR with TAF 101 and TAF104 to ligate the products together. This product was digested with EcoRI and XbaI and cloned directionally into pBluescript to create pMMK8. Cloning of the same PCR product into pEX18Ap elicited pMMKB. Insertion of the kanamycin resistance gene from pKD4 via amplification with primers TAF300 and TAF301 into the center of the overlap product of pMMK8 by the engineered BamHI site created pMMK16 (Table 3).

Construction of Δ*manBA* deletion mutants. Marked deletions of *manBA* were not created in *B. abortus*. Instead, unmarked deletions were created directly by electroporation of pMMK31 into 2308. Mutants were plated directly onto carbenicillin to select for co-integration of the plasmid. These colonies were replica-patched onto sucrose, to identify colonies that were carbenicillin resistant but sucrose sensitive. These colonies were then grown in sucrose; colonies that survived were plated onto TSA and tested via acriflavin agglutination to identify rough mutants. The unmarked strain is referred to as BAΔ*manBA*.

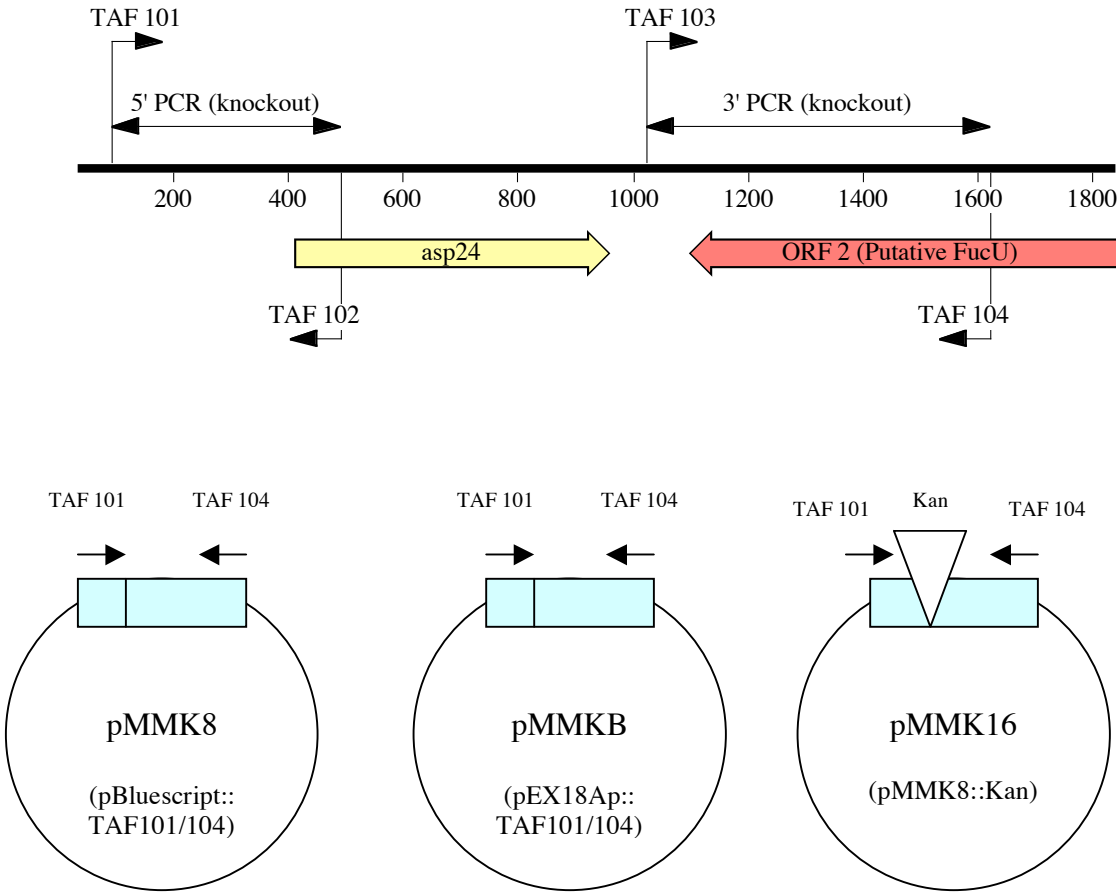

FIG. 4. Construction of plasmids pMMK8, pMMKB, and pMMK16 for *asp24* deletion mutants. The region 5' of *asp24* was amplified using primers TAF 101/ TAF 102 and the region 3' of the gene was amplified using primers TAF 103/TAF 104 as described in the text. The two products were joined together in a second round of PCR using primers TAF 101/ TAF 104. The product was cloned into pBluescript with EcoRI and XbaI to elicit pMMK8, and into pEX18Ap with EcoRI and XbaI to elicit pMMKB. A kanamycin cassette was amplified from pKD4 with TAF300/301 and cloned into pMMK8 with BamHI, which was engineered at the overlapping region that joined the 5' and 3' products together, to elicit pMMK16.

Marked deletions of *manBA* were created in *B. melitensis* via electroporation of pMMK33 into 16M. Knockouts were selected by resistance to kanamycin and sensitivity to carbenicillin. Marked deletions are referred to as BMΔ*manBA*::Kan. Unmarked deletions of *manBA* were created in *B. melitensis* via electroporation of pMMK31 into BMΔ*manBA*::Kan. Mutants chosen for sucrose counter-selection were kanamycin resistant, carbenicillin resistant, and sucrose sensitive. These colonies were grown in sucrose; colonies that survived the selection that were kanamycin resistant were unmarked deletion mutants. The unmarked deletion mutants are referred to as BMΔ*manBA*. Deletion mutants were verified by PCR (Fig. 5).

Construction of Δ*virB2* deletion mutants. Plasmid constructs to engineer deletions of *virB2* in *Brucella* species as well as primers to PCR verify the deletions and produce a probe for Southern blotting were kindly given by Renee Tsolis's lab (18). Marked deletions of *virB2* were created *B. abortus* via electroporation of 2308 with pAV2.2. Knockouts were selected by growth on kanamycin and lack of growth on carbenicillin. Marked deletions are referred to as BAΔ*virB2*::Kan. Unmarked deletions of *virB2* were created in *B. abortus* via electroporation of pAS1.1 into BAΔ*virB2*::Kan. Mutants chosen for sucrose counter-selection were kanamycin resistant, carbenicillin resistant, and sucrose sensitive. These colonies were grown in sucrose; colonies that survived the selection that were kanamycin resistant were unmarked deletion mutants. Unmarked deletions were named BAΔ*virB2*. Mutants were verified by PCR (Fig. 6) and Southern blot (Fig. 7).

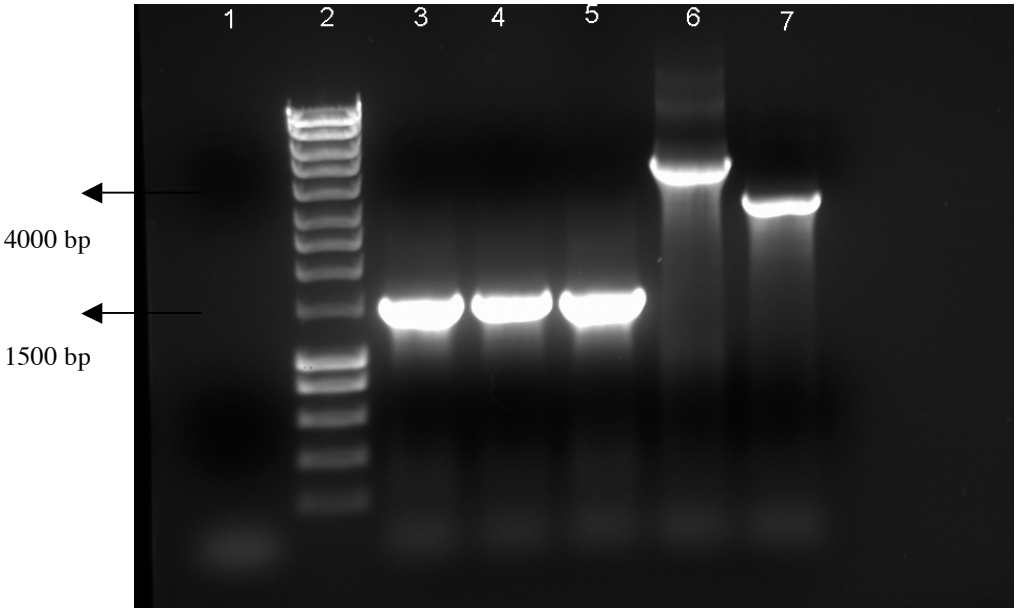

FIG. 5. PCR validation of *B. melitensis manBA* deletion mutants. Genomic DNA was prepared as described in the text and amplified with TAF 356/TAF 359 for BMΔ*manBA* (1465 bp), lanes 3-5; 16M (4410), lane 6; BMΔ*manBA*::Kan (3065), lane 7. A negative PCR control was run in lane 1, and Hyperladder I molecular weight marker was run in lane 2.

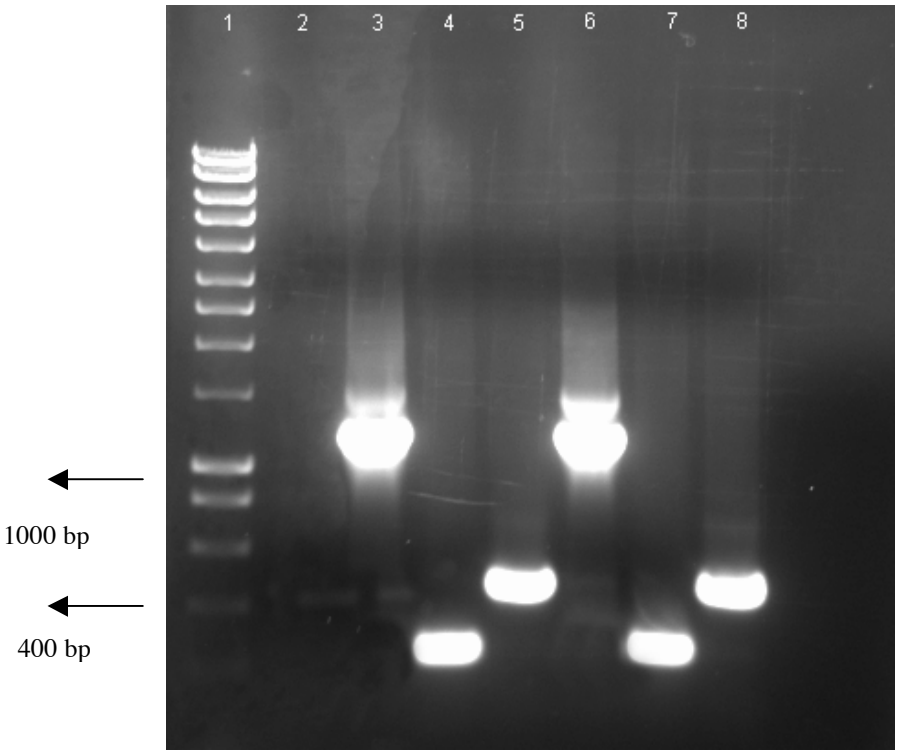

FIG. 6. PCR of *virB2* deletion mutants. Genomic DNA was prepared as described in the text and amplified with VirB1540F/1973R (18) from BAΔ*virB2*::Kan (~1000 bp), lane 3; BAΔ*virB2* (199 bp), lane 4; 2308 (433 bp), lane 5; BMΔ*virB2*::Kan (~1000 bp), lane 6; BMΔ*virB2* (199 bp), lane 7; 16M (433 bp), lane 8. Hyperladder I molecular weight marker was run in lane 1, and a negative PCR control in lane 2.

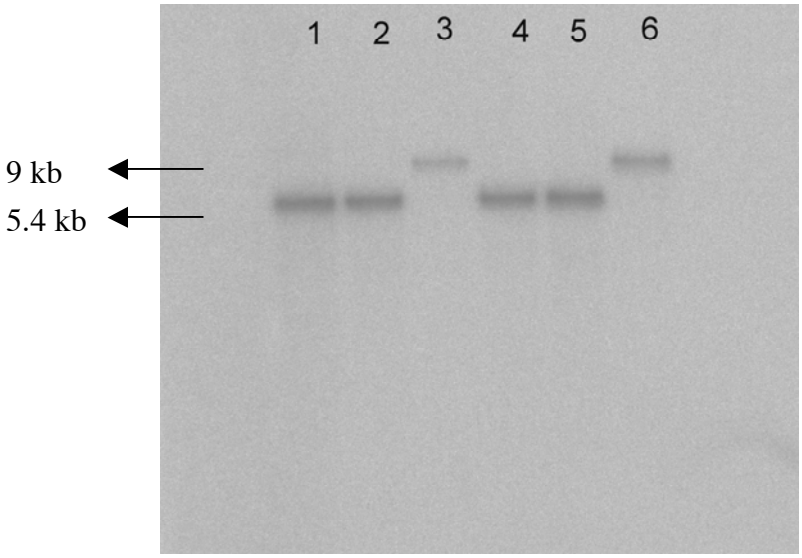

FIG. 7. Southern blot validation of *virB2* deletion mutants digested with EcoRI. Genomic DNA was isolated as described in the text and digested with EcoRI from BAΔ*virB2*::Kan (5.4 kb), lane 1; BAΔ*virB2 (*5.4 kb), lane 2; 2308 (9 kb), lane 3; BMΔ*virB2*::Kan (5.4 kb), lane 4; BMΔ*virB2 (*5.4 kb), lane 5; 16M (9 kb), lane 6. Both marked and unmarked deletions are the same size because of the location of the EcoRI site; it was engineered during the cloning of the fragment 3' of the knockout.

Marked deletions of *virB2* were created *B. melitensis* via electroporation of 16M with pAV2.2. Knockouts were selected by resistance to kanamycin and sensitivity to carbenicillin. Marked deletions are referred to as BMΔ*virB2*::Kan. Unmarked deletions of *virB2* were created in *B. melitensis* via electroporation of pAS1.1 into BMΔ*virB2*::Kan. Mutants chosen for sucrose counter-selection were kanamycin resistant, carbenicillin resistant, and sucrose sensitive. These colonies were grown in sucrose; colonies that survived the selection that were kanamycin resistant were unmarked deletion mutants. Unmarked deletions were named BMΔ*virB2*. Mutants were verified by PCR (Fig. 6) and Southern blot (Fig. 7).

Construction of Δ*cydBA* deletion mutants. Marked deletions of *cydBA* were created *B. abortus* via electroporation of 2308 with pMMK15. Knockouts were selected by growth on kanamycin and lack of growth on carbenicillin. Marked deletions are referred to as BAΔ*cydBA*::Kan. Unmarked deletions of *cydBA* were created in *B. abortus* via electroporation of pMMK11 into BAΔ*cydBA*::Kan. Deletions were verified by PCR (Fig. 8). Mutants chosen for sucrose counter-selection were kanamycin resistant, carbenicillin resistant, and sucrose sensitive. These colonies were grown in sucrose; colonies that survived the selection that were kanamycin resistant were unmarked deletion mutants. Unmarked deletions are referred to as BAΔ*cydBA*. Marked deletions of *cydBA* were created *B. melitensis* via electroporation of 16M with pMMK15. Knockouts were selected by growth on kanamycin and lack of growth on

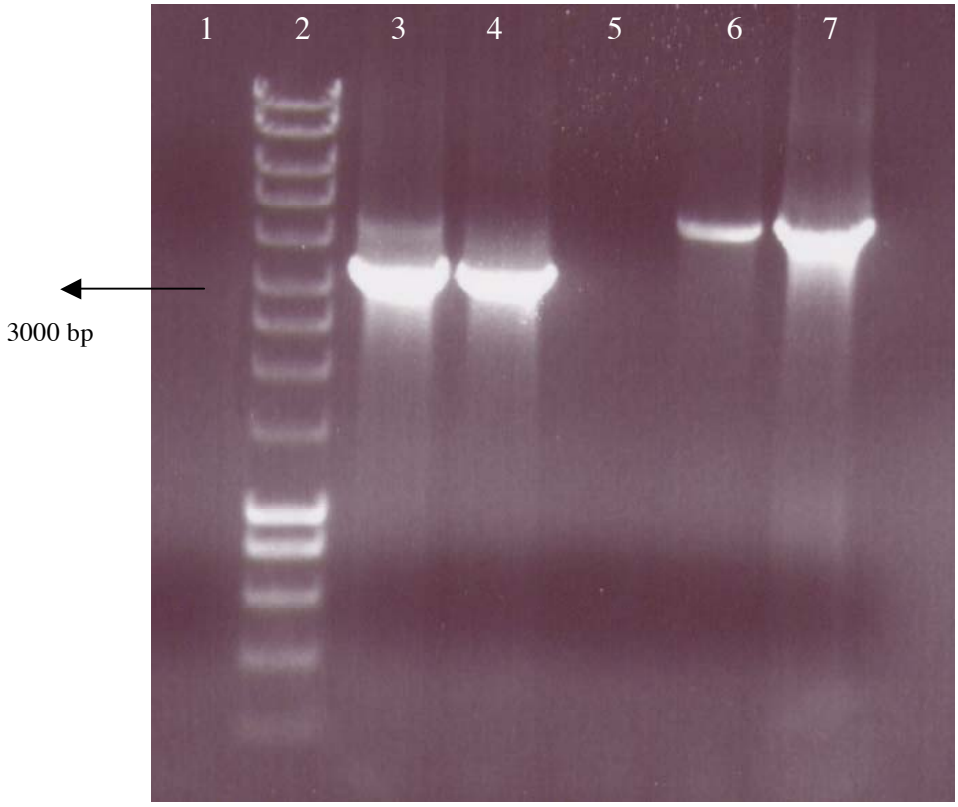

FIG. 8. PCR validation of marked *B. abortus cydBA* deletion mutants. Genomic DNA was prepared as described in the text and amplified with TAF 160-X/TAF 299 for BAΔ*cydBA::*Kan (3006 bp), lanes 3-4; 2308 (4172 bp), lanes 6-7. Negative PCR controls were run in lanes 1 and 5; Molecular weight marker Hyperladder I was run in lane 2.

carbenicillin. Marked deletions are referred to as BMΔ*cydBA*::Kan. Unmarked deletions of *cydBA* were created in *B. melitensis* via electroporation of pMMK11 into BMΔ*cydBA*::Kan. Mutants chosen for sucrose counter-selection were kanamycin resistant, carbenicillin resistant, and sucrose sensitive. These colonies were grown in sucrose; colonies that survived the selection that were kanamycin resistant were unmarked deletion mutants. Unmarked deletions are referred to as BAΔ*cydBA*. Deletions were verified by PCR (Fig. 9) and Southern blot (Fig. 10).

Construction of Δ*asp24* deletion mutants. Marked deletions of *asp24* were created *B. abortus* via electroporation of 2308 with pMMK16. Knockouts were selected by resistance to kanamycin and sensitivity to carbenicillin. Marked deletions are referred to as BAΔ*asp24*::Kan. Unmarked deletions of *asp24* were created in *B. abortus* via electroporation of pMMKB into BAΔ*asp24*::Kan. Mutants chosen for sucrose counter-selection were kanamycin resistant, carbenicillin resistant, and sucrose sensitive. These colonies were grown in sucrose; colonies that survived the selection that were kanamycin resistant were unmarked deletion mutants. Unmarked deletions are referred to as BAΔ*asp24*. Deletion mutants were verified by PCR (Fig. 11) and Southern blot (Fig. 12).

Marked deletions of *asp24* were created *B. melitensis* via electroporation of 16M with pMMK16. Knockouts were selected by resistance to kanamycin and sensitivity to carbenicillin. Marked deletions are referred to as BMΔ*asp24*::Kan. Unmarked deletions of *asp24* were created in *B. melitensis* via electroporation of pMMKB into

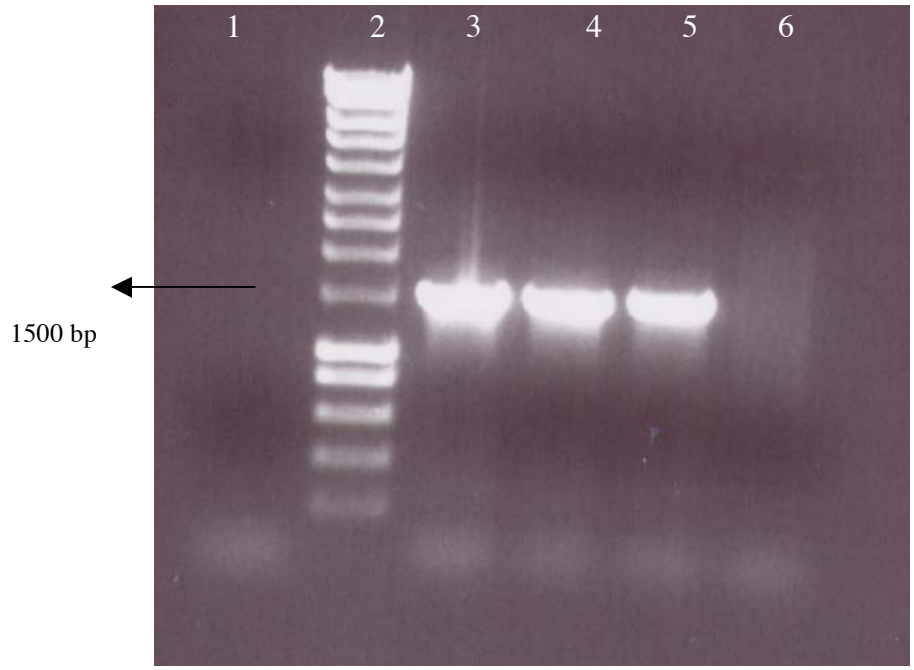

FIG. 9. PCR validation of unmarked *B. melitensis cydBA* deletion mutants. Genomic DNA was prepared as described in the text and amplified with TAF 160-X/TAF 299 for BMΔ*cydBA* (1506 bp), lanes 3-5; 16M (4172 bp), lane 6. A negative PCR control was run in lane 1, and molecular weight marker Hyperladder I was run in lane 2. The positive control 16M did not amplify under the conditions used; a longer elongation time was necessary to generate the 4172 bp product.

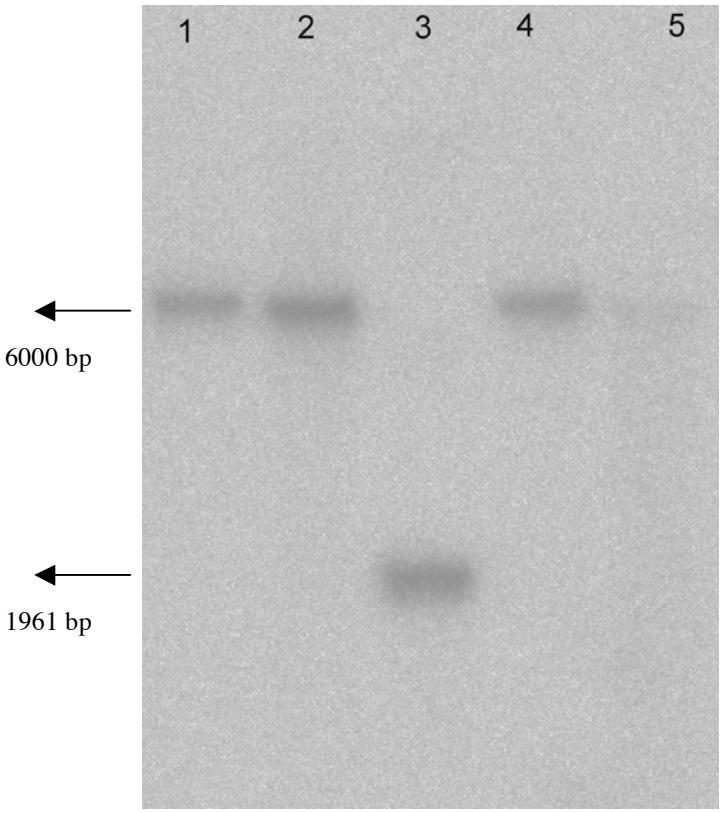

FIG. 10. Southern blot to detect marked *B. melitensis cydBA* deletion mutants digested with SphI. Genomic DNA was prepared as described in the text and digested with SphI. Lanes: 1, co-integrant plasmid pMMK15 into *B. abortus* (6000 bp); 2, 2308 (6000 bp); 3, 16MΔ*cydBA*::Kan (1961 bp); 4, pMMK15 co-integrant in 16M (6000 bp); 5, 16M (6000 bp).

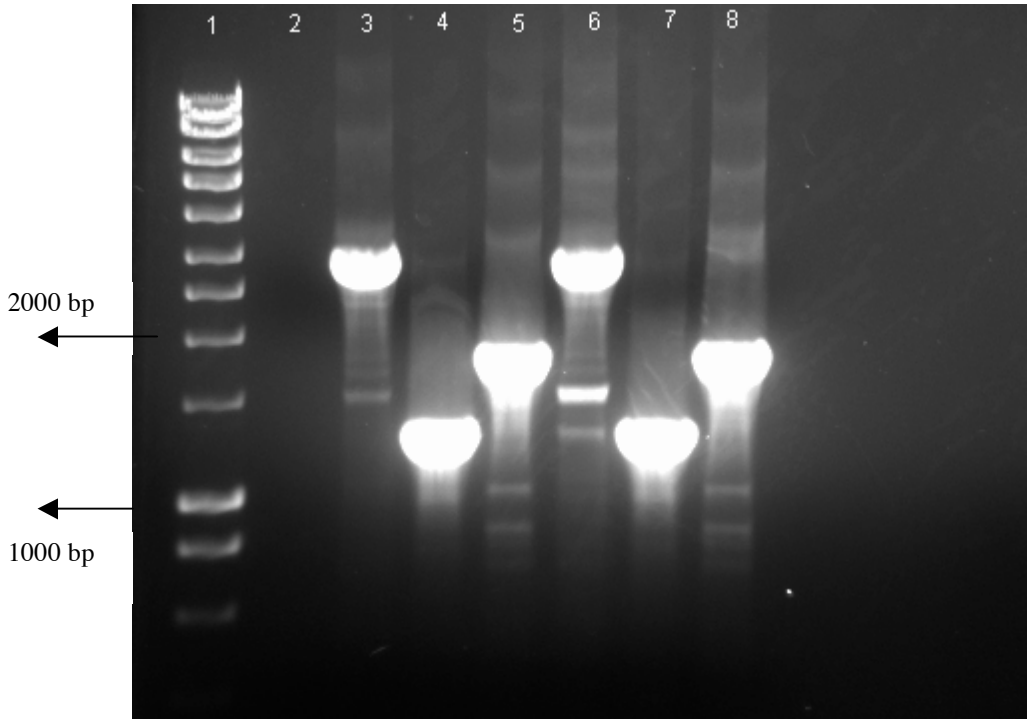

FIG. 11. PCR of *asp24* deletion mutants. Genomic DNA was prepared as described in the text and amplified with TAF 105/106 to detect knockout mutants. DNA was isolated from: BAΔ*asp24*::Kan (2849 bp), lane 3; BAΔ*asp24* (1249 bp), lane 4; 2308 (1782 bp), lane 5; BMΔ*asp24*::Kan (2849 bp), lane 6; BMΔ*asp24* (1249 bp), lane 7; 16M (1782 bp), lane 8. Hyperladder I molecular weight marker is in lane 1 and a negative PCR control is in lane 2.

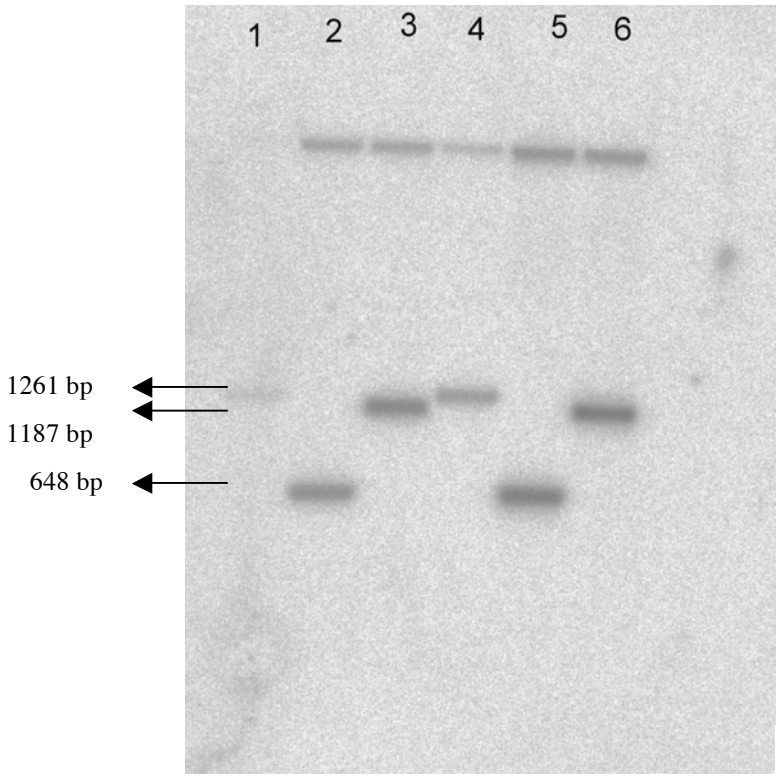

FIG. 12. Southern Blot to validate *asp24* deletion mutants digested with RsaI. Genomic DNA was prepared as described in the text and digested with RsaI for: BAΔ*asp24*::Kan (1261 bp), lane 1; BAΔ*asp24* (648 bp), lane 2; 2308 (1178 bp), lane 3; BMΔ*asp24*::Kan (1261 bp), lane 4; BMΔ*asp24 (*648 bp), lane 5; 16M (1178 bp), lane 6. The deleted region (*asp24*) is 530 bp. The probe contains one RsaI site, therefore two products are detected in the Southern blot. The size of the marked mutant is greater than wild-type organism by the kanamycin cassette (1600 bp).

BMΔ*asp24*::Kan. Mutants chosen for sucrose counter-selection were kanamycin resistant, carbenicillin resistant, and sucrose sensitive. These colonies were grown in sucrose; colonies that survived the selection that were kanamycin resistant were unmarked deletion mutants. The unmarked deletion mutant was named BMΔ*asp24*.Mutants were verified by PCR (Fig. 11) and Southern blot (Fig. 12).

DISCUSSION

Genes of interest were chosen based upon different survival characteristics of mutants, which was a random approach or to target known virulence factors as a rational approach. *Brucella abortus* rough mutants exhibit reduced virulence in the host and traffic differently than smooth organisms. They are taken up in greater numbers by macrophages than smooth strains of the same species, and resulting from this increased uptake, cause cell death of the macrophages (60). The rough mutants may therefore follow a different intracellular pathway than smooth organisms, and should be considered as vaccine strains since they are not virulent to the host (60, 64). Normal trafficking of *Brucella* involves the uptake via receptor molecules, localizing in early phagosomes, and avoidance of fusion between this *Brucella*-containing vacuole (BCV) and lysosomes (10, 35). Without this inhibition of fusion, the bacteria would be killed following the formation of the phagolysosome. *Brucella* then traffics so that it reaches the endoplasmic reticulum, where it begins replication following the formation of a replicative compartment with ER-like properties. This intracellular trafficking (late maturation events only) is controlled by the Type IV secretion system encoded by the

VirB operon. It has been demonstrated that Δ*virB* mutants are unable to sustain the interactions with the ER, and are killed when the BCV fuses with lysosomes (10). A *virB* deletion was chosen because of its inability to traffic normally and therefore exhibits reduced virulence. Mutants of *cydBA* were chosen because they represent a Class I mutant that retains all the known virulence factors. Since it clears rapidly from the host but still produces factors that may be important for proper immune stimulation, it may be a superior vaccine candidate to those currently available.

The class III mutant, *asp24*, was chosen as an example of a mutant that persists for a longer period in the host, though is an attenuated strain. Similar to the *cydBA* mutant, it also retains all the known virulence factors that may contribute to protective immunity. The contribution of Asp24 to intracellular trafficking is unknown, but may play an important role since the protein is up-regulated in infected macrophages, perhaps to establish the intracellular niche and, in turn, chronic infections.

EVALUATION OF MUTANT CANDIDATES IN THE MOUSE MODEL

INTRODUCTION

Although the mouse is not a normal host for *Brucella* species, they will develop a systemic infection after intraperitoneal (i.p.) injection with the bacterium and have been frequently utilized as a model due to this reproducible colonization (44, 61). The most frequently used strain, BALB/C, have 10 fold higher levels of recoverable bacteria in their spleens than C57BL/6 mice during the plateau phase of infection, when the CFU per spleen becomes constant. There are two main explanations regarding resistance of C57BL/6 mice to *Brucella* infection. First, BALB/C mice experience a decline in IFNγ production beginning at one week post infection that continues until the end of the plateau phase at around 6 weeks post infection. The origin of this decline is unknown (50, 51). The C57BL/6 mice, on the other hand, do not experience this decrease in IFNγ. Second, in contrast to BALB/C, C57BL/6 mice are NRAMP-susceptible strains, suggesting that control of infection originate from other genetic loci (50, 67). Overall, BALB/C that are infected with S2308 are less able to control infections compared to C57BL/6 mice, and as such, the increased susceptibility provides a sensitive model to evaluate bacterial survival. To compare any of the mutant strains, standard clearance curves of *B. abortus* S2308 and *B. melitensis* 16M were created to begin modeling the disease in the susceptible BALB/C mouse strain.

Competitive infection assays are often used to evaluate competition between the wild-type organism and the attenuated mutant in the same animal. This allows direct

comparisons of splenic colonization, and minimizes mouse-to-mouse variation while ensuring that the delivery of the dose is standardized. Signature tagged mutagenesis utilizes the same approach as competitive infections, in which more than one mutant competes in the same mouse (38). Novel marked and unmarked mutants will be compared in two ways. First, marked mutants are compared to wild-type, to demonstrate the degree of attenuation due to the genetic defect. Secondly, marked mutants are compared to unmarked mutants, to ensure that the two mutants can be used interchangeably and to identify any polar effects resulting from insertion of the kanamycin cassette.

In contrast to the competitive infection assay, the non-competitive assay mimics utilization of the mutant as vaccine in the field, and emphasize the duration of persistence of the vaccine strain under non-competitive conditions. Evaluation of colonization singly is important since the presence of wild-type organism may influence clearance of attenuated mutants from the host, and suggest enhanced rates of clearance.

To evaluate the vaccine potential of the selected unmarked mutants, the level of protection afforded against virulent challenge will be assessed as a measure of efficacy. This will be accomplished by comparing the mean CFU/spleen between naïve and vaccinated mice that are subsequently challenged with wild-type organism at various timepoints. Mutants demonstrating a significant decrease in mean bacterial colonization of the spleen relative to the control will be considered viable vaccine candidates for further evaluation. Since the mouse is not a perfect model of brucellosis in target species, vaccine candidates from the mouse will need to be re-evaluated in a target

animal model to evaluate protection against abortion. Mice do not abort in response to *Brucella* infections, though they will develop placentitis and fetal pathology (75, 76). As such, protection against abortion as a measure of vaccine efficacy for livestock and wildlife can not be assessed from the mouse model.

Immune responses to *Brucella* infections have been mainly studied in mice. In this model, protective immunity appears to be mediated by both a humoral and cellular response. Studies demonstrated that both $CD4^+$ and $CD8^+$ T lymphocytes are important for the control of infection (5, 58, 81). $CD4^+$ T cells are separated into at least two subpopulations based upon the cytokines they secrete when stimulated with antigen, either Th1 or Th2. Th1 cells, which secrete such cytokines as IL-2 and IFN-gamma, are involved in macrophage activation and attraction of inflammatory cells. This type of response is usually characterized by strong cell-mediated immunity and low humoral response. Th2 lymphocytes typically produce cytokines such as IL-4, IL-5, IL-6, and IL-10. IL-4 and IL-10 are known to downregulate the Th1 response. Th2 responses are usually associated with high titer antibody responses but poor delayed type hypersensitivity reactions (34, 82). It has been shown that lymphocytes from mice infected with live *Brucella* produce high levels of IFN-gamma and other Th1 cytokines, whereas mice immunized with heat killed bacteria or bacterial extracts seem to elicit a Th2 type response, and it is assumed that a Th1 type response is necessary to control *Brucella* infections (81, 82). Vaccine candidates are typically evaluated in mice by their induction of Th1 versus Th2 cytokines as an explanation of efficacy.

MATERIALS AND METHODS

Bacterial strains. Virulent *B. abortus* strain S2308 was obtained from Billy Deyoe at the National Animal Disease Center in Ames, Iowa, from an aborted calf and passaged once in culture, and frozen as glycerol stocks at –80°C. *B. melitensis* biovar 1 (16M) was originally acquired from Dr. Philip Elzer at Louisiana State University in Baton Rouge, Louisiana, from an aborted goat fetus. After usage of this strain in the 2002 goat safety trial, another virulent isolate was obtained from a Texas A&M aborted goat fetus. This strain was cultured, stored as frozen stocks, and used as the parental strain to generate deletion mutants, as well as virulent challenge strain in efficacy studies. Strain 19 was obtained from the National Veterinary Services Laboratory in Ames, Iowa. Rev 1 INRA was obtained from Dr. Menachem Banai. All wild-type, vaccine and unmarked deletion strains were routinely grown on tryptic soy agar (TSA; Difco Laboratories) or Farrell's media (TSA supplemented with Oxoid *Brucella* supplement) at 37°C and 5% CO_2. Marked deletion mutants were grown on TSA supplemented with 100 μg/ml kanamycin. Bacteria were grown to confluence on solid media for 72 hours and harvested into PBS (Gibco, pH 7.4) for each experiment. Cell density is estimated using a Klett meter and the strains are resuspended to the appropriate concentrations for each experiment. All bacteria are listed in Table 4.

Clearance of wild-type *Brucella* from BALB/c mice. Groups of 4-5 mice at 4-6 weeks of age were infected intraperitoneally with either $1x10^5$ CFU/animal or $1x10^6$

TABLE 4. *Brucella* vaccine strains and deletion mutants used in the mouse model

B. abortus Strain	*B. melitensis* Strain	Relevant Characteristic(s)
2308	16M	Wild-type
Strain 19 (S19)	Rev 1	Accepted vaccine strain
BAΔ*asp24*::kan	BMΔ*asp24*::kan	Marked *asp24* deletion
BAΔ*asp24*	BMΔ*asp24*	Unmarked *asp24* deletion
BAΔ*virB2*::kan	BMΔ*virB2*::kan	Marked *virB2* deletion
BAΔ*virB2*	BMΔ*virB2*	Unmarked *virB2* deletion
	BMΔ*manBA*::kan	Marked *manBA* deletion
BAΔ*manBA*	BMΔ*manBA*	Unmarked *manBA* deletion

CFU/animal and held for a time-course experiment to model the clearance of virulent 2308 and 16M from mice. At sequential timepoints post-infection, mice were sacrificed by CO_2 asphyxiation, spleens collected and weighed, homogenized in 1 ml PBS, serially diluted, and plated in duplicate onto solid media.

Competitive infection assays. For competitive infection assays, 4-6 week old female BALB/c mice purchased from Jackson Laboratories were infected via intraperitoneal injection with a mixture of bacteria in a 1.5:1 ratio of colony forming units at a final concentration of $2.5x10^5$ CFU/ml wild-type and unmarked mutants. The final ratio of input bacteria was determined after mixing the cultures together following serial dilution and plating in the presence of appropriate antibiotics. Treatment groups included wild-type *B. abortus* or *B. melitensis* with marked mutant or unmarked mutant plus marked mutant. Mice were sacrificed at selected time points post-infection, depending upon the anticipated clearance rate of each mutant. At each time point, five mice were euthanized via carbon dioxide asphyxiation. Spleens were collected, homogenized in 1 ml PBS, serially diluted, and plated in duplicate onto both TSA and TSA containing kanamycin @100μg/ml. Recovered bacteria were enumerated after 4 days of incubation. Bacteria growing on TSA represent both populations of mutants, therefore subtraction of the bacteria enumerated on kanamycin from those enumerated from TSA demonstrated the number of either wild-type or unmarked mutant. The survival index represents the output ratio (wild-type/mutant) divided by the input ratio.

Non-competitive infection assays. Survival of mutants was evaluated by vaccinating groups of 4-6 week old female BALB/c mice via intraperitoneal injection of $1x10^6$ CFU mutant or wild-type organism. Mice are vaccinated at a ten-fold higher dose in the non-competitive assays since the mutant is attenuated, and as such higher amounts or organism are used to ensure proper vaccination and presentation to the host. Treatment groups included mice vaccinated with either 16M, 2308, or unmarked deletion mutants of 16M or S2308 genetic backgrounds. Mice were sacrificed via carbon dioxide asphyxiation at varying times post-infection, depending upon the anticipated clearance rate due to the class of mutant. Spleens were collected and weighed, homogenized in 1 ml PBS, serially diluted, and plated onto TSA. Recovered bacteria were enumerated and compared to the other groups to evaluate the rate of persistence of each individual organism.

Efficacy studies. The mouse model was used to evaluate efficacies of various unmarked deletion mutants against infection. Groups of 6-10 female 4-6 week old BALB/c mice are vaccinated via intraperitoneal injection of $1x10^6$ CFU/ml of unmarked deletion mutant or PBS in naïve controls. Mice were subsequently challenged with $1x10^4$ CFU homologous wild-type strain at 12, 16, or 20 weeks after vaccination. One week after the virulent challenge, the mice were euthanized, spleens extracted and weighed, homogenized in 1 ml PBS, serially diluted and plated onto TSA. Bacterial burden in the spleen of challenge organism was used as a measure of protective

immunity [$\log_{10}$ wild-type recovered from unvaccinated mice minus $\log_{10}$ wild-type recovered from vaccinates].

Cross-*Brucella* species protection. Groups of five female 4-6 week old BALB/c mice are vaccinated via intraperitoneal injection of $1x10^6$ CFU/ml of unmarked deletion mutant or PBS in naïve controls. Mice were subsequently challenged with $1x10^4$ CFU heterologous wild-type strain at 16 weeks after vaccination. One week after the virulent challenge, the mice were euthanized, spleens extracted and weighed, homogenized in 1 ml PBS, serially diluted and plated onto TSA. Bacterial burden in the spleen of challenge organism was used as a measure of protective immunity [$\log_{10}$ wild-type recovered from unvaccinated mice minus $\log_{10}$ wild-type recovered from vaccinates].

Statistical analysis. ANOVAs were performed to compare treatment groups of mice to one another. P values less than 0.05 were considered significant.

Cytokine production. Mice were allocated to four groups of 5: (a) unvaccinated controls (b) unvaccinated for 20 weeks then challenged with wild-type for 1 week (c) vaccinated for 21 weeks and euthanized without wild-type challenge (d) vaccinated for 20 weeks then challenged with wild-type for 1 week. This was performed for both *B. melitensis* and *B. abortus* strains, as well as for three of the vaccine candidates. For the memory response from splenocytes, mice were euthanized and spleens collected.

Spleens were then pressed through fine mesh into a sterile Petri plate, and then flushed with PBS. Cells were pelleted at 1700 x g for 10 minutes; to the supernatant 10ml ACK red blood cell lysis buffer (0.15M NH_4Cl, 10.0mM $KHCO_3$, 0.1mM Na_2EDTA) was added for 5 minutes. Splenocytes were pelleted and washed three times with PBS and then enumerated using a hemacytometer. Splenocytes were resuspended in RPMI complete [RPMI 1640, 10% (v/v) FBS, 1% (v/v) sodium pyruvate, 0.1% (v/v) β-mercaptoethanol (v/v), 1% penicillin-streptomycin (v/v),] and seeded at a density of $2x10^5$ cells/well (73). Samples were stimulated with $1x10^8$ heat killed *Brucella*. Controls included unstimulated cells for a baseline level, as well as cells stimulated with ConA, a positive control mitogen, at a concentration of 2μg/ml in each well. All samples were analyzed in triplicate. Cells were incubated for three days of growth in the presence of the stimulant. Supernatants were collected for cytokine evaluation at day three and then frozen at –80°C.

Cytokine ELISAs. Enzyme-linked immunosorbent assays (ELISAs) were performed to characterize the cytokines produced by the splenocytes in response to the various stimulants (Peprotech, New Jersey). Plates were coated overnight at room temperature with 50 μl capture antibody at 1 μg/ml for IFN-gamma, IL-4, Il-10, Il-12, and 2μg/ml for IL-2. Wells were washed four times with 300μl PBS-T (0.05% Tween-20 in PBS) to remove capture antibody. The blocking reagent (1% BSA in PBS) was added to each well at a volume of 300μl and incubated overnight at 4°C. Plates were washed four times with PBS-T to remove the excess blocking reagent. Cytokine

standards were 2-fold serially diluted to generate a standard curve; the specificity differed between cytokines. Tissue culture supernatants were also added in duplicate to wells at a volume of 50μl and incubated at 4°C overnight. Plates were washed four times with PBS-T to remove residual supernatant. Detection antibody was added to wells at a concentration of 0.5μg/ml for IFN-gamma, IL-4, and IL-10, and at 0.25μg/ml for IL-2 and IL-12 and incubated for two hours at room temperature. Plates were washed four times with PBS-T to remove the detection antibody. Avidin peroxidase (50μl of a 1:2000 dilution) was then applied to each well, and after incubating for 30 minutes at room temperature, the plates were washed seven times with PBS-T for complete removal of the compound. ABTS liquid substrate (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, Sigma Catalog # A3219) was added to each well (100μl, ready-to-use concentration), and the plates were incubated at room temperature until the highest standard reached 1.2 O.D. units or the zero standard reached 0.2 O.D. units, and the reaction was subsequently stopped with 1% SDS. Plates were read at 405 nm with wavelength correction set at 650 nm.

RESULTS

Clearance of wild-type *Brucella* from BALB/c mice. The kinetics of clearance of wild-type *Brucella* was evaluated in mice to establish baseline levels of splenic colonization for comparison of mutant survival. *B. abortus* displayed chronic persistence in BALB/c mice injected with $1x10^5$ CFU i.p. (Fig. 13). *B. abortus*

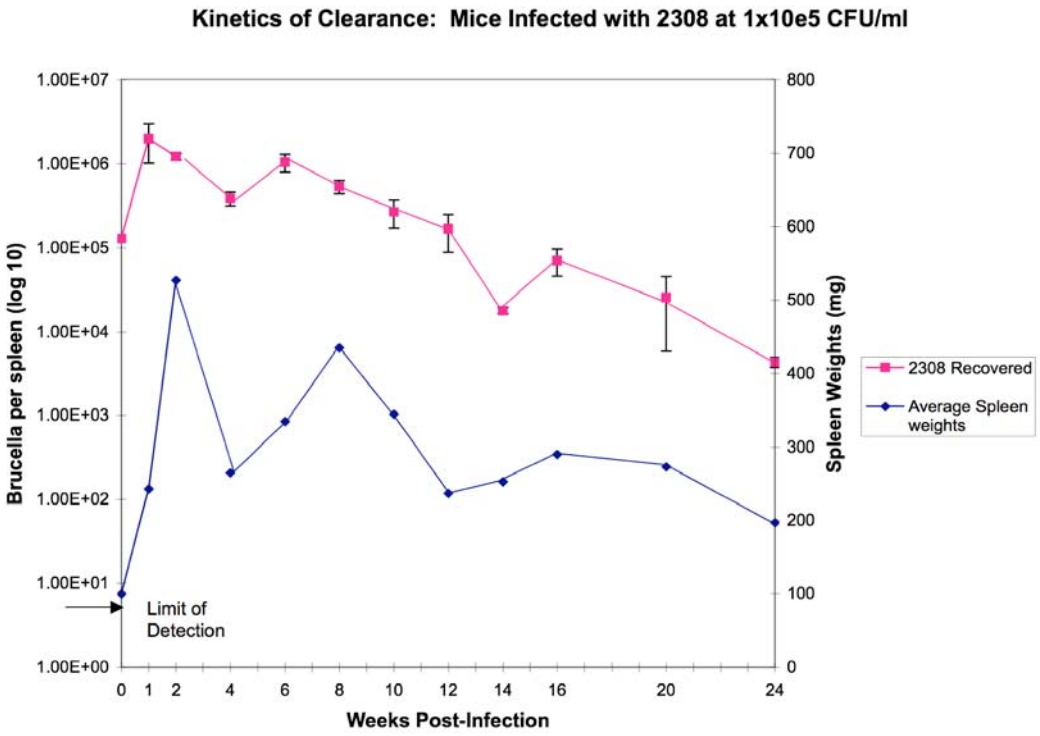

FIG. 13. Time-course of clearance of $1x10^5$ CFU *B. abortus* 2308 from mice. Mice were infected with 2308 at $1x10^5$ CFU/animal I.P. for various durations to monitor the clearance of the organism. Mice were euthanized at the various timepoints post-infection via CO_2 asphyxiation, spleens extracted and weighed, then serially diluted in 1 ml peptone saline. Serial dilutions were performed and plated to determine the CFU in the spleen at each timepoint. Degree of splenomegaly from the challenge inoculum was determined by the weight of the spleen at each timepoint. Data was plotted as the average CFU recovered per spleen +/- standard error of the mean.

colonizes and replicates in the spleen rapidly, then evolves into a plateau period where the bacteria persist in the spleen without dramatic decreases in numbers. Survival of the organism gradually decreases over a period of several months. *B. melitensis* does not cause chronic disease in BALB/c mice similar to *B. abortus*, however *B. melitensis* infection in mice caused a similar trend early in infection as *B. abortus* (Fig. 14). Though it was able to establish a severe acute infection, *B. melitensis* steadily cleared from the host, a phenotype that differs from *B. abortus*. Several aspects of infection were common to both, however. The greatest degree of bacterial colonization of the spleen was detectable at 1 week post-infection, and the greatest degree of splenomegaly was observed at two weeks post-infection for both species.

Competitive infections of *manBA* deletion mutants. In order to ensure deletion stains were attenuated, marked mutants were compared competitively to wild-type strains, and unmarked mutants were compared to marked mutant strains of the same genetic defect. Using this experimental model, a discrepancy was observed. In the presence of wild-type organism, BMΔ*manBA*::Kan was severely attenuated by 1 week post-vaccination, and completely cleared by 2 weeks (Fig. 15). In contrast, marked and unmarked BMΔ*manBA* and BAΔ*manBA* deletions persisted until 2 weeks post-vaccination, though they were cleared by 5 weeks, when mice were not co-infected with wild-type 16M (Fig. 16). This suggests an inability of the rough strain to compete for an intracellular niche when wild-type organism is present. As a result, a false rate of clearance is observed for the mutant strains, though they are still attenuated. The use of

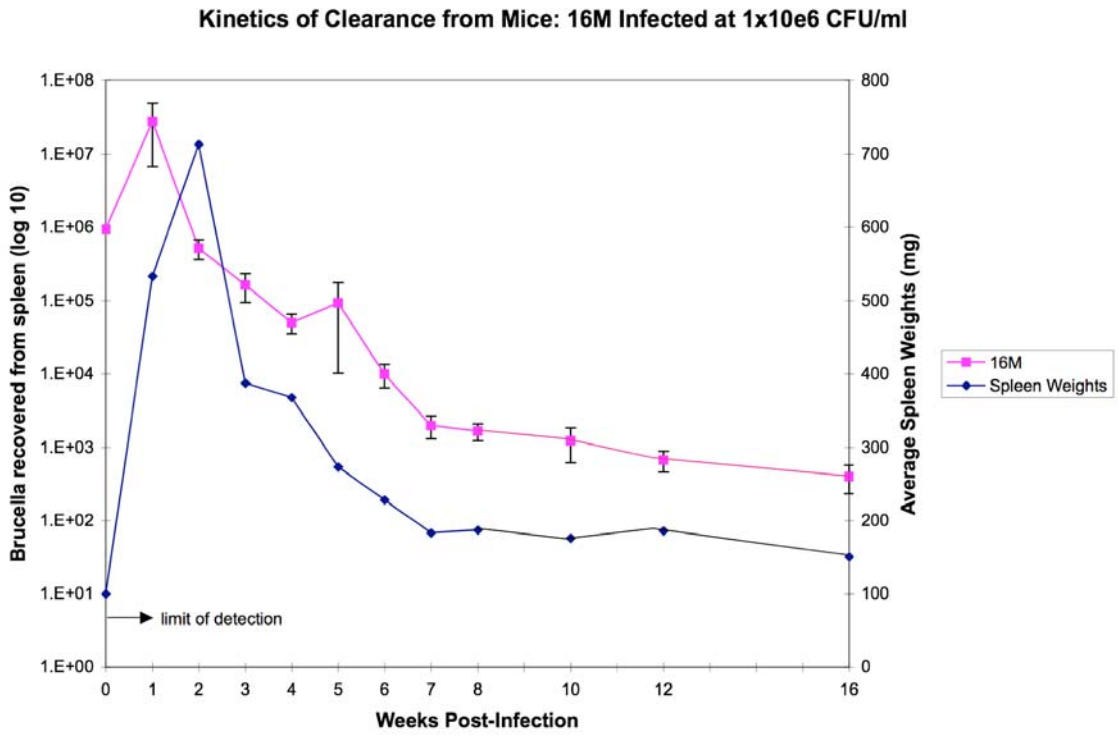

FIG. 14. Time-course of clearance of $1x10^6$ CFU *B. melitensis* 16M from mice. Mice were infected with 16M at $1x10^6$ CFU/animal I.P. for a timecourse study. Mice were euthanized at the various timepoints post-infection via CO_2 asphyxiation, spleens extracted and weighed, then serially diluted in 1 ml peptone saline. Serial dilutions were performed to determine the CFU in the spleen at each timepoint. Degree of splenomegaly from the challenge inoculum was determined by the weight of the spleen at each timepoint. Data was plotted as the average CFU recovered per spleen +/- standard error of the mean.

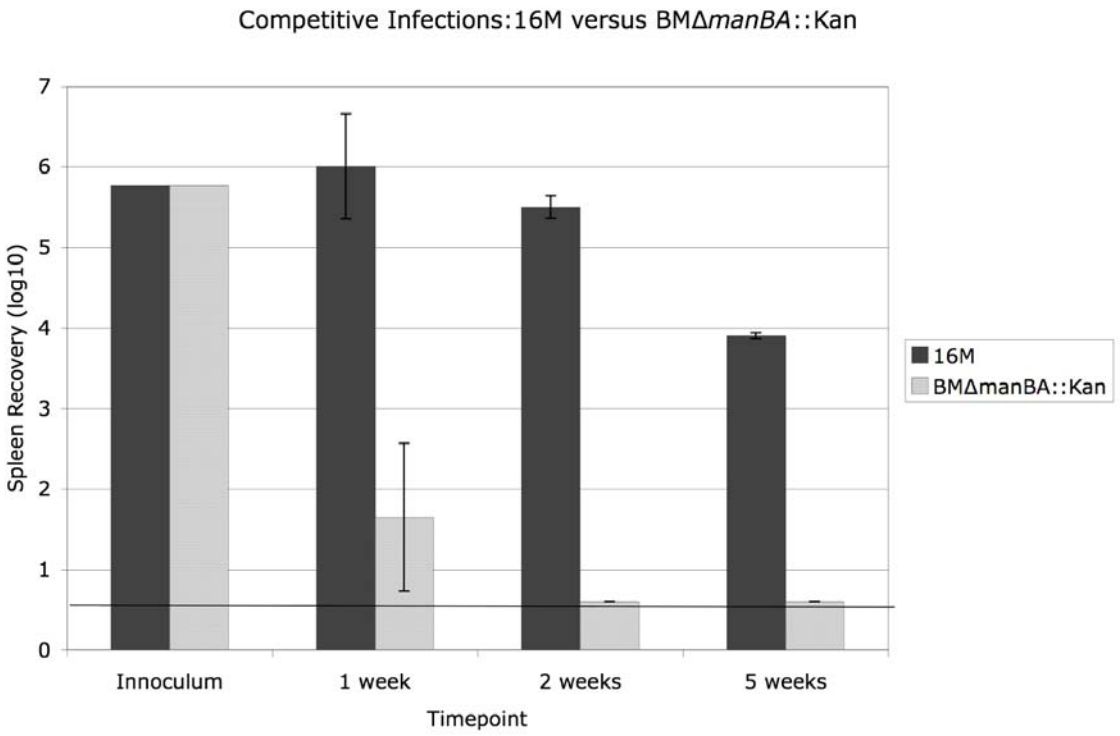

FIG. 15. Competitive clearance of marked BMΔ*manBA::*kan deletion mutants from mice in the presence of 16M. Mice were co-infected with 16M and BMΔ*manBA*::Kan at a ratio of 1.5:1 for several weeks. At each timepoint, mice were euthanized, spleens extracted, and homogenized in 1 ml peptone saline. Aliquots were plated on TSA and TSA/Kan@100 to determine the persistence of the bacteria in the spleen. Bacteria that grew on the plates containing Kan represent the remaining marked mutant strain; bacteria growing on TSA represent both 16M and the marked mutant, therefore subtraction of the CFU from the Kan plates from the CFU on TSA equals the remaining 16M. Data was plated as the average CFU recovered from spleens +/- standard error of the mean.

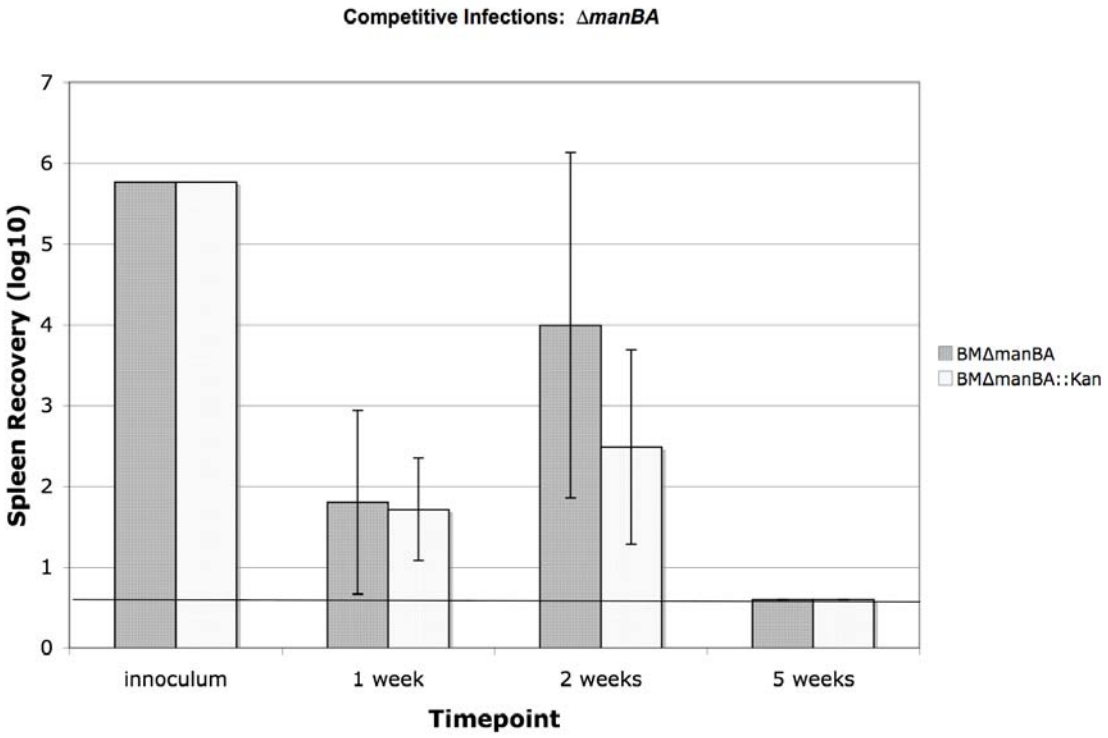

FIG. 16. Competitive clearance of unmarked BMΔ*manBA* and marked BMΔ*manBA*::Kan deletion mutants from mice. Mice were co-infected with BMΔ*manBA* and BMΔ*manBA*::Kan at a ratio of 1.5:1 for several weeks. At each timepoint, mice were euthanized, spleens extracted, and homogenized in 1 ml peptone saline. Aliquots were plated on TSA and TSA/Kan@100 to determine the persistence of the bacteria in the spleen. Bacteria that grew on the plates containing Kan represent the remaining marked mutant strain; bacteria growing on TSA represent both unmarked and marked strains, therefore subtraction of the CFU from the Kan plates from the CFU on TSA equals the remaining BMΔ*manBA*. Data was plated as the average CFU recovered from spleens +/- standard error of the mean.

these strains as vaccines would display a varied pattern of clearance, when the mutant is administered to an animal alone.

Clearance of unmarked *manBA* deletion mutants. Infection of mice non-competitively demonstrates the clearance of the vaccine strain without influence of fully virulent wild-type strain, to model field vaccination conditions. Compared to 2308, the unmarked *B. abortus manBA* deletion mutant (BAΔ*manBA*) was significantly attenuated by 3.39 logs at 1 week post-infection ($p<0.0001$), 2.44 logs at 2 weeks post-infection ($p<0.0001$), and undetected by 5 weeks post-infection ($p<0.0001$) (Fig. 17). Between 1 and 2 weeks post-infection, however, the levels of detectable vaccine strain in the spleens remained unchanged, indicating the ability of the organism to evade killing by the host during this time. Unmarked *B. melitensis* deletion mutants of *manBA* (BMΔ*manBA*) demonstrated a similar trend as BAΔ*manBA* (Fig. 18). Compared to wild-type 16M, the mutant was attenuated 2.53 logs at 1 week ($P=0.01$), 1.33 logs at 2 weeks ($P=0.001$), and undetected by 5 weeks post-infection ($P=0.001$). Numbers of bacteria in the spleen remained unchanged between 1 and 2 weeks post-infection, as was observed in the *B. abortus* mutant. From these data, we consider the *manBA* unmarked deletion highly attenuated.

Clearance of unmarked *virB2* deletion mutants. Mice infected with unmarked *B. abortus virB2* (BAΔ*virB2*) deletion mutants were attenuated 1.62 logs at 2 weeks post-infection ($P=0.003$), though the attenuation compared to 2308 is not as severe as

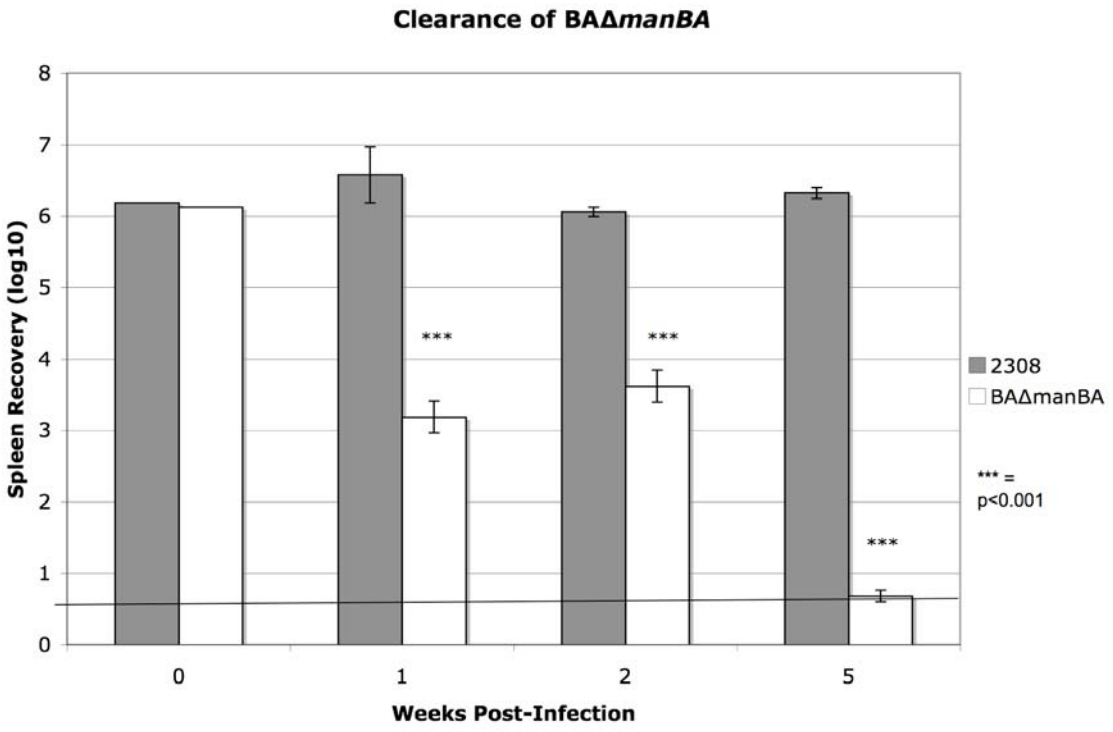

FIG. 17. Clearance of $1x10^6$ CFU BAΔ*manBA* from mice. Mice were infected with BAΔ*manBA* at $1x10^6$ CFU/animal. At each timepoint, mice were euthanized via CO_2 asphyxiation, spleens collected, the homogenized in 1 ml peptone saline. The quantities of mutant remaining in the spleens were determined by serial dilutions of the spleen homogenates, and were represented as CFU per spleen +/- standard error of the mean.

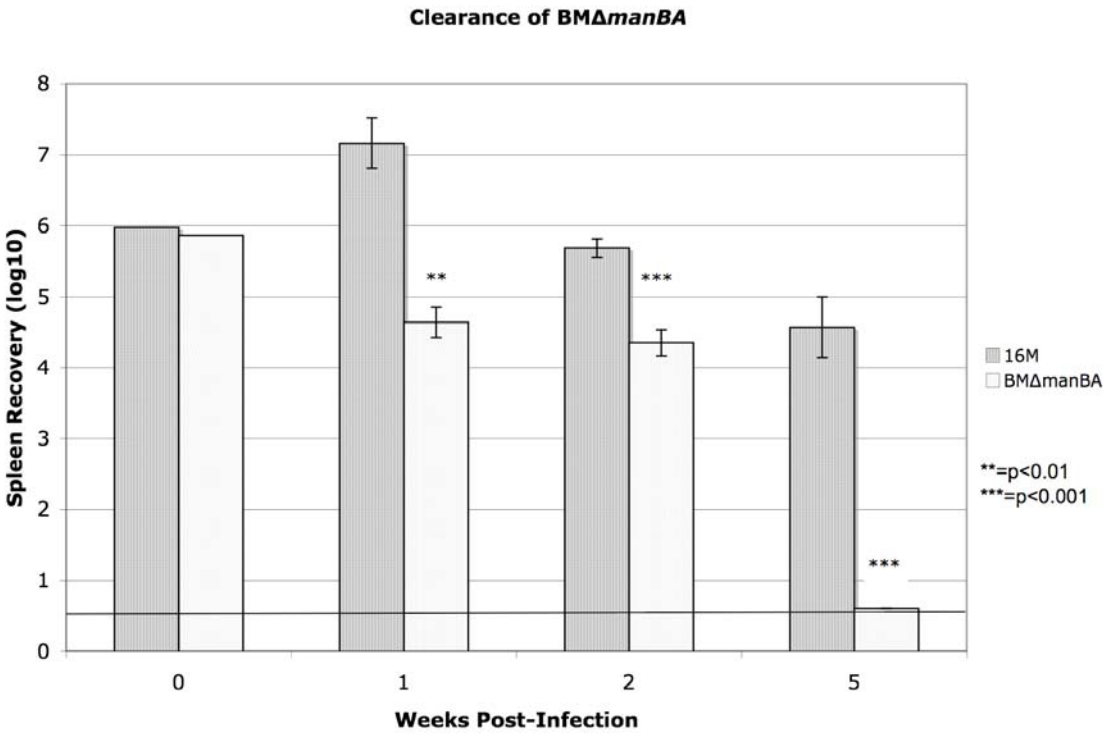

FIG. 18. Clearance of $1x10^6$ CFU BMΔ*manBA* from mice. Mice were infected with BMΔ*manBA* at $1x10^6$ CFU/animal. At each timepoint, mice were euthanized via CO_2 asphyxiation, spleens collected, the homogenized in one ml peptone saline. The quantities of mutant remaining in the spleens were determined by serial dilutions of the spleen homogenates, and were represented as CFU per spleen +/- standard error of the mean.

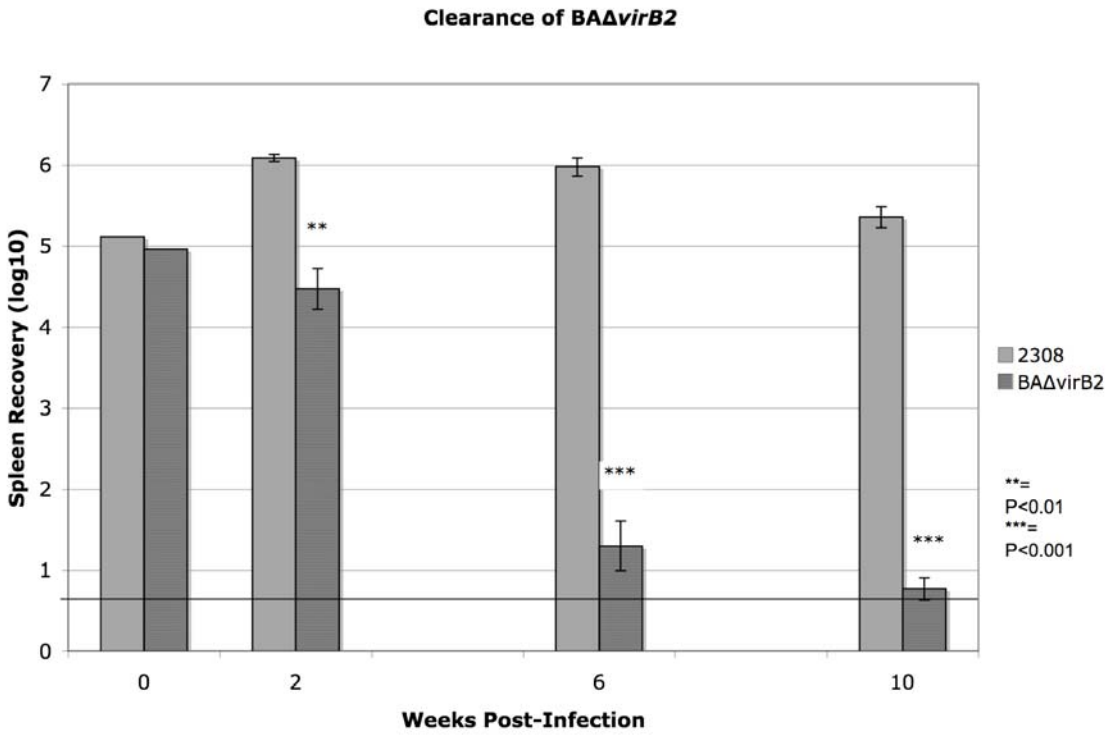

FIG. 19. Clearance of $1x10^5$ CFU BAΔ*virB2* from mice. Mice were infected with BAΔ*virB2* at $1x10^5$ CFU/animal. At each timepoint, mice were euthanized via CO_2 asphyxiation, spleens collected, the homogenized in one ml peptone saline. The quantities of mutant remaining in the spleens were determined by serial dilutions of the spleen homogenates, and were represented as CFU per spleen +/- standard error of the mean.

what was demonstrated with the BAΔ*manBA* mutant at the same timepoint (Fig. 19). BAV2 was attenuated 4.68 logs at 6 weeks post-infection (P<0.0001), which was barely greater than the limit of detection. It was undetected at 10 weeks post-infection (P<0.0001).

Unmarked *B. melitensis virB2* (BMΔ*virB2*) demonstrated similar survival characteristics to the *B. abortus* form, although the degree of attenuation was slightly higher (Fig. 20). In this case, the mutant was attenuated by 2 weeks as well, however it was 2.15 logs reduced compared to 16M (P=0.001). By 6 weeks post-infection, the mutant strain was no longer detected from the mouse spleens (P<0.0001), which held constant at 10 weeks as well (P=0.002). From these data, we consider both BAΔ*virB2* and BMΔ*virB2* to be attenuated at a moderate rate when compared to wild-type strains since they are not cleared as rapidly as *manBA* deletion mutants.

Clearance of unmarked *asp24* deletion mutants. The two *asp24* deletion mutants were the first set of gene deletions that portrayed varied survival phenotypes in the mouse model depending upon in which species of *Brucella* the deletion was created. For the *B. abortus* version (BAΔ*asp24*), the mutant was highly attenuated (4.6 logs) by 6 weeks post-infection (P<0.0001), barely detectable and 4.98 logs reduced at 8 weeks (p<0.0001) and below the limit of detection at 10 weeks (P<0.0001) (Fig. 21). In *B. melitensis*, however, BMΔ*asp24* is not reduced in the spleen at a significant level until 16 weeks post infection, where it persists at 1.22 logs lower than 16M (P=0.04) (Fig. 22). One difficulty in assessing the difference between 16M and the knockout arises

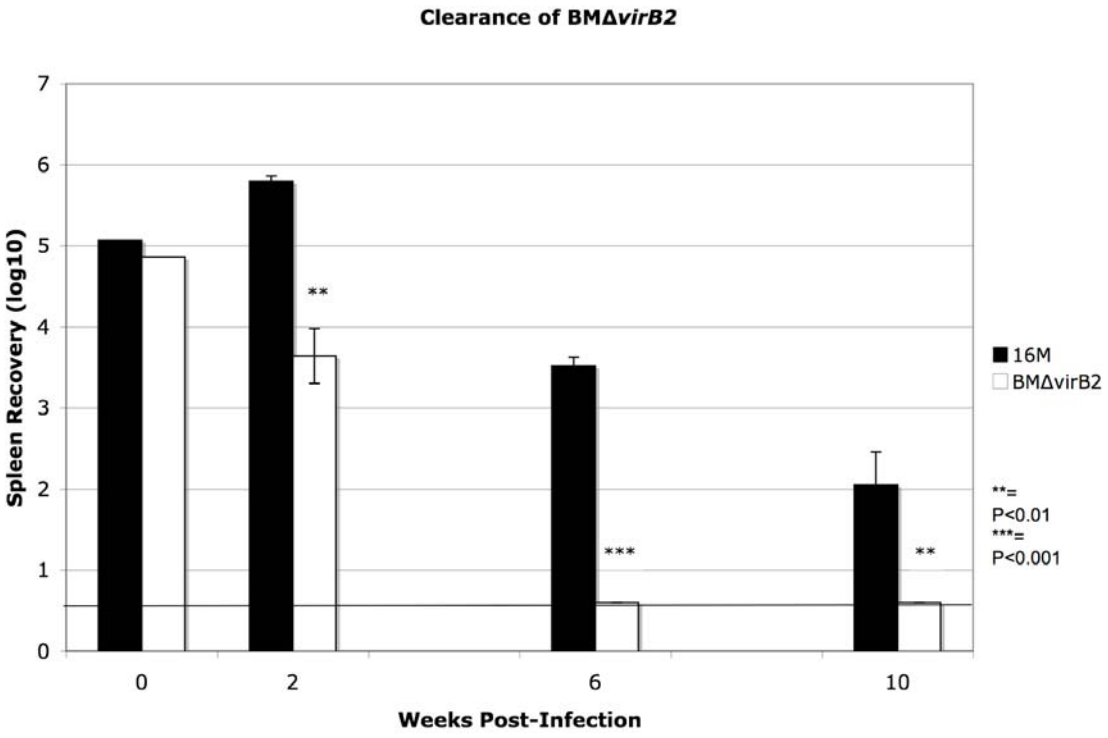

FIG. 20. Clearance of $1x10^5$ CFU BMΔ*virB2* from mice. Mice were infected with BMΔ*virB2* at $1x10^5$ CFU/animal. At each timepoint, mice were euthanized via CO_2 asphyxiation, spleens collected, the homogenized in one ml peptone saline. The quantities of mutant remaining in the spleens were determined by serial dilutions of the spleen homogenates, and were represented as CFU per spleen +/- standard error of the mean.

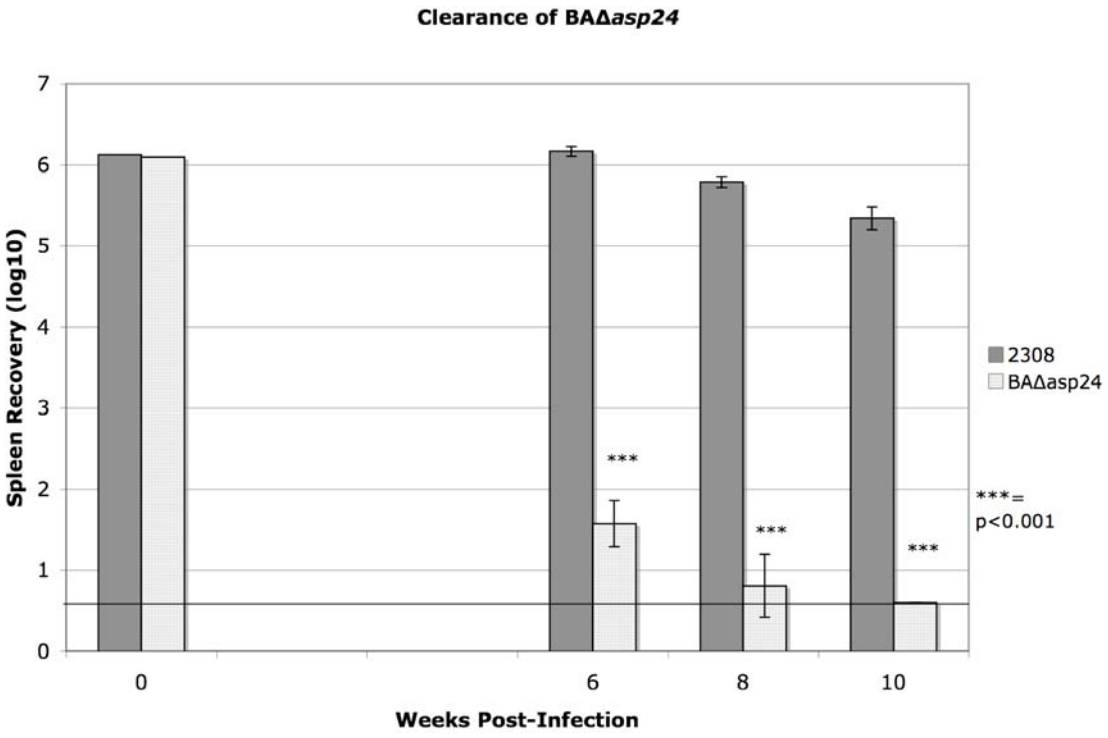

FIG. 21. Clearance of $1x10^6$ CFU BAΔ*asp24* from mice. Mice were infected with BAΔ*asp24* at $1x10^6$ CFU/animal. At each timepoint, mice were euthanized via CO_2 asphyxiation, spleens collected, the homogenized in one ml peptone saline. The quantities of mutant remaining in the spleens were determined by serial dilutions of the spleen homogenates, and were represented as CFU per spleen +/- standard error of the mean..

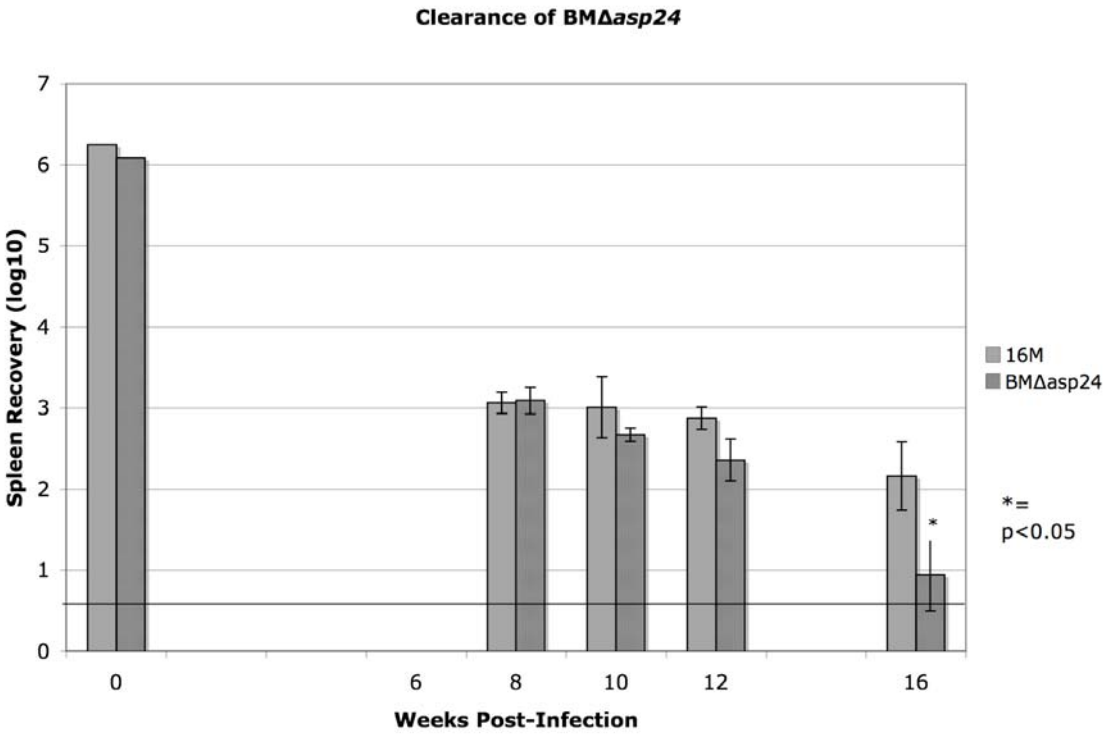

FIG. 22. Clearance of $1x10^6$ CFU BMΔ*asp24* from mice. Mice were infected with BMΔ*asp24* at $1x10^6$ CFU/animal. At each timepoint, mice were euthanized via CO_2 asphyxiation, spleens collected, the homogenized in one ml peptone saline. The quantities of mutant remaining in the spleens were determined by serial dilutions of the spleen homogenates, and were represented as CFU per spleen +/- standard error of the mean.

from the fact that 16M clears at a rapid rate compared to 2308 in mice. As such, differences between 16M and knockouts are not as apparent for mutants that are attenuated yet persistent, such as BMΔ*asp24*.

Protection against homologous 2308 challenge infection. To evaluate the vaccine potential of the selected unmarked mutants, the level of protection afforded against virulent challenge was assessed. Degree of efficacy was determined by subtracting the mean CFU/spleen recovered from mice after vaccination from the mean CFU/spleen recovered from age-matched non-vaccinated but challenged controls. This value is referred to as the U value, for units of protection, and is only relative to the non-vaccinated controls challenged with the respective wild-type at each particular time point.

At 13 weeks post-vaccination, mice were protected most significantly against 2308 challenge at 4.72 U by BAΔ*asp24* ($P<0.0001$). S19 vaccinated mice were protected at 4.28 U ($P<0.0001$) (Fig. 23). Mutants that clear more quickly from the host, BAΔ*virB2* and BAΔ*manBA*, protected mice to a lesser degree at 1.47 and 0.99 U, respectively ($P=0.004$ and $P=0.001$). At 16 weeks post-vaccination, challenge bacteria were recovered barely above the limit of detection from only one of the four mice vaccinated with BAΔ*asp24* ($P<0.0001$). S19 protected mice to a great degree at this time point as well, at 4.89 U ($P<0.0001$). Mice vaccinated with BAΔ*virB2* exhibited only 1.49 U protection ($P=0.002$) or 1.33 U with BAΔ*manBA* ($P=0.03$). This suggests again that the persistent yet attenuated mutant strains protect mice better than rapidly

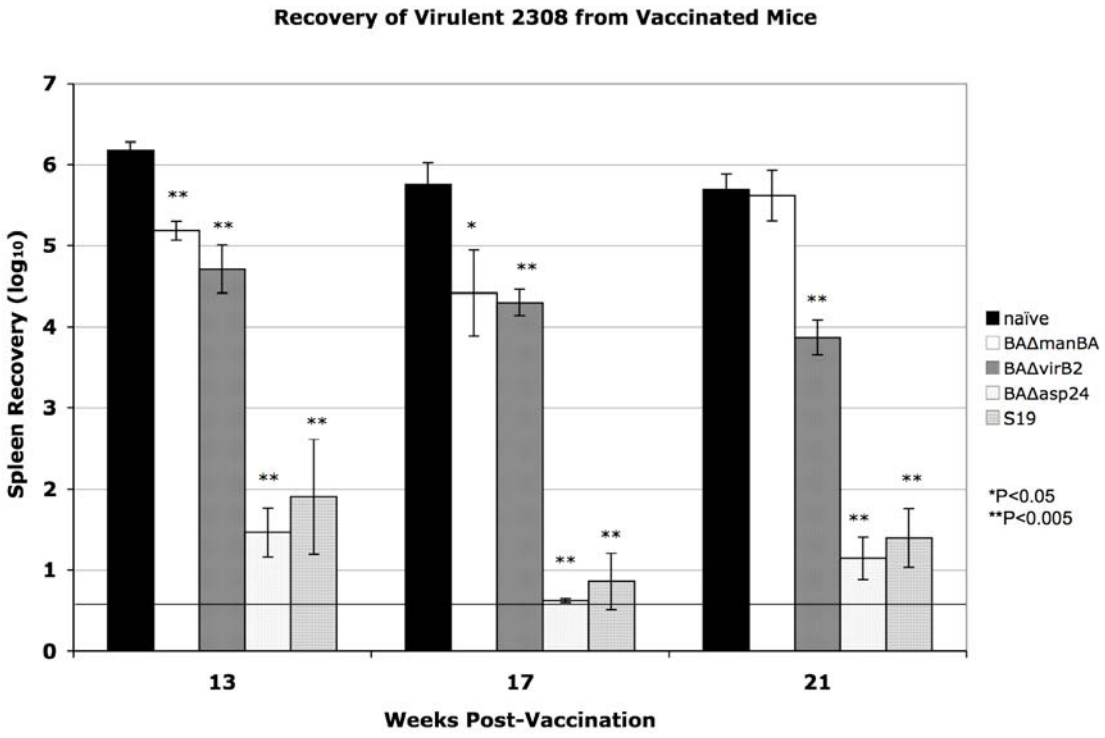

FIG. 23. Protection afforded to mice by *B. abortus* unmarked deletion mutants against virulent 2308 challenge. Mice were vaccinated with $1x10^6$ CFU unmarked *B. abortus* deletion mutants for 12, 16, or 20 weeks. After the vaccination period, the mice were challenged with $1x10^4$ CFU wild-type 2308 for 1 week. Mice were then euthanized, and spleens extracted and homogenized in 1 ml peptone saline. Homogenates were serially diluted to determine the remaining CFU bacteria in the spleens.

cleared mutants do. At 21 weeks post-vaccination, BAΔ*asp24* protected mice 4.54 U (P<0.0001), S19 at 4.29 U (P=0.0001), BAΔ*virB2* at 1.82 U (P=0.001), and BAΔ*manBA* at 0.07 U (P=0.86). Overall, the BAΔ*asp24* mutant protects mice against virulent 2308 challenge to the greatest degree even compared to another persistent mutant strain (and patented vaccine strain), S19. BAΔ*virB2* and BAΔ*manBA* protect mice to a moderate, though significant degree at most time points tested, except for BAΔ*manBA* after 20 weeks of vaccination, which elicited no significant protection.

Protection against homologous 16M challenge infection. Mice were vaccinated with *B. melitensis* unmarked deletion mutants and challenged with 16M to determine the protection afforded against subsequent infection due to the vaccine strains. At 13 weeks post vaccination, BMΔ*asp24* vaccinated mice were protected most significantly from 16M challenge, at 3.74 U (P<0.0001) (Fig. 24). Rev1 vaccinated mice were protected at 2.42 U (P=0.002). Unlike BAΔ*manBA*, whose degree of protection was less than BAΔ*virB2*, the BMΔ*manBA* mutant protected mice better than BMΔ*virB2*, at 1.87 U compared to 1.53 U, respectively (P=0.04 for both strains). At 17 weeks post-vaccination, BMΔ*asp24* mice were protected 5.6 U (P<0.0001), Rev1 at 4.12 U (P<0.0001), BMΔ*manBA* at 3.85 U (P=0.0002), and BMΔ*virB2* at 3.8 U (P=0.0001). At 21 weeks post-vaccination, BMΔ*asp24* affords the greatest degree of protection at 4.69 U (P<0.0001), followed by Rev1 at 4.39 U (P=0.0002). At this timepoint, the BMΔ*manBA* mutant protects mice better than BMΔ*virB2*, similar to the trend

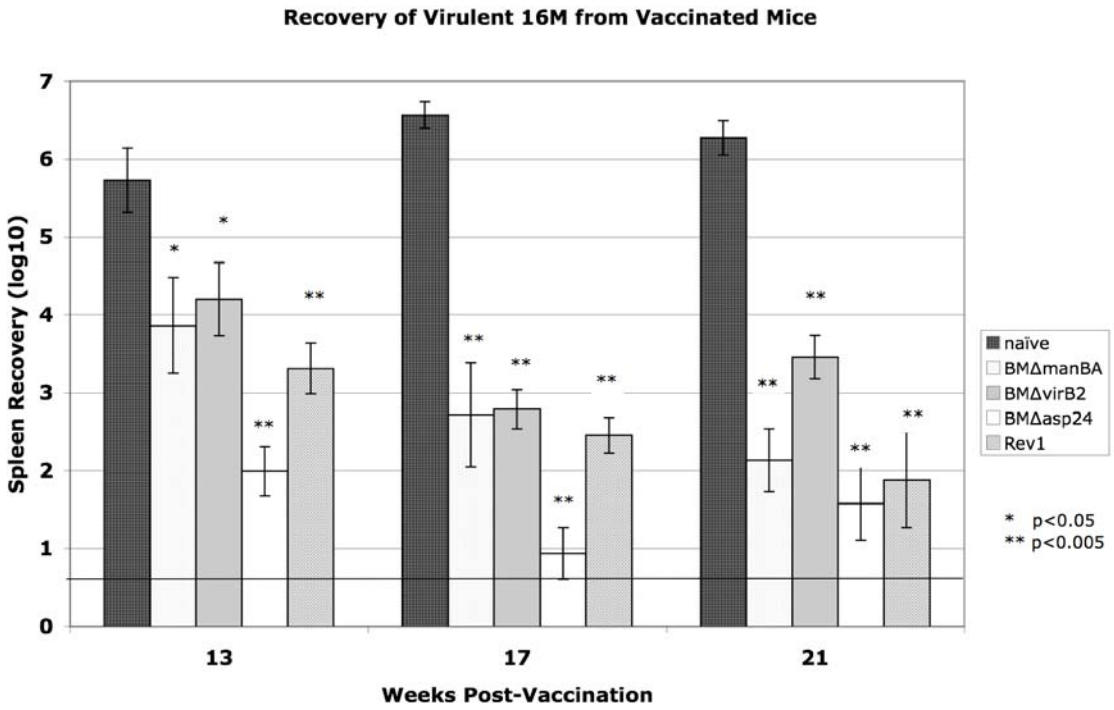

FIG. 24. Protection afforded to mice by *B. melitensis* unmarked deletion mutants against virulent 16M challenge. Mice were vaccinated with $1x10^6$ CFU unmarked *B. melitensis* deletion mutants for 12, 16, or 20 weeks. After the vaccination period, the mice were challenged with $1x10^4$ CFU wild-type 16M for 1 week. Mice were then euthanized, and spleens extracted and homogenized in 1 ml peptone saline. Homogenates were serially diluted to determine the remaining CFU bacteria in the spleens.

demonstrated at 13 weeks post-vaccination, at 4.14 U versus 2.82 U, respectively (P=0.0001 and P<0.0001). Although all vaccine strains protected mice significantly, the BMΔ*asp24* mutant afforded mice the greatest protection against 16M challenge in these experiments.

Protection against heterologous challenge: Cross *Brucella*-species protection. Mice were challenged with a heterologous wild-type strain to determine if the vaccine would be protective against another species of *Brucella*. When challenged with 2308, the BMΔ*asp24* mutant afforded the greatest degree of protection, at 4.19 U (P<0.0001) (Fig. 25). For this experiment, BMΔ*virB2* afforded more protection against 2308 challenge (3.30 U, P<0.0001), and BMΔ*manBA* the least at 1.60 U (P=0.02). Mice that were vaccinated with BAΔ*asp24* were protected against challenge with 16M to a very high degree, at 5.06 U (P<0.0001) (Fig. 26). The BAΔ*virB2* mutant, though significant as compared to the mock-vaccinated mice at 2.11 U (P=0.0001), did not protect as well as BAΔ*asp24*. Mice vaccinated with BAΔ*manBA* were protected 2.15 U (P=0.002), which is similar to the BAΔ*virB2* mutant. These data indicate that, similar to the homologous efficacy data, an attenuated mutant that persists for a longer period in the host and contains all the known virulence factors protects better than mutants without these characteristics.

IFN-gamma production from mouse splenocytes. To evaluate a major component of the Th1 response in vaccinated mice, splenocytes were assayed for levels

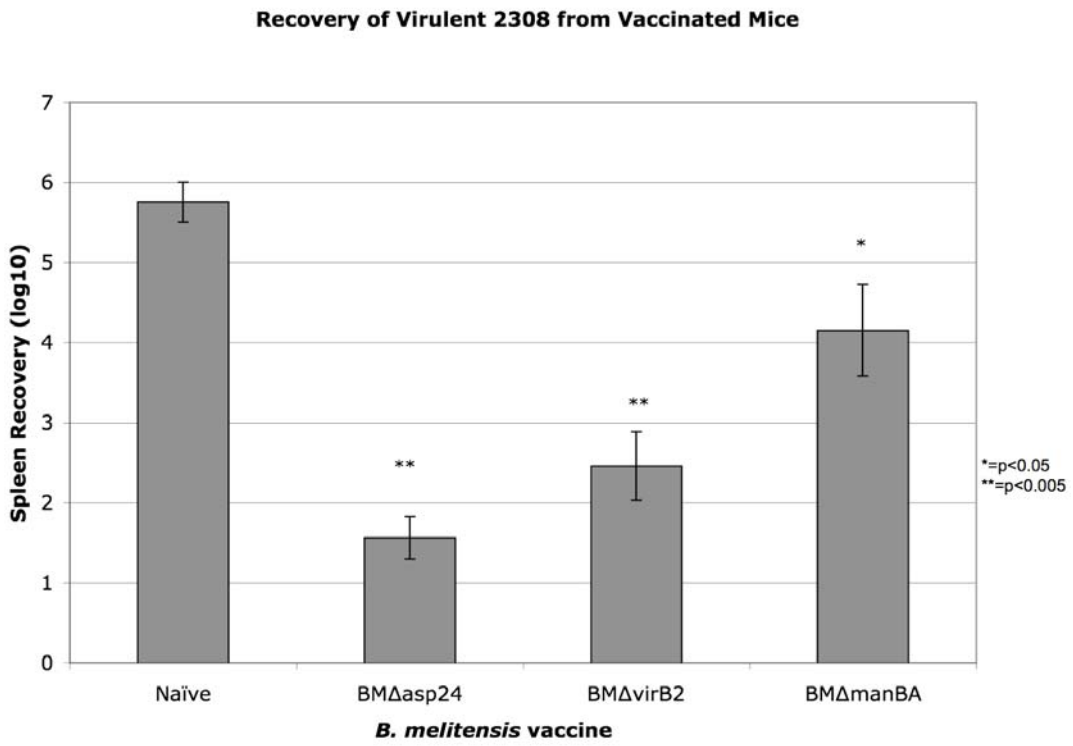

FIG. 25. Protection afforded to mice by *B. melitensis* unmarked deletion mutants against virulent 2308 challenge. Mice were vaccinated with $1x10^6$ CFU unmarked *B. melitensis* deletion mutants 16 weeks. After the vaccination period, the mice were challenged with $1x10^4$ CFU wild-type 2308 for 1 week. Mice were then euthanized, and spleens extracted and homogenized in 1 ml peptone saline. Homogenates were serially diluted to determine the remaining CFU bacteria in the spleens.

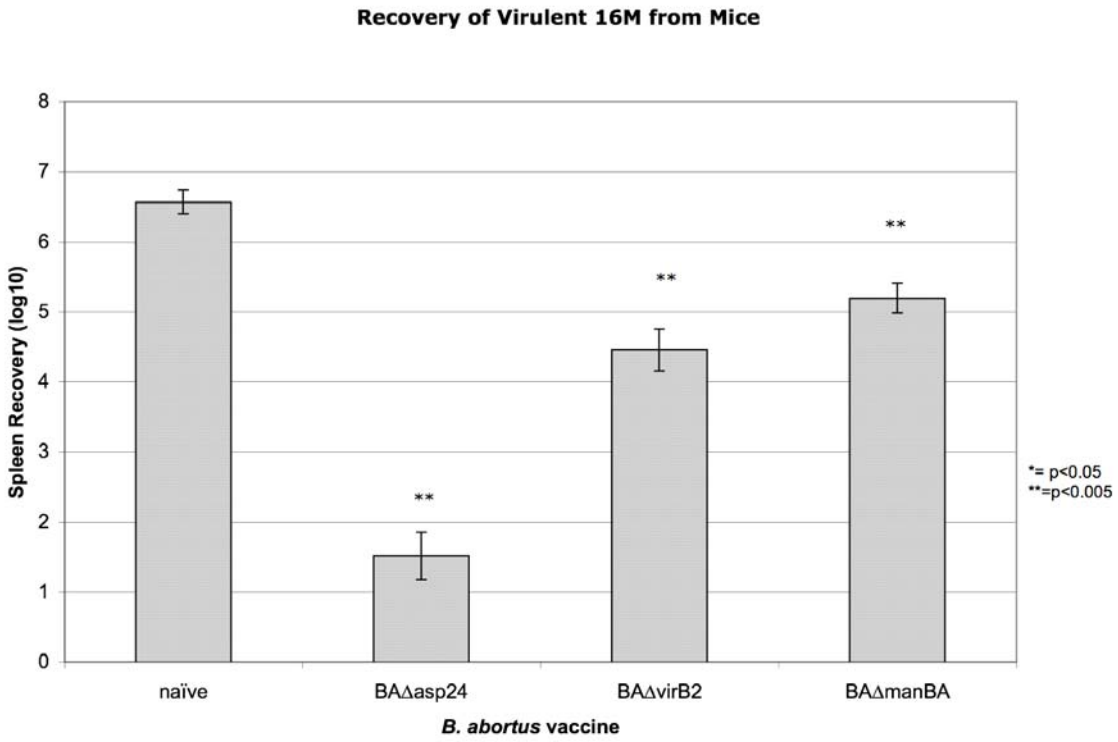

FIG. 26. Protection afforded to mice by *B. abortus* unmarked deletion mutants against virulent 16M challenge. Mice were vaccinated with $1x10^6$ CFU unmarked *B. abortus* deletion mutants for 16 weeks. After the vaccination period, the mice were challenged with $1x10^4$ CFU wild-type 16M for 1 week. Mice were then euthanized, and spleens extracted and homogenized in 1 ml peptone saline. Homogenates were serially diluted to determine the remaining CFU bacteria in the spleens.

of IFN-gamma, which is known to aid the regulation of *Brucella* infections in these animals. Production of IFN-gamma from splenocytes of vaccinated and vaccinated/challenged mice were evaluated by ELISA. Heat killed *Brucella* was used as the stimulant to compare *Brucella*-specific proliferation and cytokine production from the splenocytes of the mice. Splenocytes from unvaccinated mice that were either challenged with 2308 or 16M did not produce detectable levels of IFN-gamma *in vitro* when compared naïve mice in this study (Fig. 27). Splenocytes from mice vaccinated with BAΔ*asp24* and BMΔ*asp24* produced a significant amount of IFN-gamma (P=0.0002 and P=0.006, respectively) in response to heat killed *Brucella* (Fig. 28).

After challenge, the two strains exhibited different patterns. The level of IFN-gamma dropped significantly in BAΔ*asp24* vaccinates after challenge with 2308 (P=0.03). For mice vaccinated with BMΔ*asp24*, the level of IFN-gamma did not decrease after challenge with 16M and there was no significant change between the two groups. The BMΔ*asp24* vaccinated and challenged group was significantly greater than naïve mice for IFN-gamma production (P=0.001). The *virB2* mutants also exhibited an interesting change in IFN-gamma levels after challenge, where both BAΔ*virB2* and BMΔ*virB2* exhibited down-regulation of IFN-gamma *in vitro*, though the level was not significant due to the variation between mice (Fig. 29). The rough BMΔ*manBA* mutant did not produce significant amounts of IFN-gamma as compared to control mice (Fig. 30).

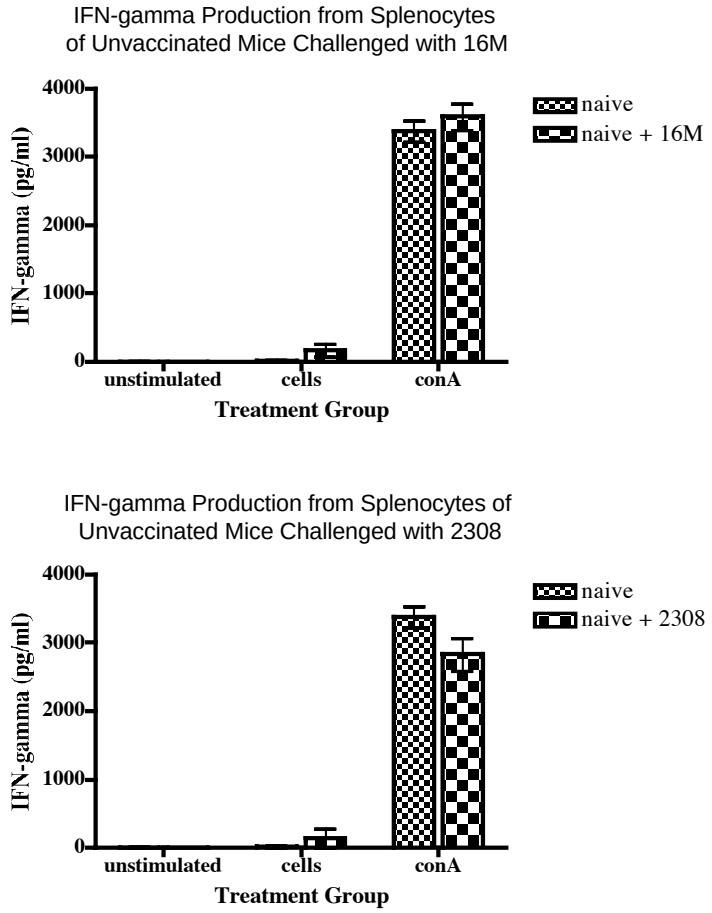

FIG. 27. IFN-gamma production from unvaccinated mice with and without challenge. Mice were not vaccinated and allowed to rest for 20 weeks before they were challenged with $1x10^4$ 16M or 2308. One group of naïve mice was left unchallenged for a baseline. Spleens were extracted and splenocytes were isolated as described in the text. Cells were either unstimulated, stimulated with $1x10^8$ heat killed *Brucella*, or stimulated with ConA. Supernatants were collected after three days and assayed by ELISA for the production of IFN–gamma.

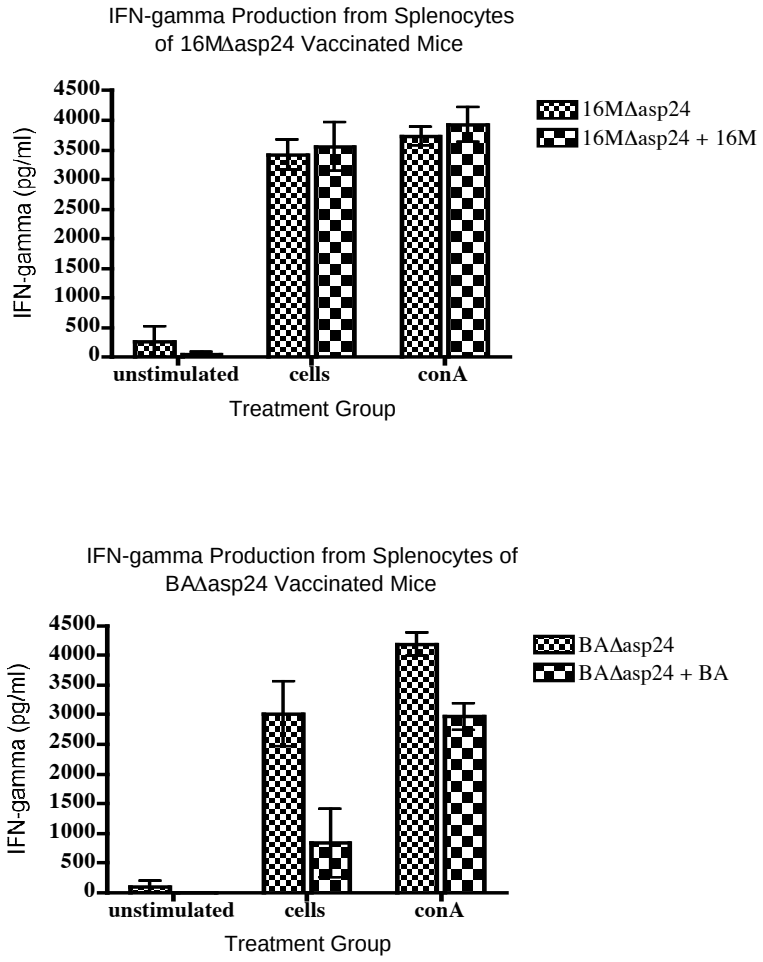

FIG. 28. IFN-gamma production from mice vaccinated with Δ*asp24* deletion mutants. Mice were vaccinated for 20 weeks with $1x10^6$ CFU unmarked *asp24* deletion mutants before they were challenged with $1x10^4$ 16M or 2308. Spleens were extracted and splenocytes were isolated as described in the text. Cells were either unstimulated, stimulated with $1x10^8$ heat killed *Brucella*, or stimulated with ConA. Supernatants were collected after three days and assayed by ELISA for the production of IFN–gamma.

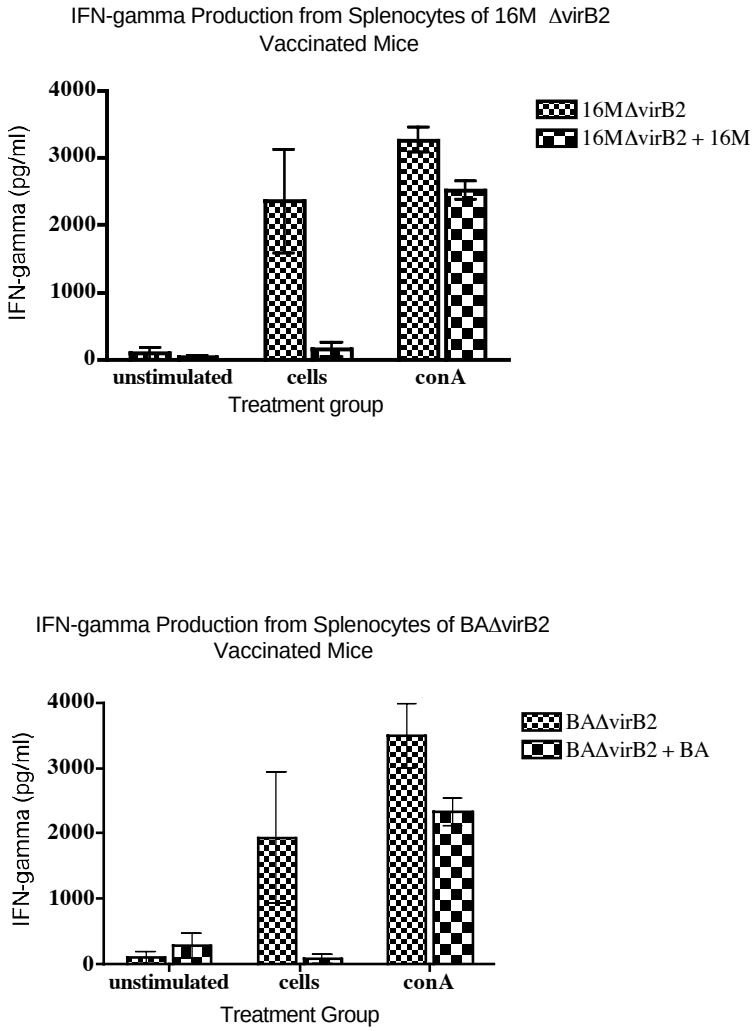

FIG. 29. IFN-gamma production from mice vaccinated with Δ*virB2* deletion mutants. Mice were vaccinated for 20 weeks with $1x10^6$ CFU unmarked *virB2* deletion mutants before they were challenged with $1x10^4$ 16M or 2308. Spleens were extracted and splenocytes were isolated as described in the text. Cells were either unstimulated, stimulated with $1x10^8$ heat killed *Brucella*, or stimulated with ConA. Supernatants were collected after three days and assayed by ELISA for the production of IFN–gamma.

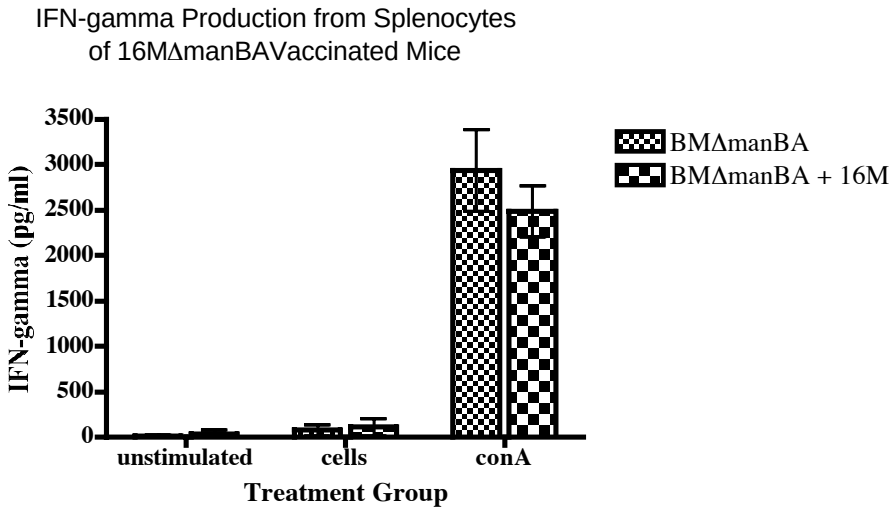

FIG. 30. IFN-gamma production from mice vaccinated with BMΔ*manBA*. Mice were vaccinated for 20 weeks with $1x10^6$ CFU unmarked BMΔ*manBA* before they were challenged with $1x10^4$ 16M or 2308. Spleens were extracted and splenocytes were isolated as described in the text. Cells were either unstimulated, stimulated with $1x10^8$ heat killed *Brucella*, or stimulated with ConA. Supernatants were collected after three days and assayed by ELISA for the production of IFN–gamma.

IL-10 production from mouse splenocytes. To evaluate a cytokine that typically counteracts IFN-gamma with anti-inflammatory properties, levels of IL-10 were assayed from splenocytes of vaccinated mice. The overall trend for IL-10 production was that naïve mice challenged with 2308 or 16M produced the cytokine in greater amounts *in vitro* after challenge (Fig. 31), as was evident for BAΔ*asp24* and BMΔ*asp24* mutants after challenge (Fig. 32). BAΔ*virB2* and BMΔ*virB2*, however, produced less IL-10 overall after challenge (Fig. 33). IL-10 production from BMΔ*manBA* vaccinated mice with and without challenge remained unchanged (Fig. 34). Except for the unvaccinated group challenged with 16M (P=0.003), there was no significant difference between mice challenged and not challenged for the same vaccine strain. When compared to the naïve controls, however, IL-10 levels from BMΔ*asp24* vaccinates were significantly increased before challenge (P=0.02) as well as after challenge (P=0.005), and BAΔ*asp24* was greater before challenge (P=0.05).

IL-12 production from mouse splenocytes. Extremely low amounts of IL-12 were detectable for mice vaccinated with 16MΔ*asp24* before challenge, though not significant. IL-12 was significantly detected only from naïve mice after 16M challenge (P=0.009). There was not any significant production of IL-12 for any of the *B. abortus* groups.

IL-2 production from mouse splenocytes. IL-2 production was increased for mice vaccinated with BMΔ*asp24* both before and after challenge (Fig. 35). No other

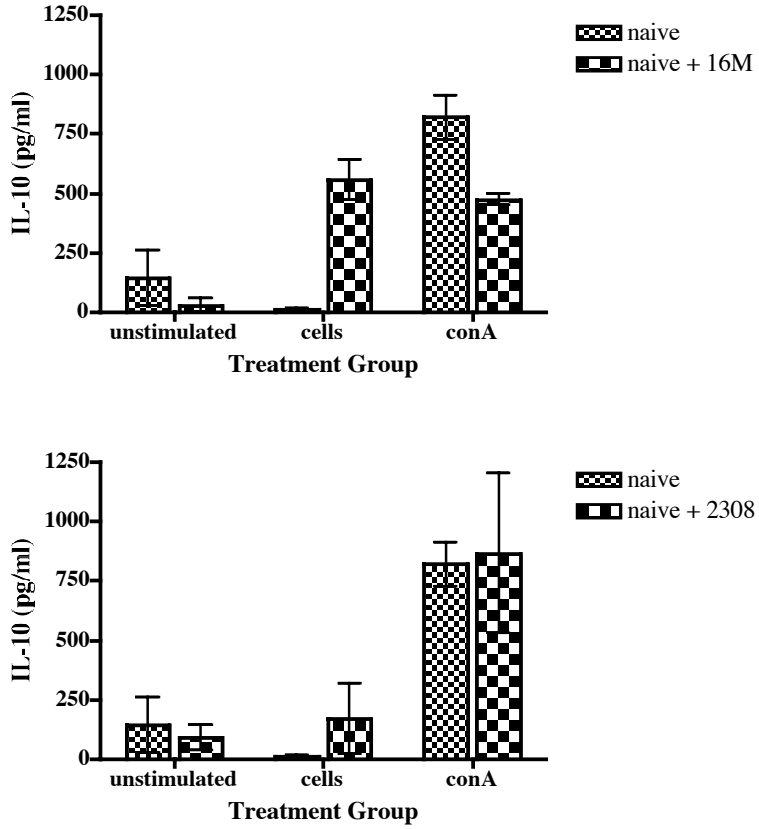

FIG. 31. IL-10 production from unvaccinated mice with and without challenge. Mice were unvaccinated and allowed to rest for 20 weeks with before they were challenged with $1x10^4$ 16M or 2308. One group of mice was left unchallenged for a baseline. Spleens were extracted and splenocytes were isolated as described in the text. Cells were either unstimulated, stimulated with $1x10^8$ heat killed *Brucella*, or stimulated with ConA. Supernatants were collected after three days and assayed by ELISA for the production of IL-10.

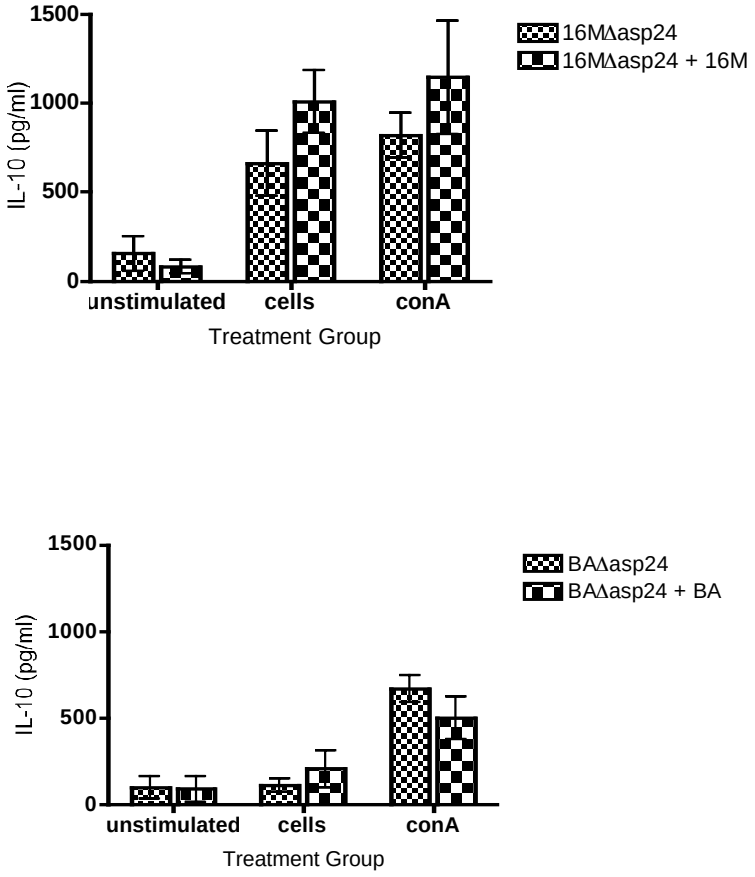

FIG. 32. IL-10 production from mice vaccinated with Δ*asp24* deletion mutants. Mice were vaccinated for 20 weeks with $1x10^6$ CFU unmarked *asp24* deletion mutants before they were segregated into two groups, either unchallenged or challenged with $1x10^4$ 16M or 2308. Spleens were extracted and splenocytes were isolated as described in the text. Cells were either unstimulated, stimulated with $1x10^8$ heat killed *Brucella*, or stimulated with ConA. Supernatants were collected after three days and assayed by ELISA for the production of IL-10.

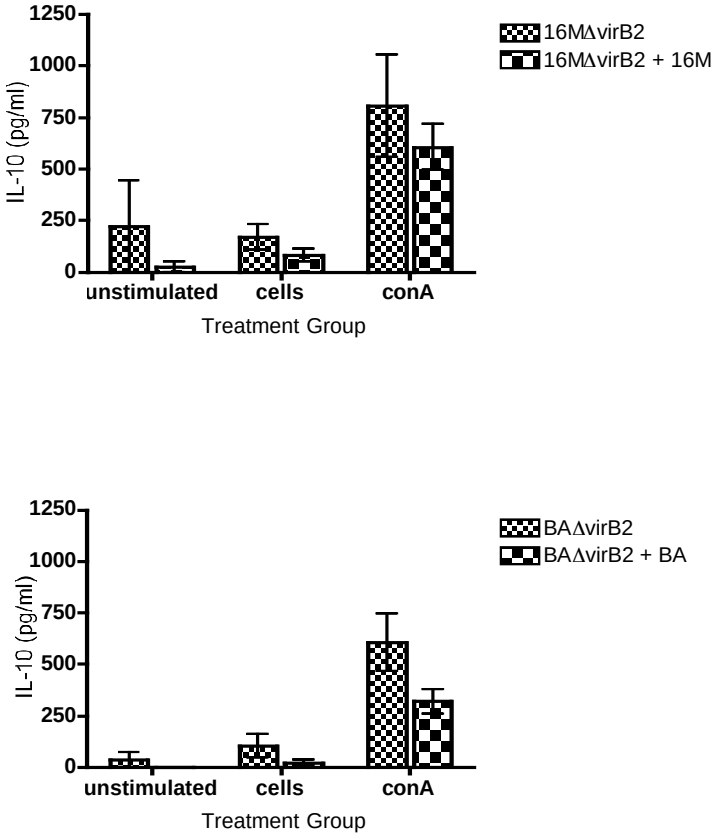

FIG. 33. IL-10 production from mice vaccinated with Δ*virB2* deletion mutants. Mice were vaccinated for 20 weeks with $1x10^6$ CFU unmarked *virB2* deletion mutants before they were segregated into two groups, either unchallenged or challenged with $1x10^4$ 16M or 2308. Spleens were extracted and splenocytes were isolated as described in the text. Cells were either unstimulated, stimulated with $1x10^8$ heat killed *Brucella*, or stimulated with ConA. Supernatants were collected after three days and assayed by ELISA for the production of Il-10.

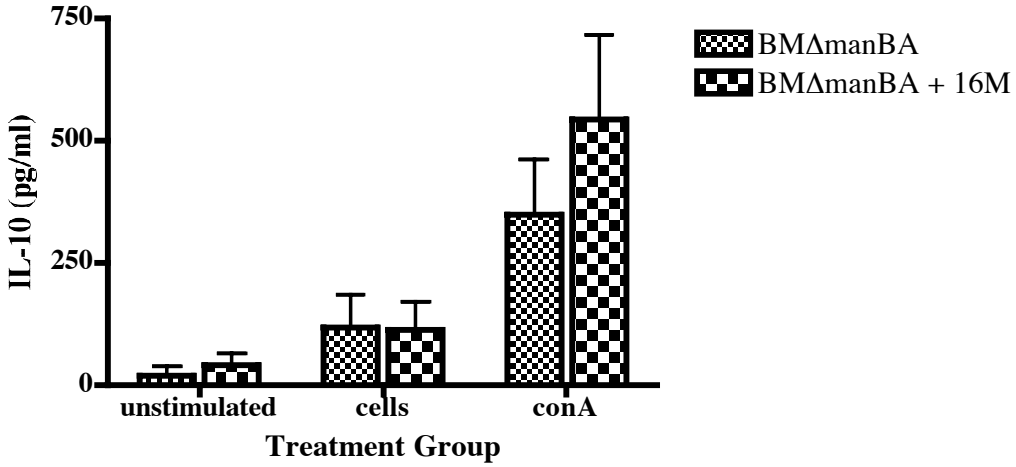

FIG. 34. IL-10 production in mice vaccinated with BMΔ*manBA*. Mice were vaccinated for 20 weeks with $1x10^6$ CFU unmarked BMΔ*manBA* before they were segregated into two groups, either unchallenged or challenged with $1x10^4$ 16M. Spleens were extracted and splenocytes were isolated as described in the text. Cells were either unstimulated, stimulated with $1x10^8$ heat killed *Brucella*, or stimulated with ConA. Supernatants were collected after three days and assayed by ELISA for the production of IL-10.

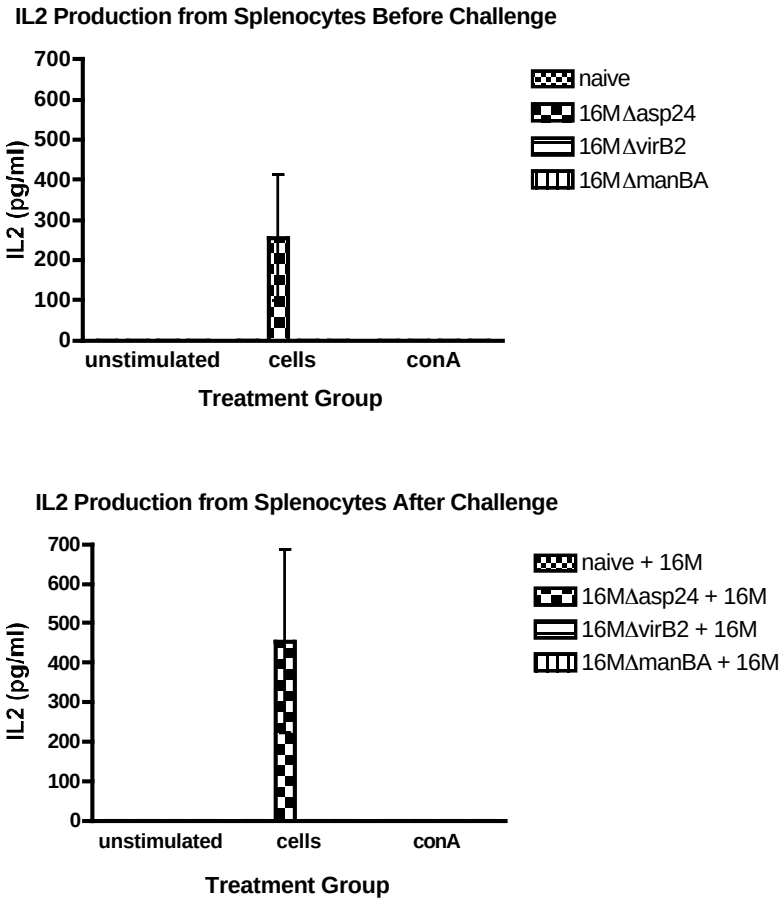

FIG. 35. IL-2 production from mice vaccinated with *B. melitensis* deletion mutants. Mice were vaccinated for 20 weeks with $1x10^6$ CFU unmarked *B. melitensis* deletion mutants before they were segregated into two groups, either unchallenged or challenged with $1x10^4$ 16M. Spleens were extracted and splenocytes were isolated as described in the text. Cells were either unstimulated, stimulated with $1x10^8$ heat killed *Brucella*, or stimulated with ConA. Supernatants were collected after three days and assayed by ELISA for the production of IL-2.

groups demonstrated an increase in production. The group vaccinated with BAΔ*virB2* displayed an elevated average of IL-2 before challenge, but due to the variation between mice, this was not significant. There was no detectable production of IL-2 after challenge for the *B. abortus* group.

IL-4 production from mouse splenocytes. Overall, there was no significant production of IL-4 from splenocytes stimulated *in vitro* with heat killed *Brucella*.

DISCUSSION

Competitive infection assays have been used historically to evaluate competition between the wild-type organism and the attenuated mutant in the same animal. This allowed for direct comparisons of splenic colonization, and minimized mouse-to-mouse variation while ensuring that the delivery of the dose was standardized. Signature tagged mutagenesis utilizes the same approach competitive infections, in which multiple mutants compete with one another in the same mouse (38). Novel marked and unmarked mutants were compared in two ways. First, marked mutants were compared to wild-type, to demonstrate the degree of attenuation due to the genetic defect. Secondly, marked mutants are compared to unmarked mutants, to ensure that the two mutants can be used interchangeably and to identify any polar effects resulting from insertion of the kanamycin cassette. The downfall of this assay is that the degree of attenuation of particular mutants is, in our well documented example, exacerbated by the presence of wild-type organism. Since both types of bacteria were competing for the

same intracellular niche, the attenuated organism may have been out-competed by the better-equipped wild-type strain. This was the case for both BAΔ*manBA* and BMΔ*manBA* mutant strains, whose presence was rapidly eliminated from mice in a competitive infection scenario with wild-type organism, but not when mice were vaccinated with the mutant strains alone. For assays such as signature tagged mutagenesis, this effect is useful for identifying mutants that are attenuated, but it is not accurate for identifying survival characteristics.

In contrast to the competitive infection assay, the non-competitive clearance assay evaluated non-competitive growth of attenuated mutants in the mouse. These experiments mimicked utilization of the mutant as vaccine in the field, and emphasized the duration of persistence of the vaccine strain under non-competitive conditions. Evaluation of colonization singly was important since the presence of wild-type organism influenced clearance of attenuated mutants from the host, and suggested enhanced rates of clearance. In non-competitive experiments, deletion mutants exhibited different rates of clearance from mice. The rough mutants, BAΔ*manBA* and BMΔ*manBA*, were significantly attenuated by 2-3 logs as quickly as 1 week post-infection but requiring 5 weeks post-infection to be undetectable. The *virB2* mutants, BAΔ*virB2* and BMΔ*virB2,* cleared at a more gradual rate than the *manBA* deletions, suggesting that their defect in survival occurs at a later stage of infection. Lastly, BAΔ*asp24* and BMΔ*asp24* cleared from mice at a greatly delayed rate, with BAΔ*asp24* (between 8 and 10 weeks post-infection) and BMΔ*asp24* (after 16 weeks), suggesting that Asp24 is only required for persistence of the organism. The differences in survival

exhibited by these mutants favored their use in evaluating the correlation between protection and persistence of the vaccine strain.

Protection against homologous challenge (i.e. identical vaccine and challenge species) was most successful with the persistent BAΔ*asp24* and BMΔ*asp24* mutants. These mutants protected mice against infection at or near sterile levels at all timepoints examined, most of which were well past the time of vaccine clearance. We consider these mutants to be the best candidates for novel live vaccine strains, particularly since they protect mice better than presently utilized vaccine strains. BAΔ*virB2*, BMΔ*virB2*, BAΔ*manBA*, and BMΔ*manBA*, although providing significant levels of immunity, did not protect mice from homologous challenge as well as the Δ*asp24* mutants or currently available vaccine strains. However, the rapid clearance of these mutants enhances their safety, and may support their use if delivered using controlled release where the duration and presentation to the host can be extended to improve efficacy.

In protection studies against heterologous challenge, both BAΔ*asp24* and BMΔ*asp24* again protected mice significantly against challenge, just barely above the limit of detection in both situations. These results suggest that survival beyond 8-10 weeks does not enhance protection, and since prolonged survival may have side-effects, the use of BAΔ*asp24* as a vaccine strain may be sufficient. BMΔ*virB2* afforded better protection against 2308 challenge than BAΔ*virB2* afforded against 16M challenge. These data indicate that vaccination of animals with BAV2 may not protect against a *B. melitensis* exposure, which is possible in the event of a bioterrorist attack or exposure

from infected small ruminant species (such as goats). In this situation, Δ*virB* mutants may not be sufficient to protect the host from more than one species of *Brucella*.

The degree of protection afforded by different mutant types as vaccine candidates was compared with the cytokine profiles elicited from splenocytes both before and after challenge in order to characterize any direct correlated of protective immunity. This method was used to evaluate the long-term immune response and mimics field conditions, where animals are vaccinated and at some unknown period are exposed to wild-type organism, and subsequently mount a memory response.

IFN-gamma, a Th1 cytokine involved in pathogen clearing from activated macrophages, is well defined as an important factor for controlling *Brucella abortus* infections in mice. IFN-gamma is well documented as an aid to control infection through activation of macrophages against intracellular bacteria and protozoa (5, 33, 56). Infection in mice is more severe when the animals are treated with antibody to IFN-gamma, and brucellosis becomes a fatal infection in IFN-gamma knockout mice. This data suggests that IFN-gamma is critical in the early stages of infection (5, 50, 51, 56). Since IFN-gamma has been shown to be a strong component of the host immune responses against *Brucella* infections, it was necessary to evaluate the production elicited by the different mutant strains.

The importance of IFN-gamma in controlling infection is confirmed by the data presented here. The best protection was provided by the mutant that elicited the strongest IFN-gamma response, BMΔ*asp24*. Both before and after challenge, the levels of this cytokine produced by splenocytes remained very high. For BAΔ*asp24*,

BAΔ*virB2*, and BMΔ*virB2* IFN-gamma production was high in mice before challenge when stimulated *in vitro,* reflecting a memory response and correlating with subsequent protection against challenge. When the mice were challenged, however, it is possible that in these groups, where the vaccine strain had cleared approximately 10 weeks before challenge, that the memory T cells left the spleen for general circulation in response to the infection. It is known that mice infected with S19 or 2308 experienced depletion of splenic lymphoid tissue when examined histologically, with decreased percentages of splenic T cells (24). It is possible that culturing splenocytes one week post- challenge to stimulate an *in vitro* response will not reflect what is observed when mice are vaccinated solely. Mice that were vaccinated with RB51 for 6 weeks produced high levels of IFN-gamma, but the levels dropped after challenge for one week with 2308, which was consistent with these results for smooth mutants (57). IFN-gamma was not induced significantly in mice vaccinated with a rough mutant, BMΔ*manBA*, and the levels remained unchanged before and after challenge. It is important to note, however, that RB51 and *manB* mutants contain different defects in LPS, correlating with improved protection from RB51 as compared to *manB* vaccination in mice, which may account for the observed differences in cytokine profiles (13, 48, 69).

IL-10 is chiefly an anti-inflammatory molecule that may aid in the control of negative effects due to pro-inflammatory cytokines, such as IFN-gamma (71). IL-10 is actually produced as part of a Th2 response, but may be involved in negative feedback control of *B. abortus* induced cytokine secretion, such as IL-12. For many organisms, it affects the Th1 response by down-regulating IL-12 affinity receptors thus decreasing

IFN-gamma (5, 26). For *Brucella*, IL-10 does not seem to down-regulate IFN-gamma production, however. Perhaps for this disease, the effect of IL-10 on the immune response is not counterbalance the production of Th1 cytokines, but rather to limit exaggerated pro-inflammatory responses of the host. IL-10 is known to help limit the extent and strength of inflammatory responses (71). Since BALB/c mice are known to mount a very strong inflammatory response early in infection, Th2 cytokines such as IL-10 are necessary to prevent overwhelming the host (5, 57). This might also benefit the host by limiting the influx of phagocytic cells and potential spread of the disease, though this would not necessarily benefit the bacteria. Recently, it has been shown that IL-10 develops pro-inflammatory qualities during active inflammatory responses (71). The fact that IL-10 was generally increased after challenge in these experiments may suggest an attempt of the host to down-regulate the inflammatory response that is normally induced by *Brucella*. IL-10 knockout mice do not tolerate *B. abortus* infection well, also demonstrating its importance in down-regulating the pro-inflammatory response (33). Levels of IL-10 in mice vaccinated with *asp24* mutants increased after challenge, similar to levels of IL-10 in naïve mice challenged with wild type organism. This suggests that the *asp24* deletions elicit similar biologic effects as wild type organism, and may explain the superior protection afforded by these strains.

Surprisingly, IL-12 was not detected from any of the infected splenocytes stimulated with heat killed *Brucella* except for naïve mice challenged with 16M. IL-12 plays a pivotal role in the immune cascade after its release from macrophages, including activation and differentiation of NK cells, and T and B cells. *B. abortus* produced IL-12

p40 in human monocytes, as well as in mouse models (33, 57). It is important to note that for these experiments, culture supernatants were collected at three days post-stimulation. It is possible that this timepoint is not optimal for the detection for certain cytokines, which may be produced either rapidly or late *in vitro*. The same results were observed for IL-2 and IL-4. IL-4, however, is typically upregulated when mice are not infected with living bacteria, but rather soluble *Brucella* proteins or heat killed bacteria, so this result may not be surprising (82).

Another possible explanation for the data is that due to the experimental timepoints chosen, the mice were vaccinated for longer periods of time than have been looked at in the past. Numerous studies have considered the effect of *Brucella* infection on cytokine production and immune response, but none have looked at time points as late as 20 weeks, but rather much earlier in infection (26, 39, 50, 57, 72). In cases of acute human brucellosis, patients also display a Th1-type response similar to the previous mouse studies, producing IFN-gamma and IL-2; patients experiencing chronic infections do not. The diminished Th1 response as *Brucella* infection progresses may explain the establishment of chronic infections (31). It has also been demonstrated that chronic brucellosis is accompanied by a significant, general immunosuppression (29, 31) therefore persistent mutants whose attenuation isn't marked compared to wild-type until later in mouse infections (typically greater than 8 weeks), and could possibly be considered a more chronic infection, may not display a strong Th1 response. In this study, mice were vaccinated for 20 weeks, and it is very possible that the numbers of T cells were diminished or virtually non-existent, and so we would not see the "typical"

immune pattern previously published for acute mouse models of infection: Th1, with up-regulation of IFN-gamma, IL-2, and IL-12. Since previous research has never considered such late time points in the mouse, direct comparisons can not be made.

Overall, the mouse has proven to be a sensitive and popular model for evaluating brucellosis. Since the virulence of *Brucella* is linked to its ability to survive in macrophages, as it is able to do in the mouse model, virulence in other animal hosts such as ruminants can be predicted. In addition, this model has proved invaluable for characterization of genetic components necessary for virulence, as well as an affordable and consistent method for evaluation of potential vaccine candidates, by assessment of mutant survival (23, 28, 38). The mouse model cannot be used, however, to gauge the effect of persistence on the colonization of reproductive tissues, thus the need for a natural ruminant model after the initial murine screen. Since the mouse is not an accurate model of disease in target species, vaccine candidates from the mouse will need to be re-evaluated in a suitable ruminant model. The *asp24* deletion mutants were the most protective in the mouse screen against both homologous and heterologous challenge infections, and perhaps their protective ability in target ruminants may be superior to the other candidates in that model as well.

SAFETY AND EFFICACY OF UNMARKED MUTANTS IN THE PREGNANT GOAT MODEL

INTRODUCTION

Experimental infections of the goat with *B. abortus* S2308 result in disease similar to cattle, regarding clinical symptoms, distribution of the organism within the reticuloendothelial and reproductive systems, and serological responses (47). Infection with S2308 in the goat is not efficiently reproducible. Pregnant dams abort 30-50% of the time and dam/kid pairs are colonized approximately 50-70% of the time. In contrast, pregnant goats experimentally inoculated with *B. melitensis* 16M abort fetuses 70-100% of the time, with 90-100% of the dam/kid pair colonized. In artificially controlled infection of goats, 16M therefore elicits a more reproducible experimental effect. This effect directly mimics field infection, since pregnant goats naturally infected with *B. melitensis* often abort in the last trimester of pregnancy (21).

The vaccine currently used in goats is Rev 1, a 16M derivative whose genetic basis for attenuation is undefined. Rev 1 may induce abortions if administered to a pregnant goat and does not prevent spread of the disease in flocks endemically infected prior to vaccination (19). This vaccine does, however, protect approximately 80-100% of goats from abortion and colonization of dam/kid pairs after challenge with wild-type 16M in an experimental setting, and therefore Rev 1 is a model vaccine to compare protection provided by novel vaccine strains, and improved levels of safety (21).

For all these reasons, the goat model is useful for evaluating the *in vivo* phenotype of various mutants, by comparing abortions caused by infection with attenuated unmarked deletions to the wild-type 16M, thus assessing their degree of virulence. The response of the pregnant goat to vaccination with attenuated 16M mutants is therefore a sensitive model to evaluate safety of the mutant for use as a vaccine in some target species.

The caprine model of brucellosis (or *Brucella* infection) is well documented, offering many advantages over the murine model to assess attenuation of mutants in a ruminant species (3, 19, 21, 47). For example, more goats can be used in restricted spaces due to their smaller size, they cost less than larger ruminants, and the gestation period is only approximately five months as compared to nine in the cow, and therefore results are more timely. In addition, the conjunctival route of inoculation mimics the natural route of infection, allowing the bacteria to invade mucosal sites (21).

MATERIALS AND METHODS

The pregnant goat model of pathogenesis. To compare the pathogenesis of BMΔ*cydBA*, BMΔ*asp24*, and BMΔ*virB2* to the virulent parental strain 16M, 4 groups of 6 each pregnant female goats averaging 110 days of gestation were infected at $1X10^7$ CFU/ml by placing 50 μl of either 16M or the three selected 16M unmarked deletion mutants into each conjunctival sac. This challenge dose is commonly used and has demonstrated consistent abortion and colonization in the goat (21). Animals were monitored daily; birth status was recorded as aborted fetuses or live kid(s). Serum samples were collected prior to vaccination and at necropsy for serological studies. The

fetuses were necropsied with samples of lung, liver, spleen, and abomasal fluid collected for bacteriology and histology. Approximately 30 days after delivery, all dams were euthanized and necropsied; samples of liver, spleen, mammary gland, supramammary lymph nodes, and parotid lymph nodes were collected for bacteriology and histopathology.

Culturing of tissues. For primary isolation of *Brucella* from tissues, a piece of the tissue to be cultured was washed in 70% ethanol and flamed to remove external contaminants. The tissue was then sliced open, and the cut surface was macerated with a sterile scalpel. The macerated tissue surface was then streaked across the surface of a Farrell media plate (25). All tissue-inoculated plates were incubated for up to ten days to detect the presence of *Brucella*. Positivity is based upon a scale of plus or minus, with isolation of ≥1 CFU per plate determining the tissue to be positive.

Biotyping. All colonies arising on Farrell's media from bacteriology were first identified by size, shape, and color, to be consistent with *Brucella*. Colonies were also Gram stained to ensure that they are gram-negative coccobacilli, also consistent with *Brucella*. Isolates were then biotyped as per the standard biotyping protocols. This included growth on dyes, including thionin and basic fuchsin, hydrogen sulfide production, carbon dioxide requirements, growth on erythritol, lysis by phage, and agglutination by monospecific sera. These tests were completed to ensure the colonies were modified *B. melitensis* biovar 1.

Immune responses in the pregnant goat. Serum samples were collected from goats at several timepoints post-infection. Approximately 10 ml of blood was collected into vacutainers without additive, allowed to clot overnight, and then and spun at 1700 x g for 25 minutes at room temperature. Serum was decanted into a 15ml conical tube for testing and subsequent freezing. *Brucella* card tests (USDA Ames, Iowa) were performed on the serum samples to determine seroconversion.

Enzyme-linked immunosorbent assays (ELISAs) were preformed on serum samples collected at various times post-infection to detect the IgG response of infected goats. Ninety six well plates were coated with 100μl of 1μg/ml *Brucella* lysate. Lysate was prepared from heat killed liquid cultures of 16M (approximately 4 x 10^{10} CFU, washed in PBS) by sonication. Protein concentration was determined to be 10μg/ml using BioRad Protein Assay Reagent as described by the manufacturer. A 1:10,000 dilution of the bacterial lysate in carbonate buffer was used to coat the wells overnight at 4°C. Wells were blocked for three hours with 300μl 3% gelatin (w/v) in PBS, and then plates were washed three times with PBS to remove residual blocking buffer. For pre-bleeds, goat sera was first diluted 1:8 with 0.3% gelatin (w/v) in PBS-T, and then added to the first well of each row (A through H) and 2-fold serially diluted across the plate in successive wells. For post-vaccination samples, sera were diluted to 1:128 and then two-fold serial dilutions were prepared as described above. Following a one-hour incubation, the wells were washed three times with PBS-T to remove all traces of sera. Peroxidase labeled rabbit anti-goat IgG (H+L) secondary antibody (KPL) was added at a 1:2500 dilution (0.2 μg/ml) in 100μl PBS-T per well, and the plates were rocked for 1 hour at

room temperature. The wells were washed seven times with PBS-T to remove any residual secondary antibody. ABTS (Sigma) substrate was added at 100μl per well according to the manufacturer's protocol, and color development continued at room temperature for 10-15 minutes before the reaction was stopped with 100μl 1% SDS (w/v). The samples were read at 405 nm using a visible wavelength spectrophotometer.

Cut-off values for ELISA were established first with known negative goat samples. Cut-off values were determined to be the fifth two fold serial dilution (1:32), which was the first dilution where changes in OD values were detectable that would be above background. Baseline titers were therefore set at a value of 5. Titers for samples were based upon the first dilution in the series that the OD reading was below the cut-off OD value. The IgG levels are therefore represented by the two fold increases in IgG levels over the duration of infection.

Histology. Samples of all tissues collected from both the dams and kids were fixed in 10% buffered formalin, trimmed with a scalpel so that they fit into tissue cassettes. Tissues were paraffin embedded and stained with a routine H&E stain by the Histology lab at the Texas A&M College of Veterinary Medicine. Histological differences of the H&E slides were microscopically assessed by ACVP Board Certified pathologist Dr. L. Garry Adams to determine differences between vaccine groups.

Vaccination efficacy. Mature non-pregnant goats were vaccinated in groups of eight with either $1x10^9$ CFU unmarked 16M deletion mutant, saline or $1x10^6$ CFU Rev 1

vaccine strain. Four weeks after vaccination, dams were housed with males and allowed to breed. Pregnancy was confirmed via ultrasound, and at approximately 110 days of gestation, dams were challenged with $1x10^7$ CFU in 100μl 16M via bilateral conjunctival inoculation. At birth, the status of the kid(s) was recorded as live, weak, born live but found dead, or dead on arrival, and samples of spleen and abomasal fluid were collected for bacteriology. Within 14 days of delivery, dams were euthanized; samples of liver, spleen, mammary gland, parotid lymph node, and supramammary lymph node were collected and cultured (19, 21). Serum samples were collected at vaccination, five weeks post-vaccination, at the date of challenge (18 weeks post-vaccination), and at necropsy (7 weeks post-challenge) for serological studies. Protection due to vaccinations with mutant strains was compared to protection conferred by Rev-1 vaccine strain.

RESULTS

Pilot goat safety study. The first set of pregnant goat safety experiments to determine safety of unmarked deletion mutants in pregnant animals were held at Texas A&M University in the spring of 2002. Goats were divided into four different treatment groups: (a) 10-16M inoculated (b) 10-BMΔ*virB2* inoculated, (c) 10-BMΔ*cydBA* inoculated, or (d) 10-uninfected controls. The purchased goats were to arrive at approximately 90-100 days of gestation. Several goats gave birth prior to arrival, and one immediately delivered after arrival. Those goats were moved to the unchallenged control group. Overall, the majority of goats were well past 110 days of gestation at the

day of infection, and as such, accurate assessments of abortion and safety could not be determined (Figs. 36, 37, and 38). All goats were necropsied for bacterial isolation from tissues (Tables 5 and 6). Isolation of ≥1 CFU *Brucella* from any goat tissue assigned that animal as positive. Goats were also bled weekly to detect *Brucella*-specific antibodies by card test from exposure to the vaccine strain.

In the 16M challenged group, several goats were culture positive (#9, 32, and 51). One kid was culture positive as well in all organs tested (#51-A). These isolates were cultured for isolation directly from the tissue; this one-passage isolate was frozen for the lab's future virulent 16M stock. The acquisition of the virulent fetal isolate was a vital asset to the lab for future experimentation and mutant production.

The biohazard buildings were useful for containing these experimental goats that posed health risks to the human workers due to the presence of *Brucella* in their tissues. None of the staff was accidentally infected, and these buildings will be an excellent facility to continue large animal experiments.

Evaluation of vaccine safety. In the spring of 2003, a goat safety study was performed at Louisiana State University (LSU) to determine the safety of unmarked deletion mutants in pregnant goats. Treatment groups included (a) 6-16M inoculated goats, (b) 6-BMΔ*asp24* inoculated goats, (c) 6-BMΔ*virB2* inoculated goats, and (d) 6-BMΔ*cydBA* inoculated goats. Birth statuses of kids or fetuses were recorded by the staff at LSU. The 16M infected group did not demonstrate marked abortions or abnormal

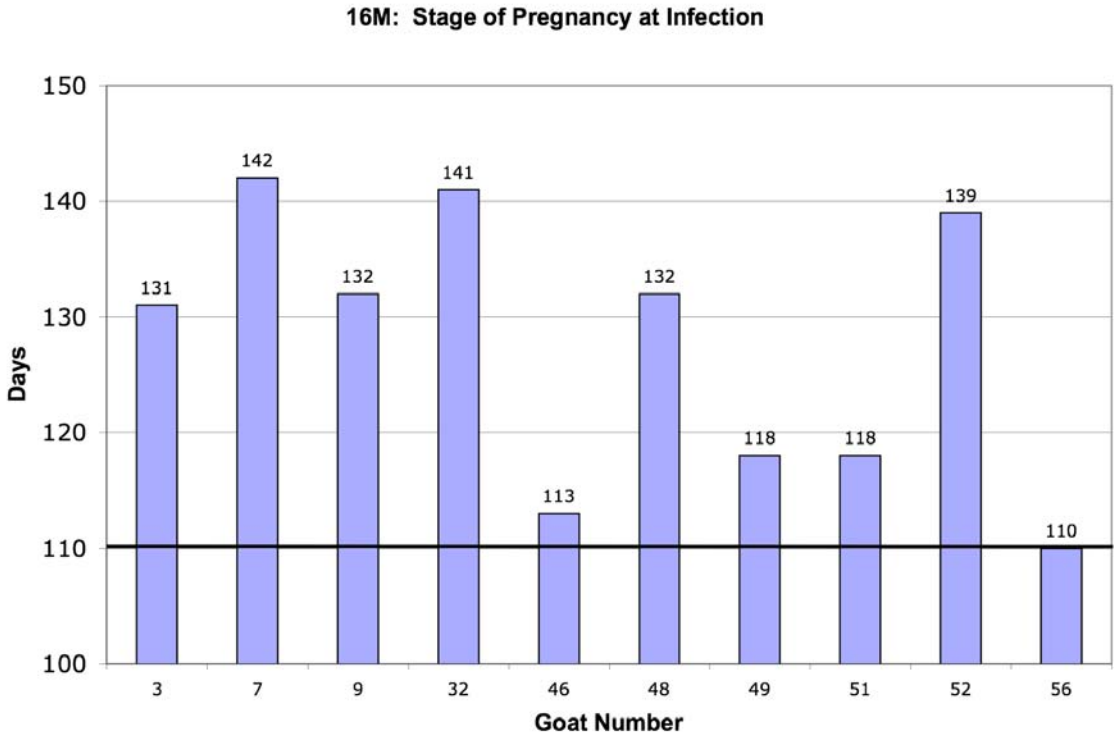

FIG. 36. Day of gestation at challenge for 16M treatment group: pilot goat safety. Goats were infected with $1x10^7$ CFU 16M conjunctivally in the third trimester of pregnancy. The gestation period of the goat is approximately 150 days, therefore subtracting the number of days elapsed since infection from 150 equals the approximate stage of gestation at infection. Bars represent the approximate day of gestation that each goat was vaccinated. The solid line at 110 days represents the target stage of gestation for experimental challenge infection. In this group, 6/10 goats were challenged after the target infection period of 110 days +/- 10 days.

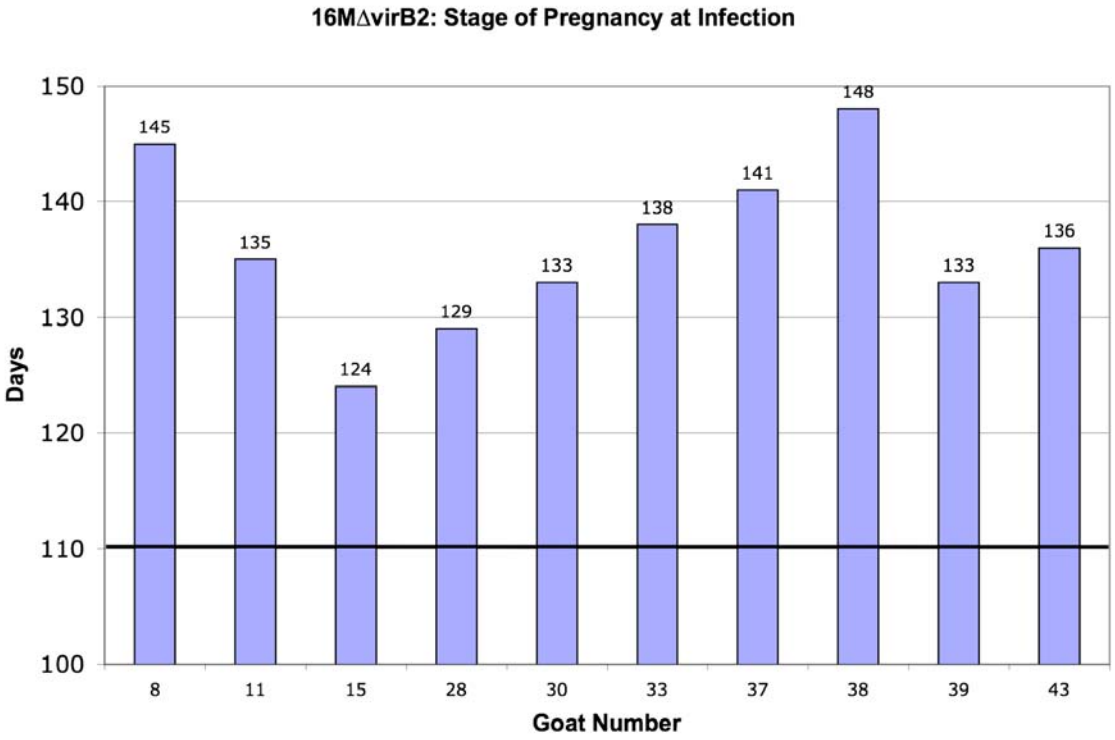

FIG. 37. Day of gestation at challenge for BMΔ*virB2* treatment group: pilot goat safety. Goats were vaccinated with $1x10^7$ CFU unmarked deletion BMΔ*virB2* conjunctivally in the third trimester of pregnancy. The gestation period of the goat is approximately 150 days, therefore subtracting the number of days elapsed since infection from 150 equals the approximate stage of gestation at infection. Bars represent the approximate day of gestation that each goat was vaccinated. The solid line at 110 days represents the target stage of gestation for experimental challenge infection. In this group, 10/10 goats were challenged after the target infection period of 110 days +/- 10 days.

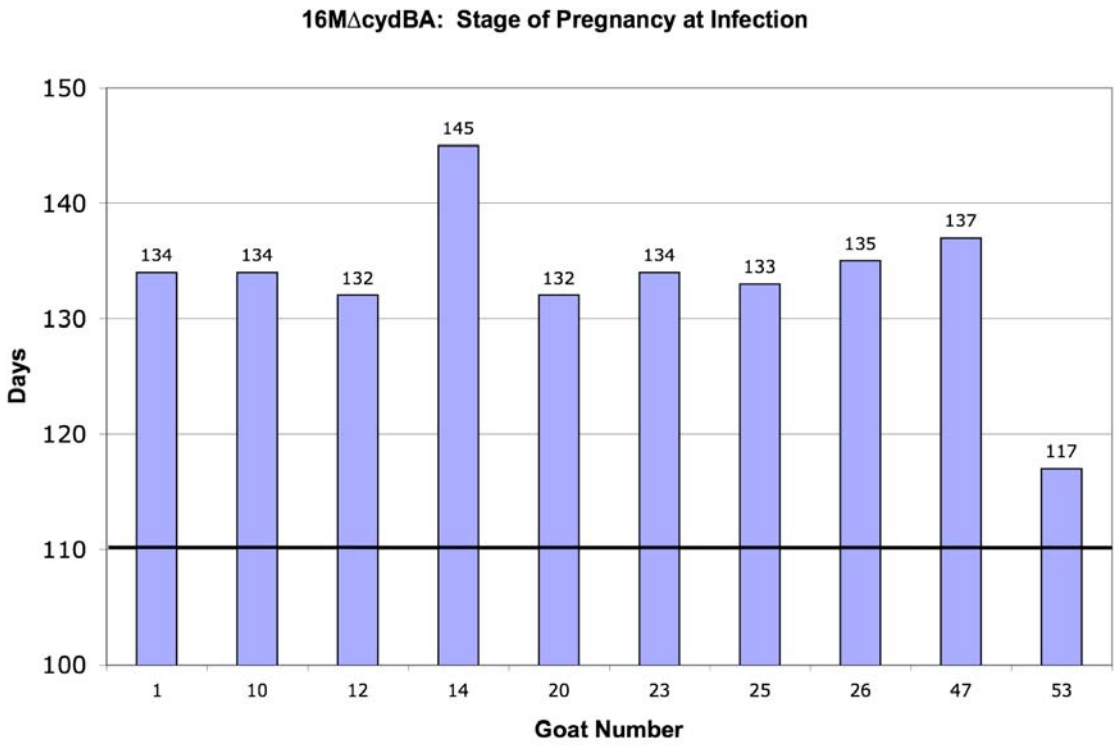

FIG. 38. Day of gestation at challenge for BMΔ*cydBA* treatment group: pilot goat safety. Goats were vaccinated with $1x10^7$ CFU unmarked BMΔ*cydBA* conjunctivally in the third trimester of pregnancy. The gestation period of the goat is approximately 150 days, therefore subtracting the number of days elapsed since infection from 150 equals the approximate stage of gestation at infection. Bars represent the approximate day of gestation that each goat was vaccinated. The solid line at 110 days represents the target stage of gestation for experimental challenge infection. In this group, 9/10 goats were challenged after the target infection period of 110 days +/- 10 days.

TABLE 5. Culture and card test results from pilot goat safety study control groups

16M			
Goat Number	Dam	Kid	Card Test
3	-	-	+
7	-	-	+
9	+	-	+
32	+	-	+
46	-	-	+
48	-	-	-
49	-	-	+
51	+	+	+
52	-	-	+
56	-	-	-

saline			
Goat Number	Dam	Kid	Card Test
13	-	-	-
18	-	-	-
19	-	-	n/a
34	-	-	-
35	-	-	-
40	-	-	n/a
50	-	-	-
54	-	-	-
55	-	-	-
57	-	-	-

TABLE 6. Culture and card test results from pilot goat safety study *B. melitensis* mutant groups

BMΔ*virB2*			
Goat Number	Dam	Kid	Card Test
8	-	-	n/a
11	-	-	-
15	-	-	-
28	-	-	-
30	-	-	-
33	-	-	-
37	-	-	-
38	-	-	-
39	-	-	-
43	-	-	-

BMΔ*cydBA*			
Goat Number	Dam	Kid	Card Test
1	-	-	-
10	-	-	-
12	-	-	-
14	-	-	-
20	-	-	-
23	-	-	-
25	-	-	-
26	-	-	-
47	-	-	-
53	-	-	-

birth statuses in this study; the challenge stock was from LSU, and was not the virulent isolate acquired at Texas A&M (Table 7). Only 1/8 kids (#28-A) was DOA and 1/8 (#49-A) was never born. For BMΔ*cydBA*, 3/7 kids were considered questionable birth statuses (found dead, or died shortly after birth), and 1/7 was never born (#43-A). The group vaccinated with BMΔ*asp24* experienced the most recorded abnormal birth statuses, with 8/8 kids born live but found dead or were DOA. BMΔ*virB2* vaccinates had high numbers of abnormal births as well, with 4/8 dead *in utero* or born live but found dead. The majority of the kids from both the BMΔ*asp24* and BMΔ*virB2* groups, though dead, were not considered abortions by the group at LSU, since the mornings that they were born were extremely cold and rainy. Based on these criteria, the deaths in the BMΔ*asp24* and BMΔ*virB2* mutant groups were attributed to the weather and not to the infections. Goats marked with N/A represent dams that were euthanized due to injury or illness before parturition.

In order to obtain objective data, bacteriologic culture of tissues was performed. In the 16M infection group, 4/5 dams were culture positive and 3/5 of the corresponding kids were positive (Table 8). Isolation of ≥1 CFU *Brucella* from any goat tissue assigned that animal as positive. Culture positive animals were also seropositive using the card test. The BMΔ*cydBA* mutant appeared to be as virulent in the pregnant goat model as wild-type 16M. In the BMΔ*cydBA* group, 5/6 of the dams were culture and card test positive, and 4/6 of the kids were positive. It was for this reason that the *cydBA* mutants were dropped from the mouse studies, since the 2003 goat efficacy study

TABLE 7. Birth status of goats from the 2003 safety study

16M	Birth Status
#9	2 live
#28	1 DOA
#29	2 live
#46	N/A
#48	2 live
#49	Not born

BMΔ*asp24*	Birth Status
#8	2 BLFD
#12	1 BLFD
#15	1 DOA
#35	2 BLFD
#45	1 BLFD
#47	1 BLFD

BMΔ*cydBA*	Birth Status
#1	1 BLFD
#17	2 live
#22	1 live, 1 BLFD
#26	N/A
#32	1 BLFD
#43	Never gave birth

BMΔ*virB2*	Birth Status
#4	1 born live
#5	2 born live
#7	1 live, 1BLFD
#13	1 dead in utero
#23	2 DOA
#44	N/A

TABLE 8. Culture and card test results for the 2003 goat safety study

16M Recovered	Dam	Kid	Card Test
#9	-	-	-
#28	+	+	+
#29	+	-	+
#48	+	+	+
#49	+	+	n/a

BMΔ*virB2* Recovered	Dam	Kid	Card Test
#4	-	-	-
#5	-	-	-
#7	-	-	-
#13	-	-	-
#23	-	-	-
#44	-	-	-

BMΔ*cydBA* Recovered	Dam	Kid	Card Test
#1	+	+	+
#17	-	+	+
#22	+	+	+
#26	+	+	+
#32	+	-	+
#43	+	-	n/a

BMΔ*asp24* Recovered	Dam	Kid	Card Test
#8	+	-	+
#12	+	-	+
#15	+	-	+
#35	-	-	-
#45	+	-	-
#47	+	-	+

occurred first. The *cydBA* mutants will not be improved vaccine candidates since they are not safe in pregnant goats. The BMΔ*virB2* mutant did not colonize any of the maternal or fetal tissues and did not cause seroconversion. BMΔ*virB2* was therefore considered an improved vaccine strain for efficacy trials since it was safe in pregnant goats. The BMΔ*asp24* mutant showed the most interesting phenotype in the pregnant goats. Though it was able to colonize maternal tissues (5/6) and cause seroconversion (4/6), it was not cultured from any of the fetal tissues. The maternal phenotype in goats was not surprising, since the mutant does persist in mice for extended periods. The inability of BMΔ*asp24* to colonize fetal tissues makes it a superior vaccine candidate to consider for efficacy trials, since it is considered safe for pregnant goats. The absence of *Brucella* from these groups of kids also corroborated the birth statuses as being normal though many of these kids had died.

Serologic responses in the goat safety study. The IgG titers of the goat samples as determined by ELISA confirmed the card tests, but were more specific since relative titers could be assigned, whereas the card test only provided positive or negative results. The results for 16M, BMΔ*cydBA*, and BMΔ*asp24* indicate a spread of IgG titers between animals within each group that was indistinguishable from group to group (Figs. 39, 40, and 41). The BMΔ*virB2* group (Fig. 42) did not have elevated levels of IgG, which was consistent with the negative card test results and elevated attenuation caused by this defect (Table 8).

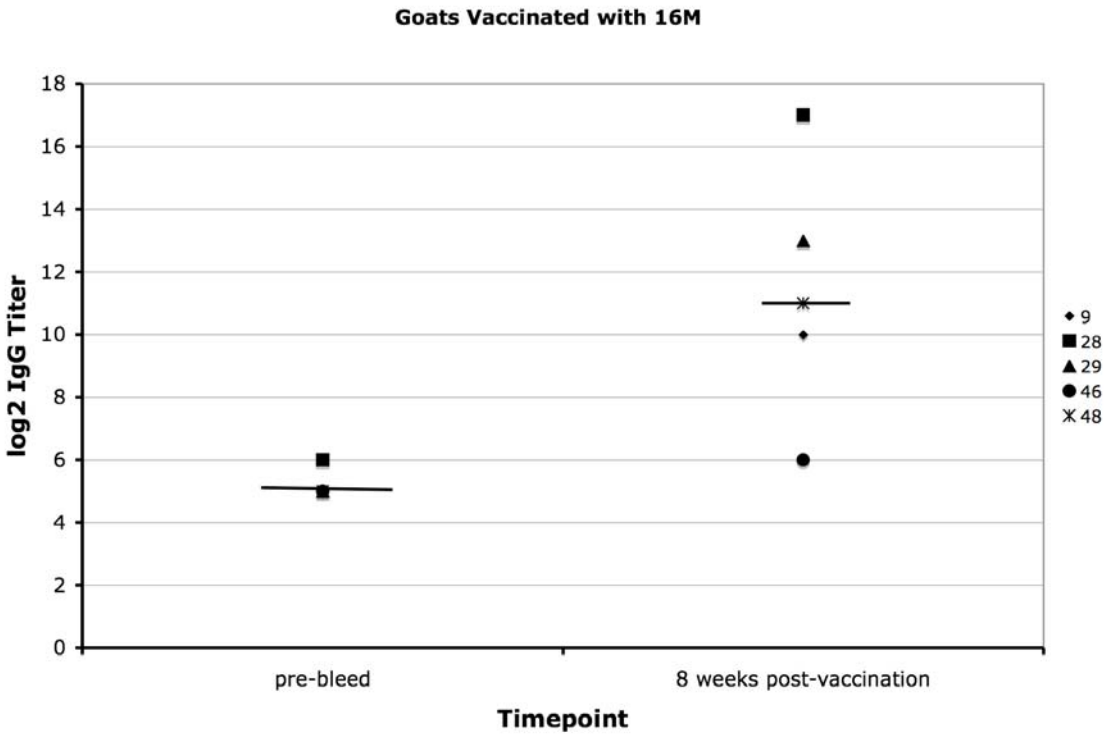

FIG. 39. 2003 safety study: IgG titers of goats challenged with 16M. Goats were vaccinated with $1x10^7$ CFU 16M conjunctivally at 110 +/- 10 days gestation. Goats were bled at two timepoints; one pre-bleed sample before challenge and one sample eight weeks post-challenge. Serum samples were assayed via ELISA to detect IgG antibodies due to the vaccination. Baseline titers were set at 5 as described in the text. Titers for samples were based upon the first dilution in the series that the OD reading was below the cut-off OD value. The IgG levels are therefore represented by the two fold increase in IgG levels over the duration of infection.

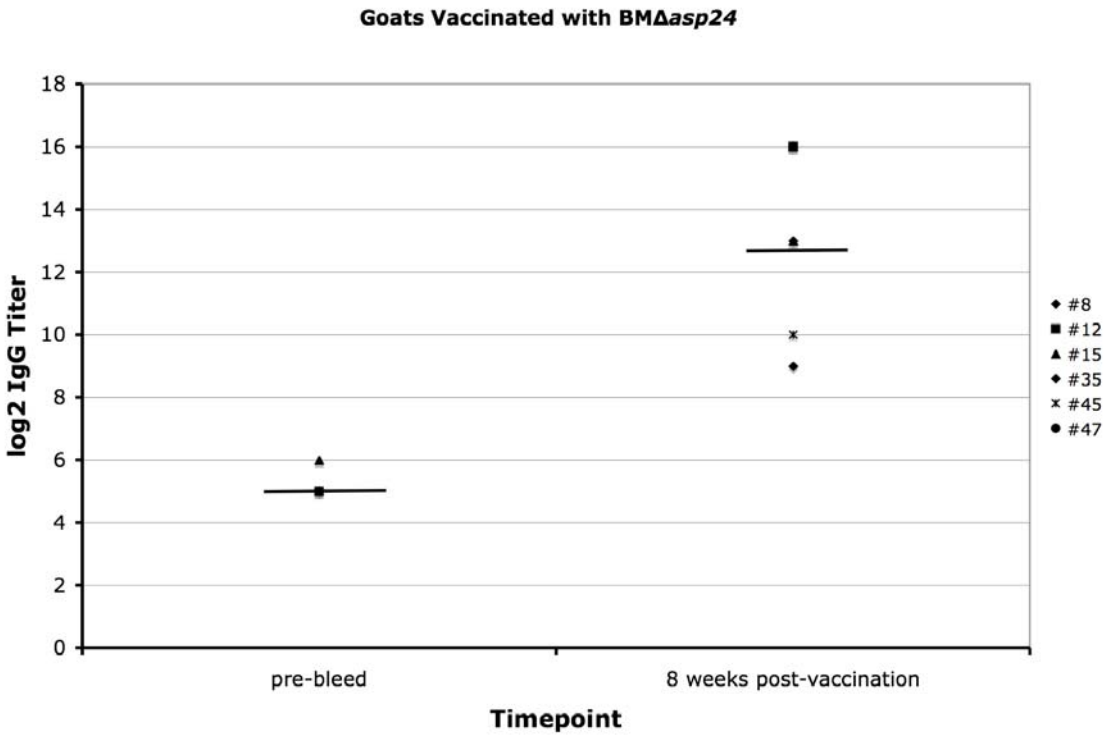

FIG. 40. 2003 safety study: IgG titers of goats vaccinated with BMΔ*asp24*. Goats were vaccinated with $1x10^7$ CFU unmarked BMΔ*asp24* conjunctivally at 110 +/- 10 days gestation. Goats were bled at two timepoints; one pre-bleed sample before challenge and one sample eight weeks post-challenge. Serum samples were assayed via ELISA to detect IgG antibodies due to the vaccination. Baseline titers were set at 5 as described in the text. Titers for samples were based upon the first dilution in the series that the OD reading was below the cut-off OD value. The IgG levels are therefore represented by the two fold increase in IgG levels over the duration of infection.

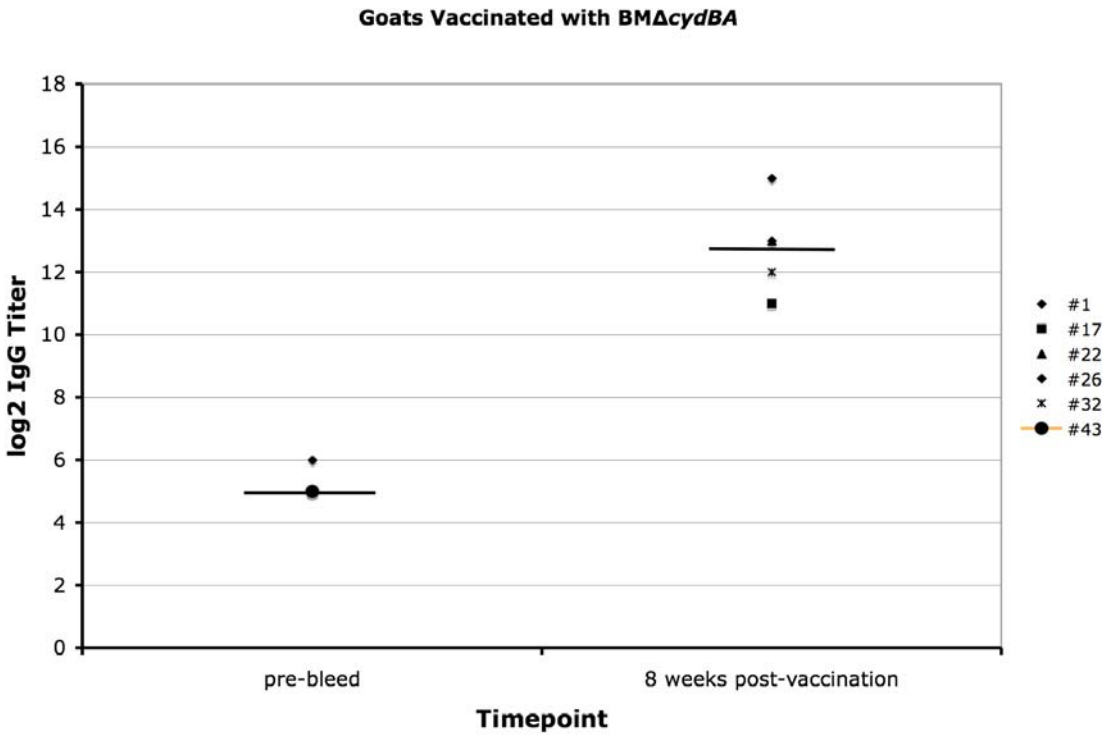

FIG. 41. 2003 safety study: IgG titers of goats vaccinated with BMΔ*cydBA*. Goats were vaccinated with $1x10^7$ CFU unmarked BMΔ*cydBA* conjunctivally at 110 +/- 10 days gestation. Goats were bled at two timepoints; one pre-bleed sample before challenge and one sample eight weeks post-challenge. Serum samples were assayed via ELISA to detect IgG antibodies due to the vaccination. Baseline titers were set at 5 as described in the text. Titers for samples were based upon the first dilution in the series that the OD reading was below the cut-off OD value. The IgG levels are therefore represented by the two fold increase in IgG levels over the duration of infection.

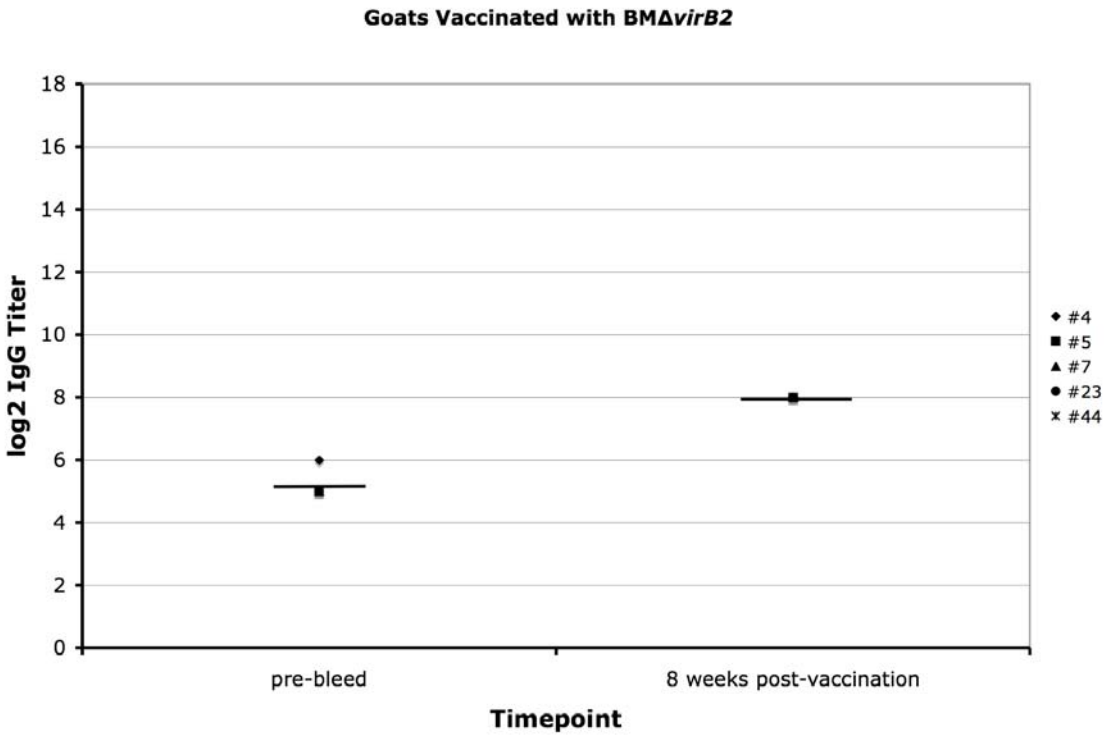

FIG. 42. 2003 safety study: IgG titers of goats vaccinated with BMΔ*virB2*. Goats were vaccinated with $1x10^7$ CFU unmarked BMΔ*virB2* conjunctivally at 110 +/- 10 days gestation. Goats were bled at two timepoints; one pre-bleed sample before challenge and one sample eight weeks post-challenge. Serum samples were assayed via ELISA to detect IgG antibodies due to the vaccination. Baseline titers were set at 5 as described in the text. Titers for samples were based upon the first dilution in the series that the OD reading was below the cut-off OD value. The IgG levels are therefore represented by the two fold increase in IgG levels over the duration of infection.

Histological changes resulting from exposure to vaccine strains. Tissues collected at time of necropsy were assessed for histological changes due to the vaccine strain or from wild-type 16M. Degree of pathology was graded on a scale of 0 to 4, with zero representing no marked change and four being the most marked. For each animal, various samples were evaluated and for each tissue, various criteria were considered.

Goats infected with 16M had a great deal of pathology associated with both maternal and fetal tissues. For the dams, there was a moderate to high degree of activity (B and T cell) in the parotid lymph nodes (PLN) and supramammary lymph nodes (SMLN) (Table 9). There was mild pathology in the livers, some degree of activity in the splenic nodules, and a moderate degree of inflammation in the mammary glands (MG). The kids experienced a moderate to high degree of B and T cell activity in the spleen (Table 10). The lungs portrayed a moderate degree of hemorrhage and a varied degree of inflammation, primarily mononuclear. The liver pathology was varied, but there was an overall moderate inflammation in the portal triad and sinusoidal areas.

Goats infected with BMΔ*cydBA* had, on average, marked pathological changes in their tissues (Table 11). Dams had mild to moderate B and T cell expansion in the PLNs and SMLNs. In the mammary glands, a high degree of inflammation was evident, with 3/5 dams having early microgranuloma formation. There was overall low pathology in the spleen except for a moderate to high degree of hemorrhage primarily in the PLNs, as well as low pathology in the livers. Spleens of kids had moderate to high B and T cell expansion (Table 12). Some kids had high degrees of congestion in the lungs with hemorrhage, and some with inflammatory cells in the liver.

TABLE 9. Histologic changes of pregnant goats infected with 16M

Dam #	Changes in Spleen	Changes in Liver	Changes in Parotid Lymph Node	Changes in Supramammary Lymph Node	Changes in Mammary Gland
#9	1 activity in nodules 1 hemorrhage PLNs 0 sinusoidal hyperplasia 0 congestion	0 congestion 0 mononuclear portal inflammation	2 B cell expansion 3 T cell expansion 1 histiocytosis	3+ B cell expansion 1 T cell expansion	Lactating 1+ mononuclear inflammation
#28	2 activity in nodules 2 hemorrhage PLNs 1 sinusoidal hyperplasia 0 congestion	1 congestion 1 mononuclear portal inflammation	4 B cell expansion 1 T cell expansion 0 histiocytosis	4 B cell expansion 1 T cell expansion 2 sinusoidal histiocytosis	minimal lactation 4+ mononuclear inflammation
#29	1 activity in nodules 2 hemorrhage PLNs (excessive PMN and hemorrhage) 1 sinusoidal hyperplasia 0 congestion	0 congestion 1 mononuclear portal inflammation	4 B cell expansion 1 T cell expansion 1 histiocytosis	4+ B cell expansion 2+ T cell expansion 2+ sinusoidal histiocytosis (active)	minimal lactation 4 inflammation (early microgranuloma)
#46	0 activity in nodules 0 hemorrhage PLNs 1 sinusoidal hyperplasia (lymphoid depletion) 4 congestion	0 congestion 0 mononuclear portal inflammation	4 B cell expansion 1 T cell expansion 1 histiocytosis	4+ B cell expansion 4 T cell expansion 1 sinusoidal hyperplasia	mild lactation 2 mononuclear inflammation (PMNs)
#48	1 activity in nodules 2 hemorrhage PLNs 0 sinusoidal hyperplasia 4 congestion	0 congestion 1 portal mononuclear inflammation	N/A	1 B cell expansion 0 T cell expansion 1 sinusoidal hyperplasia	4 mononuclear inflammation
#49	3 activity in nodules 1 hemorrhage PLNs 0 sinusoidal hyperplasia 4 congestion	0 congestion 1 portal mononuclear inflammation	1 B cell expansion 1 T cell expansion 0 histiocytosis	1 B cell expansion 0 T cell expansion 0 sinusoidal histiocytosis	N/A

TABLE 10. Histologic changes of goat fetuses infected with 16M

Kid or fetus #	Changes in Spleen	Changes in Lung	Changes in Liver
#9A	(immature)	1 atelactasis 5 congestion 2 hemorrhage 2 inflammation (90% mononuclear)	5 congestion 4 hemorrhage 1 inflammation (mild sinusoidal accumulation of mononuclear cells)
#9 B	1-2 congestion 3 B cell expansion 1 T cell expansion 0 sinusoidal hyperplasia	3 atelactasis 1 congestion 4 hemorrhage 1 inflammation (90% mononuclear)	1 congestion 0 hemorrhage 0 inflammation
#28 A	1 congestion 2 B cell expansion 3 T cell expansion 2 sinusoidal hyperplasia	1 atelactasis 4 congestion 4 hemorrhage 3-4 inflammation (50-50)	0 congestion 0 hemorrhage 3 inflammation (mild sinusoidal and marked portal triad mononuclear-macrophage infiltrate)
#29 A	0 congestion 2 B cell expansion 3 T cell expansion 2 sinusoidal hyperplasia	2 atelactasis 2 congestion 2 hemorrhage 1 inflammation (90% mononuclear)	0 congestion 0 hemorrhage 1 inflammation (mild mononuclear infiltration of portal triad
#29 B	0-1 congestion 2 B cell expansion 4 T cell expansion 0 sinusoidal hyperplasia	1 atelactasis 1 congestion 1 hemorrhage 2 inflammation (99% mononuclear)	N/A
#48 A	0 congestion 4 B cell expansion 4 T cell expansion 0 sinusoidal hyperplasia	4 atelactasis; pleural and interlobular edema 0 congestion 0 hemorrhage 0 inflammation	N/A
#48 B	0 congestion 1 B cell expansion 3 T cell expansion 0 sinusoidal hyperplasia	3 atelactasis 1 congestion 2 hemorrhage 1 inflammation (mononuclear)	0 congestion 0 hemorrhage 3 inflammation; early sinusoidal microgranuloma formation
#49 A	1 congestion 2 B cell expansion 3 T cell expansion 0 sinusoidal hyperplasia	4 atelactasis 0 congestion 1 hemorrhage 1 inflammation	0 congestion 0 hemorrhage 1 inflammation; mild mononuclear portal infiltration

TABLE 11. Histologic changes of pregnant goats infected with BMΔ*cydBA*

Dam #	Changes in Spleen	Changes in Liver	Changes in Parotid Lymph Node	Changes in Supramammary Lymph Node	Changes in Mammary Gland
#1	0 congestion 3 activity in nodules 0 sinusoidal hyperplasia 3 hemorrhage PLNs	0 congestion	2 B cell expansion 3 T cell expansion 0 histiocytosis	3+ B cell expansion 1 T cell expansion 0 sinusoidal histiocytosis	Lactation 3+ mononuclear inflammation
#17	0 congestion 0 activity in nodules 0 sinusoidal hyperplasia 0 hemorrhage PLNs	1 congestion	0 B cell expansion 0 T cell expansion 0 histiocytosis	1 B cell expansion 0 T cell expansion 1 sinusoidal histiocytosis	minimal lactation 0 mononuclear inflammation1 foamy milk granuloma
#22	0 congestion 2 activity in nodules 0 sinusoidal hyperplasia 2-3 hemorrhage PLN	1 congestion	2 B cell expansion 0 T cell expansion 0 histiocytosis	N/A	minimal lactation 2 mononuclear inflammation mononuclear, early granuloma formation
#26	0 congestion 1 degree of activity in nodules 1 sinusoidal hyperplasia 1 hemorrhage PLN	1 congestion	1 B cell expansion 0 T cell expansion 0 histiocytosis	3+ B cell expansion 1+ T cell expansion 1 sinusoidal histiocytosis	heavy lactation
#32	1 congestion 1 activity in nodules 0 sinusoidal hyperplasia 2 hemorrhage PLN: excessive PMN and hemorrhage	1 congestion	0 B cell expansion 0 T cell expansion 0 histiocytosis	1 B cell expansion 1 T cell expansion 4 sinusoidal histiocytosis	4+ mononuclear inflammation, early microgranuloma formation (PMNs at 1)
#43	4+ congestion 2 degree of activity in nodules: inflammatory	1 congestion	3 B cell expansion 1 T cell expansion 0 histiocytosis	2 B cell expansion 1 T cell expansion 2 sinusoidal histiocytosis	N/A

TABLE 12. Histologic changes of goat fetuses infected with BMΔ*cydBA*

Kid or fetus #	Changes in Spleen	Changes in Lung	Changes in Liver
#1A	0-1 congestion 2+ B cell expansion 1+ T cell expansion 0 sinusoidal hyperplasia	2-3 atelactasis 4 congestion 5 hemorrhage 1 inflammation (99% mononuclear accumulation in septal capillaries)	2 congestion
#17A	1 congestion 1 B cell expansion 3-4 T cell expansion 2 sinusoidal hyperplasia	4-5 atelactasis, 2-3 congestion 0 hemorrhage 0 inflammation	0 congestion 0 inflammation
#17B	1 congestion 3 B cell expansion 2 T cell expansion 1-2 sinusoidal hyperplasia	2 atelactasis 0 congestion 0 hemorrhage 0 inflammation	0 congestion 0 inflammation
#22A	2 congestion 3 B cell expansion 1 T cell expansion 0 sinusoidal hyperplasia	1 atelactasis (alveolar rupture marked) 0 congestion 1 hemorrhage 0 inflammation	0 congestion 0 inflammation
#22B	1 congestion 2 B cell expansion 3 T cell expansion 1 sinusoidal hyperplasia	1 atelactasis (alveolar rupture marked) 0 congestion 1 hemorrhage 0 inflammation	0 congestion 0 inflammation
#32A	1 congestion 3-4 B cell expansion 1 T cell expansion 1 sinusoidal hyperplasia	0 congestion 1 hemorrhage; marked agonal emphysema 0 inflammation	0 congestion 2 portal triad inflammation (mononuclear) 1 sinusoidal inflammation
#43A	2-3 congestion 3 B cell expansion 3 T cell expansion 1 sinusoidal hyperplasia	0 atelactasis 0 congestion 3-4 hemorrhage 0 inflammation	0 congestion 2-3 portal mononuclear and neutrophilic infiltrate; sinusoidal mononuclear
#43B	1 congestion 3 B cell expansion 3 T cell expansion 0 sinusoidal hyperplasia	0 atelactasis 0 congestion 1 hemorrhage 0 inflammation	0 congestion 4 sinusoidal microgranulomas around THV

Goats infected with BMΔ*asp24* overall showed low histological changes in the PLNs, livers, and spleens (Table 13). In the SMLN and mammary gland, however, there were numerous changes, with moderate to high inflammation in the MG (primarily mononuclear) and very high activity of B and T cells in the SMLN. Kids overall had moderate B and T cell expansion in the spleen with mild congestion, few changes in the livers, and mild congestion in the lungs with low levels of inflammation or hemorrhage (Table 14).

Goats infected with BMΔ*virB2* showed low overall histological change associated with the vaccination. Dams had no significant change in the livers or spleens, mild B and T cell expansion in lymph nodes and spleens, and only mild inflammation in the mammary gland (Table 15). Tissues of kids showed moderate B and T cell expansion in spleens, very mild degree of congestion for some livers, and mild hemorrhage and mononuclear inflammation in the lungs (Table 16).

Vaccine efficacy study. Efficacy studies were performed at LSU in the fall of 2003, comparing a vaccine strain, Rev1, with safe unmarked vaccine candidates BMΔ*asp24*, BMΔ*virB2*, and saline as a negative control. Goats were bled prior to vaccination and again five weeks after vaccination to confirm vaccination status. A male goat was introduced to each group at four weeks post-vaccination to impregnate the ewes. At 110 days of gestation, determined as described above, challenged with wild-type 16M. Depending upon the protection induced, goats either aborted or gave birth to kids. Kids that were found dead were not necessarily considered aborted, since

TABLE 13. Histologic changes of pregnant goats infected with BMΔ*asp24*

Dam #	Changes in Spleen	Changes in Liver	Changes in Parotid Lymph Node	Changes in Supramammary Lymph Node	Changes in Mammary Gland
#8	0 congestion 2 activity in nodules 0 sinusoidal hyperplasia 2 hemorrhage PLN	0 congestion 0 inflammation	1 B cell expansion 1 T cell expansion 1 histiocytosis	1 B cell expansion 0 T cell expansion 1 sinusoidal histiocytosis	mild lactation
#12	4 congestion 3 activity in nodules 0 sinusoidal hyperplasia 4 hemorrhage PLN	0 congestion 0 inflammation	0 B cell expansion 0 T cell expansion 0 histiocytosis	4 B cell expansion 1 T cell expansion 0 sinusoidal histiocytosis	Lactating 2+ mononuclear inflammation in interstitium
#15	0 congestion 1 activity in nodules 0 sinusoidal hyperplasia 0 hemorrhage PLN	1 congestion 0 inflammation	N/A	4 B cell expansion 2 T cell expansion 2= sinusoidal histiocytosis	lactating 4+ mononuclear inflammation PMN (interstitial); early microgranuloma
#35	1 congestion 2 degree of activity in nodules 1 sinusoidal hyperplasia 2 hemorrhage PLN	0 congestion 1+ mononuclear inflammation (portal area)	3 B cell expansion 1 T cell expansion 1 histiocytosis	4 B cell expansion 3 T cell expansion 1 sinusoidal histiocytosis	Mild lactation
#45	0 congestion 3 activity in nodules 1 sinusoidal hyperplasia 1 hemorrhage PLN	1 congestion 0 inflammation	0 B cell expansion 0 T cell expansion 0 histiocytosis	4 B cell expansion 2 T cell expansion 1 sinusoidal histiocytosis	Active lactation 3 mononuclear infiltrate; early microgranuloma
#47	0 congestion 1 degree of activity in nodules 1 sinusoidal hyperplasia 0 hemorrhage PLN	0 congestion 2+ mononuclear inflammation (portal area)	4 B cell expansion 1 T cell expansion 1 histiocytosis	4+ B cell expansion 3+ T cell expansion 1 sinusoidal histiocytosis, slight hemorrhage	4 inflammation mononuclear with large, well-formed microgranulomas, plasma cytosis, PMNs at 2

TABLE 14. Histologic changes of goat fetuses infected with BMΔ*asp24*

Kid or fetus #	Changes in Spleen	Changes in Lung	Changes in Liver
#8A	2 congestion 0 B cell expansion 2 T cell expansion 0 sinusoidal hyperplasia	1 atelactasis 2 congestion 1 hemorrhage 2 inflammation (99% mononuclear)	0 congestion 1 sinusoidal inflammation 2 portal mononuclear inflammation
#8B	1 congestion 1 B cell expansion 1 T cell expansion 0 sinusoidal hyperplasia	5 atelactasis 1 congestion 2 hemorrhage 1 inflammation (99% mononuclear)	5 congestion 0 inflammation
#12A	2-3 congestion 3-4 B cell expansion 1-2 T cell expansion 0 sinusoidal hyperplasia	4 atelactasis 3 congestion 0 hemorrhage 0 inflammation	0 congestion 1 portal mononuclear aggregation
#15A	no score putrefied	4 atelactasis 0 congestion 0 hemorrhage 2 serous inflammation	putrefied-can't assess
#35A	2 congestion 3 B cell expansion 1 T cell expansion 0 sinusoidal hyperplasia	1 atelactasis (alveolar rupture marked) 0 congestion 1 hemorrhage 0 inflammation	0 congestion 0 inflammation
#35B	1 congestion 2 B cell expansion 3 T cell expansion 1 sinusoidal hyperplasia	1 atelactasis (alveolar rupture marked) 0 congestion 1 hemorrhage 0 inflammation	0 congestion 0 inflammation
#45A	3-4 congestion 3 B cell expansion 1 T cell expansion 0 sinusoidal hyperplasia	0 atelactasis 0 congestion 0 hemorrhage 0 inflammation	1 congestion, marked hematopoeisis 0 inflammation
#47A	1 congestion 3 B cell expansion 3 T cell expansion 0 sinusoidal hyperplasia	3 atelactasis 2 congestion 1 hemorrhage 2 inflammation (90% mononuclear, 10% PMN)	2 congestion 0 inflammation

TABLE 15. Histologic changes of pregnant goats infected with BMΔ*virB2*

Dam #	Changes in Spleen	Changes in Liver	Changes in Parotid Lymph Node	Changes in Supramammary Lymph Node	Changes in Mammary Gland
#4	0 congestion 0-1 activity in nodules 0 sinusoidal hyperplasia 3 hemorrhage PLN	0 hemorrhage 0 inflammation	1 B cell expansion 0 T cell expansion 0 histiocytosis	1+ focal B cell expansion 0 T cell expansion 1 sinusoidal histiocytosis	Lactating 1+ inflammation
#5	0 congestion 3 activity in nodules 1 sinusoidal hyperplasia 2 hemorrhage PLN	0 hemorrhage 0 inflammation	0 B cell expansion 0 T cell expansion 0 histiocytosis	1 B cell expansion 2 T cell expansion 1 sinusoidal histiocytosis	Lactating 2+ lymphocytic infiltrate of granuloma with mature macrophages
#7	0 congestion 4 activity in nodules 0 sinusoidal hyperplasia 4 hemorrhage PLN	0 hemorrhage 1+ mononuclear inflammation (portal area)	1 B cell expansion 1 T cell expansion 1 histiocytosis	2 B cell expansion 2 T cell expansion 1 sinusoidal histiocytosis	Lactating 1+ mononuclear inflammation (interstitial)
#13	1 congestion 4 degree of activity in nodules 2 sinusoidal hyperplasia 1 hemorrhage PLN	0 hemorrhage 1 mononuclear inflammation (portal area)	0 B cell expansion 0 T cell expansion 0 histiocytosis	1 B cell expansion 3 T cell expansion 2 sinusoidal histiocytosis (neutrophils at 3)	N/A
#23	1 congestion 2 activity in nodules 2 sinusoidal hyperplasia 1 hemorrhage PLN	1 hemorrhage 0 inflammation	0 B cell expansion 0 T cell expansion 0 histiocytosis	2 B cell expansion 0 T cell expansion 0 sinusoidal histiocytosis	Lactating 0 inflammation
#44	1 congestion 1 degree of activity in nodules (lymphoid depletion) 0 sinusoidal hyperplasia 3 hemorrhage with neutrophilic PMNs	0 hemorrhage 1+ mononuclear inflammation (portal area)	4 B cell expansion 1 T cell expansion 0 histiocytosis	N/A	mild lactation 2 focal mononuclear inflammation with early granuloma

TABLE 16. Histologic changes of goat fetuses infected with BMΔ*virB2*

Kid or fetus #	Changes in Spleen	Changes in Lung	Changes in Liver
#4A	1 B cell expansion 2 T cell expansion	2 degree of atelactasis 4 congestion 2 hemorrhage 3 inflammation (60% mononuclear, 40% PMN	0 congestion 0.5-1 sinusoidal mononuclear aggregation
#5A	2 B cell expansion 2 T cell expansion	1 atelactasis 1 congestion 1 hemorrhage 1 inflammation (90% mononuclear, 10% PMN)	0 congestion 0 inflammation
#5B	2 B cell expansion 2 T cell expansion	2 atelactasis 3 congestion 4 hemorrhage 2 inflammation (focal-50% mononuclear, 50% PMN)	1 congestion 0.5 inflammation (sinusoidal mononuclear aggregates)
#7A	1 congestion 2 B cell expansion 1 T cell expansion	1 atelactasis 1 congestion w/ emphysema	0 congestion 0 inflammation
#7B	1 congestion 3 B cell expansion	4 atelactasis 2 congestion 2 hemorrhage 2 inflammation (90% mononuclear, 10% PMN)	0 congestion 0 inflammation
#13A	1 congestion 3 B cell expansion 2 T cell expansion	4 atelactasis 4 congestion 1 hemorrhage 4 inflammation (50-50).	5 congestion 0 inflammation
#23A	3 B cell expansion 2 T cell expansion	4 atelactasis 3 congestion 1 hemorrhage; (mild accumulation mononuclear cells)	0 congestion 0 inflammation
#23B	1 congestion 4 B cell expansion 1 T cell expansion	4 atelactasis 3 congestion 3 hemorrhage 2 inflammation (90% mononuclear, 10% PMN).	0 congestion 0 inflammation
#44A	N/A	1 atelactasis; agonal emphysema	3 congestion 0.5 inflammation
#44B	0-1 congestion 1 B cell expansion 4 sinusoidal hyperplasia	N/A	0 congestion 0.5 inflammation (mononuclear-sinusoidal)

this requires additional criteria such as culture status and approximate age of the kid to determine whether it was aborted because of challenge or died due to environmental factors (Table 17). Goats that were not vaccinated but challenged with 16M had the greatest numbers of kids that had suspect birth statuses: aborted, weak, or dead. All kids in the Rev 1 vaccine group displayed normal birth status. Goats vaccinated with BMΔ*asp24* had a few suspect births upon original designation. Upon closer inspection, however, goat # 15 aborted 16 days before it was challenged, which may have been due to environmental conditions. Goat #44 aborted only 12 days after it was challenged, which may be considered an abortion, however *Brucella* recovery from the tissues would need to be considered to confirm this point. For goats vaccinated with BMΔ*virB2*, several goats had suspect birth statuses, however two of these, #20 and #22, aborted before the date of challenge. Goat #39 aborted twins 22 days after challenge, and this may represent a lack of protection against abortion.

At one set timepoint, after all the goats had kidded, all dams were all bled then necropsied en masse. Card tests were performed on serum samples to determine the presence of *Brucella*-specific antibodies (Tables 18 and 19). Tissues were cultured for the presence of the challenge bacteria. Isolated colonies were tested to ensure it was *Brucella*, and PCR verified to determine whether it was vaccine strain or wild-type organism. In the unvaccinated/16M challenge group, the majority of the goats (7/9) and kid pairs (9/16) were culture positive from the challenge, which was an expected result (Table 20). Goats vaccinated with Rev 1 were highly protected against virulent challenge, which was also expected from this experiment. Only one goat out of seven

TABLE 17. Birth status of goats from efficacy study

Unvaccinated	
#5	1 live, 1 dead
#26	2 dead on arrival
#36	aborted
#40	1 live
#46	1 live (weak)
#48	2 live (weak)
#144	3 in utero
#164	2 dead on arrival
#172	2 live

BMΔ*asp24*	
#9	1 live
#10	1 live
#11	1 live
#12	2 live
#13	1 live
#14	2 live
#15	aborted
#44	2 aborted

Rev 1	
#2	2 live
#4	1 live
#6	1 live
#8	1 live
#27	1 live
#47	2 live
#49	2 live

BMΔ*virB2*	
#16	2 live
#17	2 live
#18	2 live
#20	aborted
#22	aborted
#33	1 live
#39	2 aborted
#42	mummy

TABLE 18: Card tests results from efficacy study controls

Saline	5 weeks post-vaccination	Pre-challenge (18 weeks post-vaccination)	Necropsy (7 weeks post-challenge)
#5	-	-	+
#26	-	-	+
#36	-	-	ns
#40	-	-	+
#46	-	-	ns
#48	-	-	+
#144	n/a	-	ns
#164	n/a	-	+
#172	n/a	-	+

Rev1	5 weeks post-vaccination	Pre-challenge (18 weeks post-vaccination)	Necropsy (7 weeks post-challenge)
#2	-	-	-
#4	-	-	-
#6	-	-	-
#8	+/-	-	-
#27	-	-	-
#47	+	-	+
#49	-	-	+

TABLE 19: Card test results for efficacy study vaccine strains

BMΔ*virB2*	5 weeks post-vaccination	Pre-challenge (18 weeks post-vaccination)	Necropsy (7 weeks post-challenge)
#16	+	+	+
#17	+	-	+
#18	+	+	+
#20	+	-	+
#22	+	+	+
#33	+	-	-
#39	+	-	ns
#42	+	+	+

BMΔ*asp24*	5 weeks post-vaccination	Pre-challenge (18 weeks post-vaccination)	Necropsy (7 weeks post-challenge)
#9	+	-	+
#10	+	+	+
#11	+	+	+
#12	+	+	+
#13	+	+	+
#14	+	+	+
#15	+	+	+
#44	+	+	+

TABLE 20. Recovery of *Brucella* from goat tissues: unvaccinated control group

Dams						Kids		
	PLN	Liver	Spleen	SMLN	MG		Abomasal fluid	lung
#5	58	0	0	0	2	#5-A/B	>1000	>1000, 500
#26	5	0	5	300	Lawn	#26-A/B	0	0,3
#36	7	0	0	0	0	#36-A	0	0
#40	0	0	0	0	0	#40-A	0	200
#46	8	1	20	0	0	#46-A	0	0
#48	0		7	3	12	#48-A/B	>1000	Lawn, 13
#144	0	0	0	0	1	#144A/B/C	0	0
#164	0	3	3	55	>1000	#164-A/B	lawn	300, 33
#172	0	0	0	0	0	#172-A/B	0	0

was culture positive and 0/10 of the kids were positive, which is a very high vaccine efficacy (Table 21). Goats vaccinated with BMΔ*asp24* were generally well protected against challenge, though not to the same degree as Rev1 vaccinates (Table 22). Of the dams, 3/8 had recovered *Brucella* from tissues, and only 1/11 kids were positive. Interestingly, dam #14, which was highly culture positive, retained the vaccine strain in the tissues as revealed by PCR (Fig. 43). The kids, however, were positive for the challenge organism in the sample of colonies used as template. These results are consistent with results from the safety study, in which the BMΔ*asp24* mutant did not colonize the fetal tissues. The absence of challenge organism in the dam was likely due to the limited sampling of bacteria for PCR. It is possible that the primary population isolated was the vaccine strain, but that challenge organism persisted in the dam as well. In this goat, the vaccine strain was able to persist for an extended period. Goats #11 and #44, which had very low numbers of recoverable bacteria, were positive only for the challenge strain. Overall, the vaccine was very protective against challenge infection. The use of elevated vaccine dose of $1x10^9$ may have contributed to this persistence and maternal pathology. A reduced dose of BMΔ*asp24* may be necessary, similar to Rev 1 that was utilized at a $1x10^6$ dose.

The majority of goats vaccinated with BMΔ*virB2* were protected against challenge infection, though not as highly as Rev 1 (Table 23). For the dams, 3/8 were culture positive for the challenge organism, and only 1/12 of the kids were culture positive. Goat #22 portrayed the highest tissue colonization levels, and this goat

TABLE 21. Recovery of *Brucella* from goat tissues: Rev 1 vaccine group

Dams						Kids		
	PLN	Liver	Spleen	SMLN	MG		Abomasal Fluid	Lung
#2	0	0	0	0	0	#2-A/B	0	0
#4	0	0	0	0	0	#4-A	0	0
#6	0	1	2	0	0	#6-A	0	0
#8	0	0	0	0	0	#8-A	0	0
#27	0	0	0	0	0	#27-A	0	0
#47	0	0	0	0	0	#47-A/B	0	0
#49	0	0	0	0	0	#49-A/B	0	0

TABLE 22. Recovery of *Brucella* from goat tissues: BMΔ*asp24* vaccine group

Dams						Kids		
	PLN	Liver	Spleen	SMLN	MG		Abomasal Fluid	Lung
#9	0	0	0	0	0	#9-A	0	0
#10	0	0	0	0	0	#10-A	0	0
#11	0	1	1	1	1	#11-A	0	0
#12	0	0	0	0	0	#12A/B	0	0
#13	0	0	0	0	0	#13-A	0	0
#14	0	1	0	>1000	Lawn	#14A/B	lawn	1, 54
#15	0	0	0	0	0	#15-A	0	n/a
#44	1	2	3	1	1	#44A/B	0	0

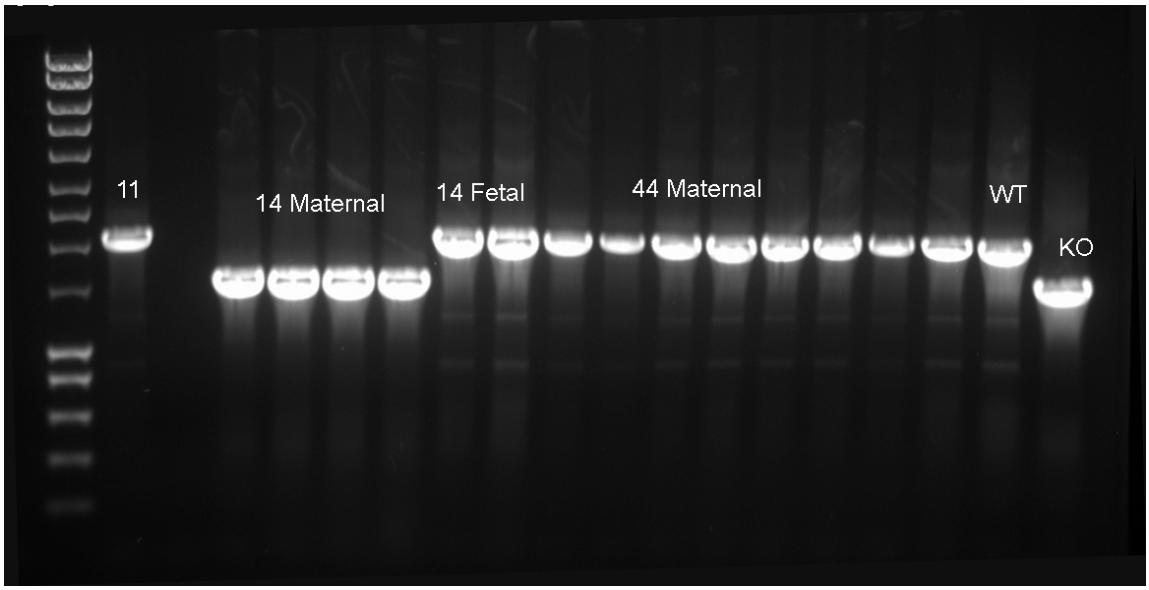

FIG. 43 . PCR validation of maternal and fetal isolates. Isolates recovered from goats vaccinated with BMΔ*asp24* and challenged with 16M were heat killed and used for PCR using primers flanking the deletion region. Products of knockout strains are smaller by approximately 530 bp. Lane 1, Hyperladder I; lane 2, dam #11 liver isolate; lane 3, negative PCR control; lanes 4-5 dam #14 SMLN; lane 6, #14 mammary gland; lane 7, #14 liver; lane 8, kid #14-A lung; lane 9, kid #14-B lung; lanes 10-17 dam #44 various tissues including PLN, SMLN, liver, and MG; lane 18, genomic DNA from wild-type 16M; lane 19, genomic DNA from BMΔ*asp24*.

TABLE 23. Recovery of *Brucella* from goat tissues: BMΔ*virB2* vaccine group

Dams						Kids		
	PLN	Liver	Spleen	SMLN	MG		Abomasal Fluid	Lung
#16	0	0	0	0	0	#16-A/B	0	0
#17	0	0	0	0	0	#17-A/B	0	0
#18	0	0	0	>1000	0	#18-A/B	200	0
#20	2	0	0	0	0	#20-A	0	
#22	27	500	400	>1000	Lawn	#22-A	0	0
#33	0	4	1	0	0	#33-A	0	0
#39	0	0	0	0	0	#39-A/B	0	0
#42	0	0	0	0	0	#42-A	0	0

aborted the day of challenge. Since there was not recovered *Brucella* from the fetus, it is possible that this goat aborted for another reason, and perhaps was more susceptible to the challenge infection due to the abortion and stressed condition. Overall, the BMΔ*virB2* mutant is a good vaccine candidate for the majority of goats, though not as significant as Rev 1, but is a safer candidate than the Rev 1 vaccine strain.

Immune responses of goats from the efficacy study. Evaluation of the humoral immune response was performed in order to determine whether IgG levels could be used as a marker for protection. As expected, the unvaccinated control group remained seronegative until after challenge (Fig. 44). Rev 1 vaccinates showed increased IgG levels after vaccination, which decreased over time and did not increased slightly after challenge (Fig. 45). Goats vaccinated with BMΔ*asp24* had increased levels of IgG at all timepoints after the vaccination, though higher IgG titers were seen after vaccination than after challenge (Fig. 46). The goat with the highest IgG levels after challenge was dam #14. This goat retained a great deal of the vaccine strain, which could explain the titer. Similarly, BMΔ*virB2* vaccinates showed increased IgG levels at all timepoints post-vaccination (Fig. 47). Dams #18 and #22 retained high numbers of challenge organism in tissues, which correlates with their high IgG levels.

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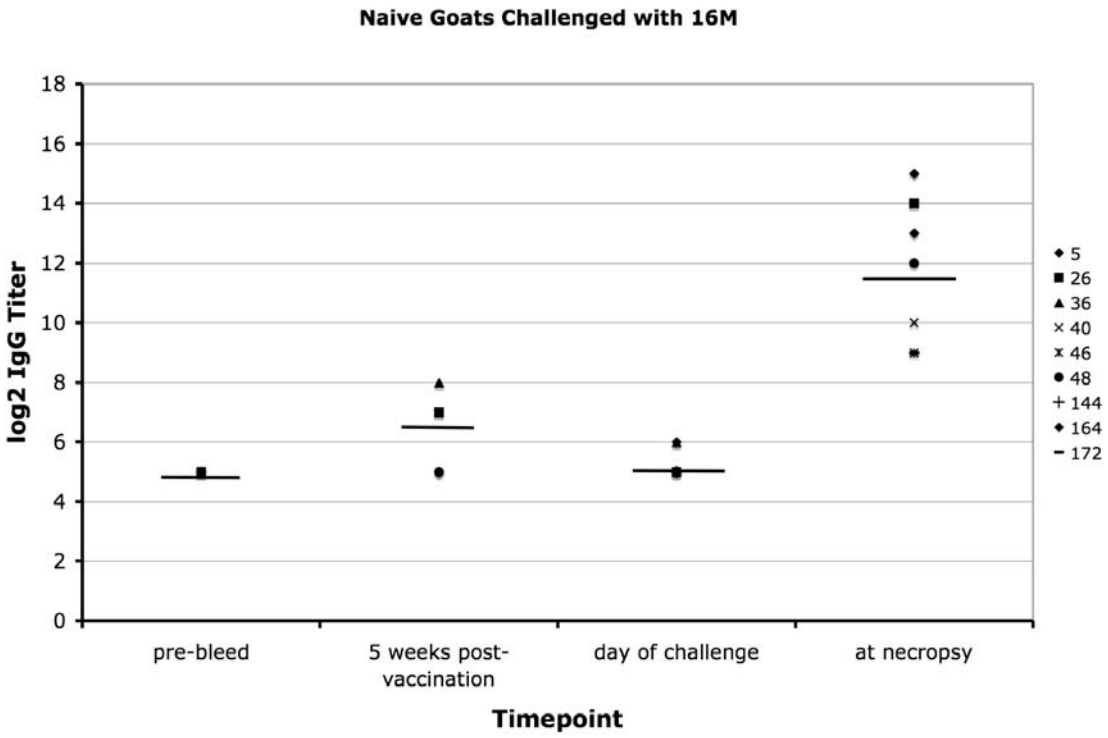


FIG. 44. Goat efficacy study: IgG titers of unvaccinated goats challenged with 16M. Goats were mock vaccinated with PBS before pregnancy, then challenged with $1x10^7$ CFU wild-type *B. melitensis* 16M conjunctivally during the third trimester of pregnancy. Goats were bled the day of vaccination, 5 weeks post-vaccination, the day of challenge (18 weeks post-vaccination), and at necropsy (7 weeks post-challenge). Serum samples were assayed via ELISA to detect IgG antibodies due to the vaccination. Baseline titers were set at 5 as described in the text. Titers for samples were based upon the first dilution in the series that the OD reading was below the cut-off OD value. The IgG levels are therefore represented by the two fold increase in IgG levels over the duration of infection.

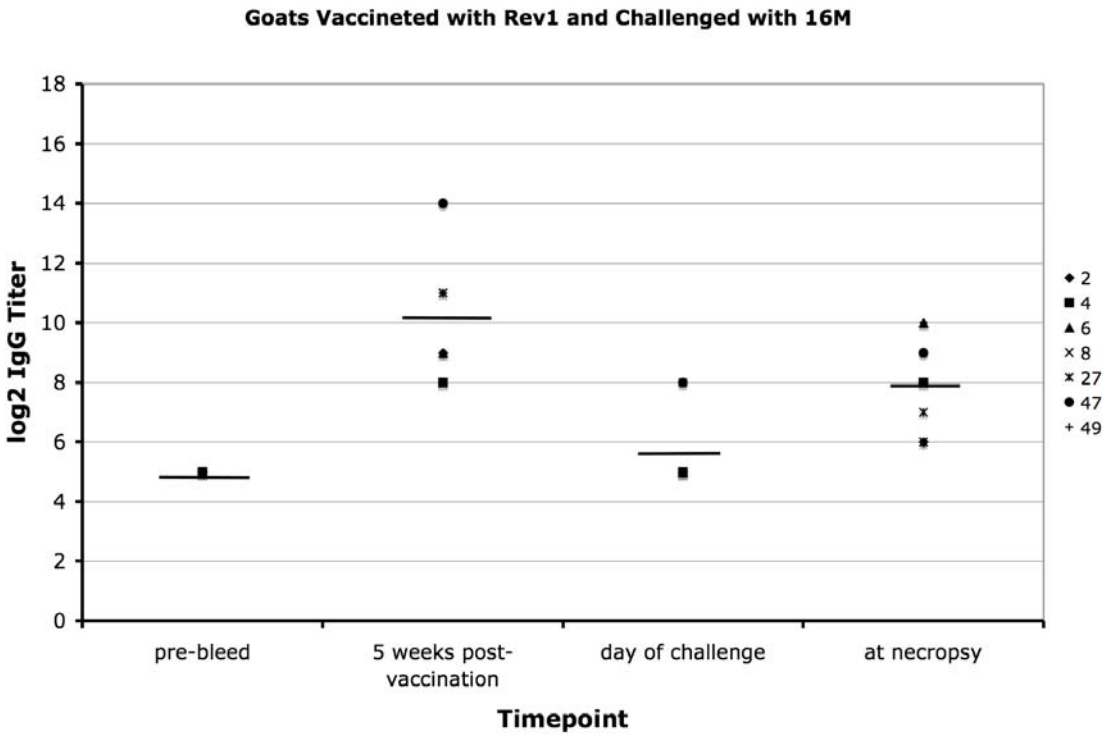


FIG. 45. Goat efficacy study: IgG titers of goats vaccinated with Rev 1 and challenged with 16M. Goats were vaccinated with Rev 1 at $1x10^6$ CFU (I.M.) before pregnancy, then challenged with $1x10^7$ CFU wild-type *B. melitensis* 16M conjunctivally during the third trimester of pregnancy. Goats were bled the day of vaccination, 5 weeks post-vaccination, the day of challenge (18 weeks post-vaccination), and at necropsy (7 weeks post-challenge). Serum samples were assayed via ELISA to detect IgG antibodies due to the vaccination. Baseline titers were set at 5 as described in the text. Titers for samples were based upon the first dilution in the series that the OD reading was below the cut-off OD value. The IgG levels are therefore represented by the two fold increase in IgG levels over the duration of infection.

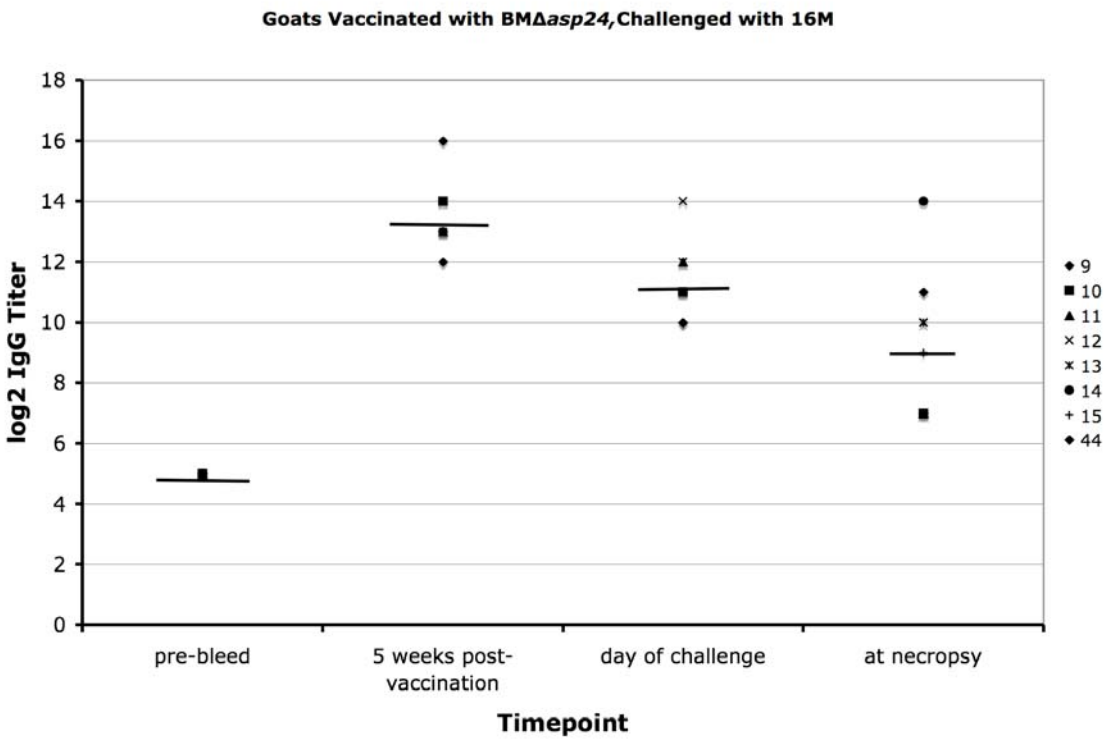


FIG. 46. Goat efficacy study: IgG titers of goats vaccinated with BMΔ*asp24* and challenged with 16M. Goats were vaccinated with$1x10^9$ CFU BMΔ*asp24* (subcutaneous) before pregnancy, then challenged with $1x10^7$ CFU wild-type *B. melitensis* 16M conjunctivally during the third trimester of pregnancy. Goats were bled the day of vaccination, 5 weeks post-vaccination, the day of challenge (18 weeks post-vaccination), and at necropsy (7 weeks post-challenge). Serum samples were assayed via ELISA to detect IgG antibodies due to the vaccination. Baseline titers were set at 5 as described in the text. Titers for samples were based upon the first dilution in the series that the OD reading was below the cut-off OD value. The IgG levels are therefore represented by the two fold increase in IgG levels over the duration of infection.

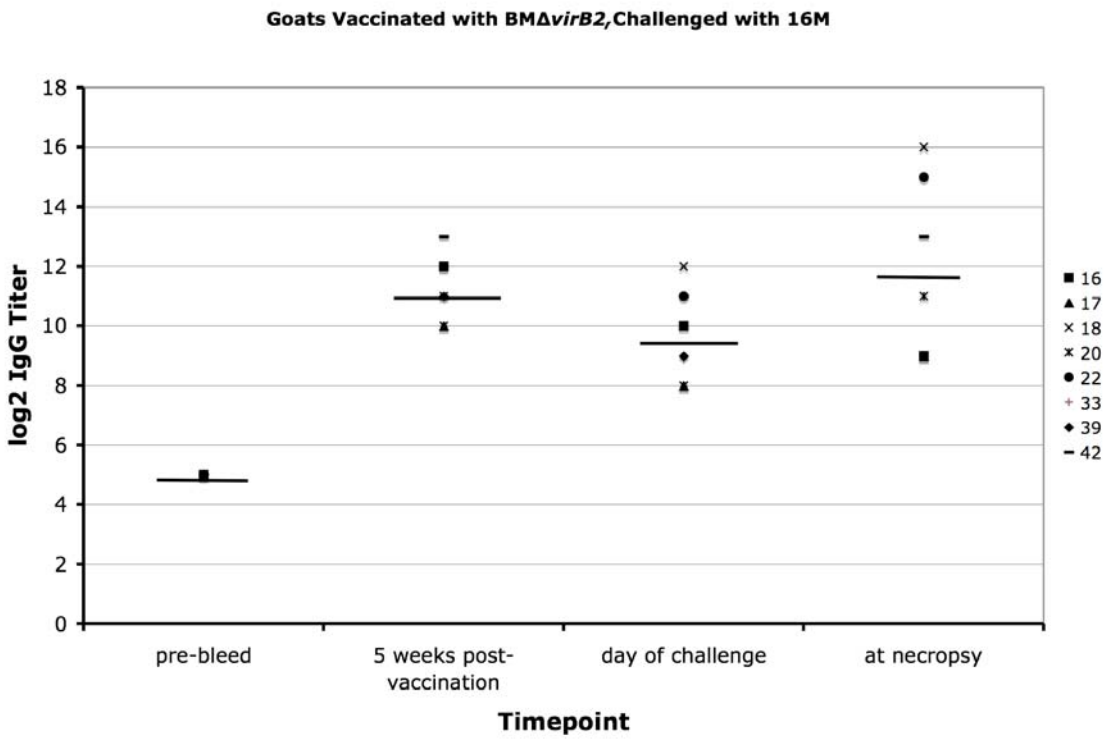


FIG. 47. Goat efficacy study: IgG titers of goats vaccinated with BMΔ*virB2* and challenged with 16M. Goats were vaccinated with$1x10^9$ CFU BMΔ*virB2* (subcutaneous) before pregnancy, then challenged with $1x10^7$ CFU wild-type *B. melitensis* 16M conjunctivally during the third trimester of pregnancy. Goats were bled the day of vaccination, 5 weeks post-vaccination, the day of challenge (18 weeks post-vaccination), and at necropsy (7 weeks post-challenge). Serum samples were assayed via ELISA to detect IgG antibodies due to the vaccination. Baseline titers were set at 5 as described in the text. Titers for samples were based upon the first dilution in the series that the OD reading was below the cut-off OD value. The IgG levels are therefore represented by the two fold increase in IgG levels over the duration of infection.

DISCUSSION

Since *Brucella* vaccine strains approved for use in livestock may elicit negative side effects upon administration or even in the long-term such as chronic shedding and repeated abortions, improved strains were considered for use in target species. The mouse model is adequate for evaluating attenuation and protection against infection, but the model's major downfall is the lack of practical information generated for target species. Specifically, protection against abortion can not be predicted, which is a major component of vaccine development for *Brucella*. The goat, a target species, was therefore used to model the effects of novel unmarked deletion strains on fetal survival and protection. The goat was chosen due to its reduced size, reproducible experimental abortions and infections, and for hosting *Brucella melitensis* infections naturally (21).

Vaccine candidates in this study were selected based upon their attenuation and protective ability demonstrated in the mouse model. The selected vaccine candidates were evaluated in pregnant ruminants to ensure the strains were safe (i.e. do not cause abortion when administered to pregnant animals) and that they would afford protection against abortion and infection upon subsequent exposure to virulent *Brucella*. For the first set of criteria, two vaccine strains, BMΔ*asp24* and BMΔ*virB2*, were safe in pregnant goats administered during mid-gestation. The BMΔ*virB2* mutant was not cultured from either dams or kids, suggesting that this strain is cleared quickly from the host at the reduced dose and $1x10^7$ CFU would therefore be a safe strain at that dose for use in pregnant goats. BMΔ*asp24* was isolated from most of the maternal tissues, suggesting a reduced rate of clearance of this strain. However, BMΔ*asp24* was not

cultured from any of the kids despite the maternal colonization. On the basis of safety of these candidate vaccine strains, BMΔ*asp24* and BMΔ*virB2* were considered to be improvements over the currently utilized vaccine strain Rev 1 which can cause abortion when given to goats during the late stages of gestation (19).

Vaccine strains may need to persist in the host for a period sufficient to evoke protective immunity. As such, the vaccine strain should ideally persist in the lymph nodes to invoke the host's immune response, but not be reactivated at sexual maturity or during pregnancy, to eliminate the possibility of abortions (12). Since the BMΔ*asp24* vaccine strain is able to persist and is not pathogenic to the fetus, it may be the best novel vaccine candidate tested. The BMΔ*virB2* mutant was safe in pregnant goats and protected the majority of the dams against subsequent infection without persistence in maternal tissues. The increased safety of this BMΔ*virB2* indicates a benefit over other strains, such as Rev 1.

Histological changes were assessed to identify pathology associated with the vaccination. BMΔ*asp24* generally had more changes associated with an inflammatory response and activity in the lymph nodes than BMΔ*virB2*. This was expected since the latter did not appear to persist in the host for as long. These changes were not as severe as goats vaccinated with either BMΔ*cydBA* or 16M, paralleling the attenuated phenotype seen in the mouse model.

The two safe vaccine candidates were then used in efficacy trials to decide whether they would provide increased levels of protection against challenge, the second important criterion. The majority of BMΔ*virB2* vaccinated goats were protected against

challenge as evidenced by lack of isolation of challenge organism from tissues, but the goats that were culture positive had very high numbers of challenge organism recovered. Increasing the dose for the BMΔ*virB2* vaccine may afford better protection to the majority of goats. Since the vaccine cleared rapidly from goats in the safety study at two logs lower of a dose, increasing the dose a log may still be safe, but would need to be considered in the event a pregnant animal received this higher vaccine dose. When cattle are given RB51 at $1x10^9$ while pregnant, they do not often suffer abortions or placentitis. When they are given the typical RB51 vaccine dose of 1-3.4 X 10^{10} organisms, however, they may suffer from placental or fetal infections (69). For this reason, even safe candidates can become pathogenic to the pregnant host at a high enough dose, and this factor will need to be assessed.

BMΔ*asp24* vaccinated goats displayed a result differing from BMΔ*virB2*. In the majority of animals vaccinated with BMΔ*asp24*, no or very low numbers of challenge organism were recovered from either the dams or kids. There was one dam in the BMΔ*asp24* group with a very high recoverable load of vaccine strain. For this strain, the dose may need to be lowered to decrease the maternal pathology and persistence of the organism. Regardless of this persistence, vaccination with $1x10^9$ still was safe for the fetuses, since vaccine strain was not isolated from the kids, another indication of altered tropism of the mutant.

Rough strains have become a huge focus in the development of cattle vaccines due to the lack of problems with standard diagnostics. These same rough strains have not showed superior protection in goats over current vaccine strains when administered

at high doses, however (20). Rough mutants are typically safe in goats, depending upon the administration dose, but they do not elicit long-term protection and will not be adequate for use in the field (65). It was hypothesized that goats need both cell-mediated as well as humoral immunity to be protected against *B. melitensis*, which may explain the protective ability of Rev 1, a smooth organism (20). It is possible that improved vaccine strains should not sacrifice the immunogenic effect of O-antigen, and instead improved diagnostics need to be considered if smooth vaccines continue to be used.

Overall, two safe vaccine candidates, BMΔ*asp24* and BMΔ*virB2*, were identified in the caprine model. The vaccines afforded protection against abortion and fetal and maternal infection in efficacy trials. The degree of pathology and infection to the dams from BMΔ*asp24,* though minor, may be lessened by decreasing the amount of vaccine strain administered from $1x10^9$ to a dose closer to the Rev 1, which is given at $1x10^6$. Both strains would need to be re-evaluated in bison, elk and cattle with *B. abortus* knockouts to ensure the protection afforded would be similar in other target species. Since there has not been an identified silver bullet vaccine, it is possible that one mutant vaccine candidate will not protect all target species. The goat model has demonstrated some positive results that need to be considered further for vaccine development.

SUMMARY AND CONCLUSION

B. abortus and *B. melitensis* are two zoonotic pathogens causing brucellosis, which is a human health threat and a disease of global economic importance. Some animals that are infected will abort during mid to late gestation, spreading the disease to others in the herd and causing reproductive losses to producers. The ability *Brucella* to persist in phagocytic cells is a central factor in the organism's survival. Recent knowledge of specific genes directly involved in the pathogenesis of the organism, such as O-antigen and the type IV secretion system, has allowed researchers to alter trafficking, and thus the fate of the bacteria *in vitro* and in the mouse model. Removing genes that are important to the organism's pathogenesis has led to the development of attenuated strains that do not cause typical disease in the host, and are possible novel live vaccine candidates for evaluation.

Brucella species defective in the *manBA*, *virB* operon, *cydBA* and *asp24* genes were previously identified in this lab as attenuated in macrophages and or mice, and were chosen as targets to create vaccine strains. Two of these genes, *manBA* and *virB2,* are involved in known virulence mechanisms of the bacteria, LPS and the T4SS, respectively. These genes were chosen not only to indicate their importance to virulence, but also to elucidate their role in protective ability of vaccine strains. Removal of the specific genes important for the organism's survival without retained possession of antibiotic resistance led to novel strains sufficient to test in animals. The

two species of *Brucella* chosen to host the deletions are both of economic importance for agricultural reasons and are both human pathogens.

In the mouse model, Δ*manBA*, Δ*virB2*, and Δ*asp24* exhibited varied survival characteristics, ranging from rapidly cleared to more persistent though attenuated. Due to the varied kinetics of clearance in mice, these mutants were considered good models to determine whether the duration of persistence of vaccine strains were directly correlated with protective ability. In general, the *Brucella* mutant that persisted the longest, BMΔ*asp24*, protected mice to the highest degree against homologous and heterologous challenge infection. BAΔ*asp24* also protected mice to a very high degree against both types of challenge, though it did not persist as long as BMΔ*asp24*. It is possible that in addition to the persistence, there is a correlation between protection and presence of known virulence factors. In particular, the *asp24* deletions possess both LPS as well as the T4SS. It is possible that the great degree of protection is due, in part, to the presence of both factors. Cytokine profiles need to be re-evaluated in another strain of mice and at different timepoints to correlate the CMI responses with the different strains.

The goat model was necessary to assess use of BMΔ*asp24*, BMΔ*cydBA*, and BMΔ*virB2* in target species that are naturally infected with *Brucella*. The goat is used as a model to assess the degree of pathology associated with the vaccine strain itself, as well as protection against abortion and infection, all of which are the criteria for *Brucella* vaccine strains for use in livestock. Both BMΔ*asp24* and BMΔ*virB2* were considered safe vaccine candidates in the pregnant goat model since they did not cause abortion or

colonize fetal tissues. BMΔ*asp24* was isolated from most of the maternal tissues in the goat safety studies, suggesting a slower rate of clearance of the vaccine strain than for BMΔ*virB2,* which was not isolated from any maternal or fetal tissues. This observation mirrors the mouse experiments, where BMΔ*asp24* is cleared from mice more slowly than BMΔ*virB2*. Both strains were protective in the majority of goats against abortion and infection. The BMΔ*asp24* vaccine strain persisted in one goat, however, and so a lowered dose may need to be considered to decrease maternal pathology while retaining the protective ability. The BMΔ*virB2* did not protect the goats as well as Rev 1 or BMΔ*asp24*, and so a higher dose may need to be considered to increase the protection levels, though this may increase the persistence. It is hoped that altering the dose will improve protection levels while maintaining the safety of the strains. Alternatively, a delivery system such as microencapsulation may offer a solution for release of safe doses the vaccine for extended periods, thus enhancing protection.

REFERENCES

1. **Adams, L. G.** 2002. The pathology of brucellosis reflects the outcome of the battle between the host genome and the *Brucella* genome. Vet Microbiol **90:**553-561.

2. **Allen, C. A., L. G. Adams, and T. A. Ficht.** 1998. Transposon-derived *Brucella abortus* rough mutants are attenuated and exhibit reduced intracellular survival. Infect Immun **66:**1008-1016.

3. **Anderson, T. D., V. P. Meador, and N. F. Cheville.** 1986. Pathogenesis of placentitis in the goat inoculated with *Brucella abortus*. I. Gross and histologic lesions. Vet Pathol **23:**219-226.

4. **Ashford, D. A., J. di Pietra, J. Lingappa, C. Woods, H. Noll, B. Neville, R. Weyant, S. L. Bragg, R. A. Spiegel, J. Tappero, and B. A. Perkins.** 2004. Adverse events in humans associated with accidental exposure to the livestock brucellosis vaccine RB51. Vaccine **22:**3435-3439.

5. **Baldwin, C. L., and M. Parent.** 2002. Fundamentals of host immune response against *Brucella abortus*: what the mouse model has revealed about control of infection. Vet Microbiol **90:**367-382.

6. **Bardenstein, S., M. Mandelboim, T. A. Ficht, M. Baum, and M. Banai.** 2002. Identification of the *Brucella melitensis* vaccine strain Rev.1 in animals and humans in Israel by PCR analysis of the PstI site polymorphism of its omp2 gene. J Clin Microbiol **40:**1475-1480.

7. **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.

8. **Boschiroli, M. L., S. Ouahrani-Bettache, V. Foulongne, S. Michaux-Charachon, G. Bourg, A. Allardet-Servent, C. Cazevieille, J. P. Lavigne, J. P. Liautard, M. Ramuz, and D. O'Callaghan.** 2002. Type IV secretion and *Brucella* virulence. Vet Microbiol **90:**341-348.

9. **Boschiroli, M. L., S. Ouahrani-Bettache, V. Foulongne, S. Michaux-Charachon, G. Bourg, A. Allardet-Servent, C. Cazevieille, J. P. Liautard, M.**

Ramuz, and D. O'Callaghan. 2002. The *Brucella suis* virB operon is induced intracellularly in macrophages. Proc Natl Acad Sci U S A **99:**1544-1549.

10. **Celli, J., C. de Chastellier, D. M. Franchini, J. Pizarro-Cerda, E. Moreno, and J. P. Gorvel.** 2003. *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J Exp Med **198:**545-556.

11. **Celli, J., and J. P. Gorvel.** 2004. Organelle robbery: *Brucella* interactions with the endoplasmic reticulum. Curr Opin Microbiol **7:**93-97.

12. **Cheville, N. F., S. C. Olsen, A. E. Jensen, M. G. Stevens, A. M. Florance, H. S. Houng, E. S. Drazek, R. L. Warren, T. L. Hadfield, and D. L. Hoover.** 1996. Bacterial persistence and immunity in goats vaccinated with a purE deletion mutant or the parental 16M strain of *Brucella melitensis*. Infect Immun **64:**2431-2439.

13. **Cloeckaert, A., M. S. Zygmunt, and L. A. Guilloteau.** 2002. *Brucella abortus* vaccine strain RB51 produces low levels of M-like O-antigen. Vaccine **20:**1820-1822.

14. **Comerci, D. J., M. J. Martinez-Lorenzo, R. Sieira, J. P. Gorvel, and R. A. Ugalde.** 2001. Essential role of the VirB machinery in the maturation of the *Brucella* abortus-containing vacuole. Cell Microbiol **3:**159-168.

15. **Cook, G. M., J. Membrillo-Hernandez, and R. K. Poole.** 1997. Transcriptional regulation of the cydDC operon, encoding a heterodimeric ABC transporter required for assembly of cytochromes c and bd in *Escherichia coli* K-12: regulation by oxygen and alternative electron acceptors. J Bacteriol **179:**6525-6530.

16. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640-6645.

17. **Davis, D. S., and P. H. Elzer.** 2002. *Brucella* vaccines in wildlife. Vet Microbiol **90:**533-544.

18. **den Hartigh, A. B., Y. H. Sun, D. Sondervan, N. Heuvelmans, M. O. Reinders, T. A. Ficht, and R. M. Tsolis.** 2004. Differential requirements for VirB1 and VirB2 during *Brucella abortus* infection. Infect Immun **72:**5143-5149.

19. **Edmonds, M. D., A. Cloeckaert, S. D. Hagius, L. E. Samartino, W. T. Fulton, J. V. Walker, F. M. Enright, N. J. Booth, and P. H. Elzer.** 2002.

Pathogenicity and protective activity in pregnant goats of a *Brucella melitensis* Deltaomp25 deletion mutant. Res Vet Sci **72:**235-239.

20. **Elzer, P. H., F. M. Enright, J. R. McQuiston, S. M. Boyle, and G. G. Schurig.** 1998. Evaluation of a rough mutant of *Brucella melitensis* in pregnant goats. Res Vet Sci **64:**259-260.

21. **Elzer, P. H., S. D. Hagius, D. S. Davis, V. G. DelVecchio, and F. M. Enright.** 2002. Characterization of the caprine model for ruminant brucellosis. Vet Microbiol **90:**425-431.

22. **Elzer, P. H., J. Smith, T. Roffe, T. Kreeger, J. Edwards, and D. Davis.** 2002. Evaluation of *Brucella abortus* strain RB51 and strain 19 in pronghorn antelope. Ann N Y Acad Sci **969:**102-105.

23. **Endley, S., D. McMurray, and T. A. Ficht.** 2001. Interruption of the cydB locus in *Brucella abortus* attenuates intracellular survival and virulence in the mouse model of infection. J Bacteriol **183:**2454-2462.

24. **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.

25. **Ewalt, D. R.** 1989. Comparison of three culture techniques for the isolation of *Brucella abortus* from bovine supramammary lymph nodes. J Vet Diagn Invest **1:**227-230.

26. **Fernandes, D. M., and C. L. Baldwin.** 1995. Interleukin-10 downregulates protective immunity to *Brucella abortus*. Infect Immun **63:**1130-1133.

27. **Ficht, T. A.** 2002. Discovery of *Brucella* virulence mechanisms using mutational analysis. Vet Microbiol **90:**311-315.

28. **Ficht, T. A.** 2003. Intracellular survival of *Brucella*: defining the link with persistence. Vet Microbiol **92:**213-223.

29. **Forestier, C., F. Deleuil, N. Lapaque, E. Moreno, and J. P. Gorvel.** 2000. *Brucella* abortus lipopolysaccharide in murine peritoneal macrophages acts as a down-regulator of T cell activation. J Immunol **165:**5202-5210.

30. **Foulongne, V., G. Bourg, C. Cazevieille, S. Michaux-Charachon, and D. O'Callaghan.** 2000. Identification of *Brucella suis* genes affecting intracellular survival in an in vitro human macrophage infection model by signature-tagged transposon mutagenesis. Infect Immun **68:**1297-1303.

31. **Giambartolomei, G. H., M. V. Delpino, M. E. Cahanovich, J. C. Wallach, P. C. Baldi, C. A. Velikovsky, and C. A. Fossati.** 2002. Diminished production of T helper 1 cytokines correlates with T cell unresponsiveness to *Brucella* cytoplasmic proteins in chronic human brucellosis. J Infect Dis **186:**252-259.

32. **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.

33. **Golding, B., D. E. Scott, O. Scharf, L. Y. Huang, M. Zaitseva, C. Lapham, N. Eller, and H. Golding.** 2001. Immunity and protection against *Brucella abortus*. Microbes Infect **3:**43-48.

34. **Golding, B., M. Zaitseva, and H. Golding.** 1994. The potential for recruiting immune responses toward type 1 or type 2 T cell help. Am J Trop Med Hyg **50:**33-40.

35. **Gorvel, J. P., and E. Moreno.** 2002. *Brucella* intracellular life: from invasion to intracellular replication. Vet Microbiol **90:**281-297.

36. **Hamdy, M. E., S. M. El-Gibaly, and A. M. Montasser.** 2002. Comparison between immune responses and resistance induced in BALB/c mice vaccinated with RB51 and Rev. 1 vaccines and challenged with *Brucella melitensis* bv. 3. Vet Microbiol **88:**85-94.

37. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77-86.

38. **Hong, P. C., R. M. Tsolis, and T. A. Ficht.** 2000. Identification of genes required for chronic persistence of *Brucella abortus* in mice. Infect Immun **68:**4102-4107.

39. **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(16MDeltapurEK). Infect Immun **67:**5877-5884.

40. **Ko, J., and G. A. Splitter.** 2003. Molecular host-pathogen interaction in brucellosis: current understanding and future approaches to vaccine development for mice and humans. Clin Microbiol Rev **16:**65-78.

41. **Kohler, S., S. Michaux-Charachon, F. Porte, M. Ramuz, and J. P. Liautard.** 2003. What is the nature of the replicative niche of a stealthy bug named *Brucella*? Trends Microbiol **11:**215-219.

42. **Kurar, E., and G. A. Splitter.** 1997. Nucleic acid vaccination of *Brucella abortus* ribosomal L7/L12 gene elicits immune response. Vaccine **15:**1851-1857.

43. **Lai, F., G. G. Schurig, and S. M. Boyle.** 1990. Electroporation of a suicide plasmid bearing a transposon into *Brucella abortus*. Microb Pathog **9:**363-368.

44. **Lestrate, P., R. M. Delrue, I. Danese, C. Didembourg, B. Taminiau, P. Mertens, X. De Bolle, A. Tibor, C. M. Tang, and J. J. Letesson.** 2000. Identification and characterization of in vivo attenuated mutants of *Brucella melitensis*. Mol Microbiol **38:**543-551.

45. **Lin, J., and T. A. Ficht.** 1995. Protein synthesis in *Brucella abortus* induced during macrophage infection. Infect Immun **63:**1409-1414.

46. **Link, A. J., D. Phillips, and G. M. Church.** 1997. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. J Bacteriol **179:**6228-6237.

47. **Meador, V. P., and B. L. Deyoe.** 1986. Experimentally induced *Brucella abortus* infection in pregnant goats. Am J Vet Res **47:**2337-2342.

48. **Monreal, D., M. J. Grillo, D. Gonzalez, C. M. Marin, M. J. De Miguel, I. Lopez-Goni, J. M. Blasco, A. Cloeckaert, and I. Moriyon.** 2003. Characterization of *Brucella abortus* O-polysaccharide and core lipopolysaccharide mutants and demonstration that a complete core is required for rough vaccines to be efficient against *Brucella abortus* and *Brucella ovis* in the mouse model. Infect Immun **71:**3261-3271.

49. **Munoz-Montesino, C., E. Andrews, R. Rivers, A. Gonzalez-Smith, G. Moraga-Cid, H. Folch, S. Cespedes, and A. A. Onate.** 2004. Intraspleen delivery of a DNA vaccine coding for superoxide dismutase (SOD) of *Brucella abortus* induces SOD-specific CD4+ and CD8+ T cells. Infect Immun **72:**2081-2087.

50. **Murphy, E. A., M. Parent, J. Sathiyaseelan, X. Jiang, and C. L. Baldwin.** 2001. Immune control of *Brucella abortus* 2308 infections in BALB/c mice. FEMS Immunol Med Microbiol **32:**85-88.

51. **Murphy, E. A., J. Sathiyaseelan, M. A. Parent, B. Zou, and C. L. Baldwin.** 2001. Interferon-gamma is crucial for surviving a *Brucella abortus* infection in

both resistant C57BL/6 and susceptible BALB/c mice. Immunology **103:**511-518.

52. **Murphy, K. C., K. G. Campellone, and A. R. Poteete.** 2000. PCR-mediated gene replacement in *Escherichia coli*. Gene **246:**321-330.

53. **Oliveira, S. C., N. Soeurt, and G. Splitter.** 2002. Molecular and cellular interactions between *Brucella abortus* antigens and host immune responses. Vet Microbiol **90:**417-424.

54. **Oliveira, S. C., and G. A. Splitter.** 1996. Immunization of mice with recombinant L7/L12 ribosomal protein confers protection against Brucella abortus infection. Vaccine **14:**959-962.

55. **Onate, A., E. Andrews, A. Beltran, G. Eller, G. Schurig, and H. Folch.** 2000. Frequent exposure of mice to crude *Brucella abortus* proteins down-regulates immune response. J Vet Med B Infect Dis Vet Public Health **47:**677-682.

56. **Paranavitana, C., E. Zelazowska, M. Izadjoo, and D. Hoover.** 2005. Interferon-gamma associated cytokines and chemokines produced by spleen cells from *Brucella*-immune mice. Cytokine **30:**86-92.

57. **Pasquali, P., R. Adone, L. C. Gasbarre, C. Pistoia, and F. Ciuchini.** 2001. Mouse cytokine profiles associated with *Brucella abortus* RB51 vaccination or B. abortus 2308 infection. Infect Immun **69:**6541-6544.

58. **Pasquali, P., A. Rosanna, C. Pistoia, P. Petrucci, and F. Ciuchini.** 2003. *Brucella abortus* RB51 induces protection in mice orally infected with the virulent strain B. abortus 2308. Infect Immun **71:**2326-2330.

59. **Paulsen, I. T., R. Seshadri, K. E. Nelson, J. A. Eisen, J. F. Heidelberg, T. D. Read, R. J. Dodson, L. Umayam, L. M. Brinkac, M. J. Beanan, S. C. Daugherty, R. T. Deboy, A. S. Durkin, J. F. Kolonay, R. Madupu, W. C. Nelson, B. Ayodeji, M. Kraul, J. Shetty, J. Malek, S. E. Van Aken, S. Riedmuller, H. Tettelin, S. R. Gill, O. White, S. L. Salzberg, D. L. Hoover, L. E. Lindler, S. M. Halling, S. M. Boyle, and C. M. Fraser.** 2002. The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. Proc Natl Acad Sci U S A **99:**13148-13153.

60. **Pei, J., and T. A. Ficht.** 2004. *Brucella abortus* rough mutants are cytopathic for macrophages in culture. Infect Immun **72:**440-450.

61. **Plommet, M., and A. M. Plommet.** 1988. Virulence of *Brucella*: bacterial growth and decline in mice. Ann Rech Vet **19:**65-67.

62. **Porte, F., J. P. Liautard, and S. Kohler.** 1999. Early acidification of phagosomes containing *Brucella suis* is essential for intracellular survival in murine macrophages. Infect Immun **67:**4041-4047.

63. **Porte, F., A. Naroeni, S. Ouahrani-Bettache, and J. P. Liautard.** 2003. Role of the *Brucella suis* lipopolysaccharide O antigen in phagosomal genesis and in inhibition of phagosome-lysosome fusion in murine macrophages. Infect Immun **71:**1481-1490.

64. **Rittig, M. G., A. Kaufmann, A. Robins, B. Shaw, H. Sprenger, D. Gemsa, V. Foulongne, B. Rouot, and J. Dornand.** 2003. Smooth and rough lipopolysaccharide phenotypes of *Brucella* induce different intracellular trafficking and cytokine/chemokine release in human monocytes. J Leukoc Biol **74:**1045-1055.

65. **Roop, R. M., 2nd, G. Jeffers, T. Bagchi, J. Walker, F. M. Enright, and G. G. Schurig.** 1991. Experimental infection of goat fetuses in utero with a stable, rough mutant of *Brucella abortus*. Res Vet Sci **51:**123-127.

66. **Roop, R. M., 2nd, G. T. Robertson, G. P. Ferguson, L. E. Milford, M. E. Winkler, and G. C. Walker.** 2002. Seeking a niche: putative contributions of the hfq and bacA gene products to the successful adaptation of the *brucellae* to their intracellular home. Vet Microbiol **90:**349-363.

67. **Sathiyaseelan, J., X. Jiang, and C. L. Baldwin.** 2000. Growth of *Brucella abortus* in macrophages from resistant and susceptible mouse strains. Clin Exp Immunol **121:**289-294.

68. **Schurig, G. G., R. M. Roop, 2nd, T. Bagchi, S. Boyle, D. Buhrman, and N. Sriranganathan.** 1991. Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet Microbiol **28:**171-188.

69. **Schurig, G. G., N. Sriranganathan, and M. J. Corbel.** 2002. Brucellosis vaccines: past, present and future. Vet Microbiol **90:**479-496.

70. **Schweizer, H. P., and T. T. Hoang.** 1995. An improved system for gene replacement and xylE fusion analysis in *Pseudomonas aeruginosa*. Gene **158:**15-22.

71. **Sharif, M. N., I. Tassiulas, Y. Hu, I. Mecklenbrauker, A. Tarakhovsky, and L. B. Ivashkiv.** 2004. IFN-alpha priming results in a gain of proinflammatory function by IL-10: implications for systemic lupus erythematosus pathogenesis. J Immunol **172:**6476-6481.

72. **Stevens, M. G., S. C. Olsen, M. V. Palmer, and G. W. Pugh, Jr.** 1996. Immune responses and resistance to brucellosis in mice vaccinated orally with *Brucella abortus* RB51. Infect Immun **64:**4534-4541.

73. **Stevens, M. G., S. C. Olsen, and G. W. Pugh, Jr.** 1995. Comparison of spleen cell proliferation in response to *Brucella abortus* 2308 lipopolysaccharide or proteins in mice vaccinated with strain 19 or RB51. Infect Immun **63:**3199-3205.

74. **Stevens, M. G., S. C. Olsen, G. W. Pugh, Jr., and D. Brees.** 1995. Comparison of immune responses and resistance to brucellosis in mice vaccinated with *Brucella abortus* 19 or RB51. Infect Immun **63:**264-270.

75. **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.

76. **Tobias, L., G. G. Schurig, and D. O. Cordes.** 1992. Comparative behaviour of *Brucella abortus* strains 19 and RB51 in the pregnant mouse. Res Vet Sci **53:**179-183.

77. **Ugalde, J. E., C. Czibener, M. F. Feldman, and R. A. Ugalde.** 2000. Identification and characterization of the *Brucella abortus* phosphoglucomutase gene: role of lipopolysaccharide in virulence and intracellular multiplication. Infect Immun **68:**5716-5723.

78. **Velikovsky, C. A., J. Cassataro, G. H. Giambartolomei, F. A. Goldbaum, S. Estein, R. A. Bowden, L. Bruno, C. A. Fossati, and M. Spitz.** 2002. A DNA vaccine encoding lumazine synthase from *Brucella abortus* induces protective immunity in BALB/c mice. Infect Immun **70:**2507-2511.

79. **Vizcaino, N., A. Cloeckaert, J. Verger, M. Grayon, and L. Fernandez-Lago.** 2000. DNA polymorphism in the genus *Brucella*. Microbes Infect **2:**1089-1100.

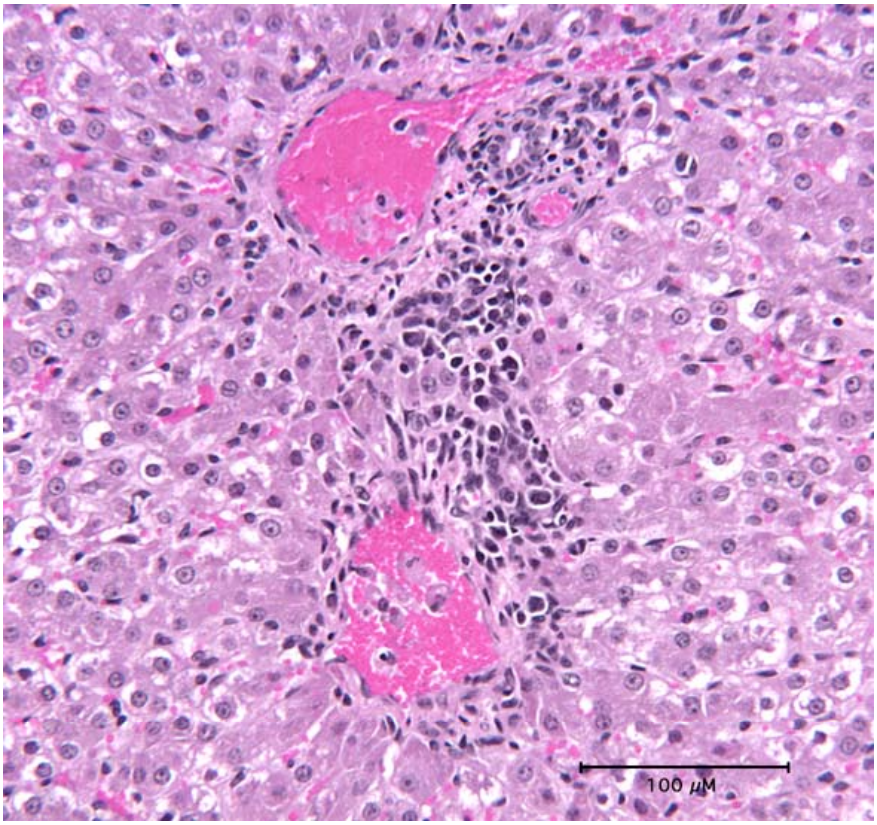
80. **Vizcaino, N., A. Cloeckaert, M. S. Zygmunt, and L. Fernandez-Lago.** 1999. Molecular characterization of a *Brucella* species large DNA fragment deleted in *Brucella abortus* strains: evidence for a locus involved in the synthesis of a polysaccharide. Infect Immun **67:**2700-2712.

81. **Zhan, Y., A. Kelso, and C. Cheers.** 1993. Cytokine production in the murine response to *brucella* infection or immunization with antigenic extracts. Immunology **80:**458-464.

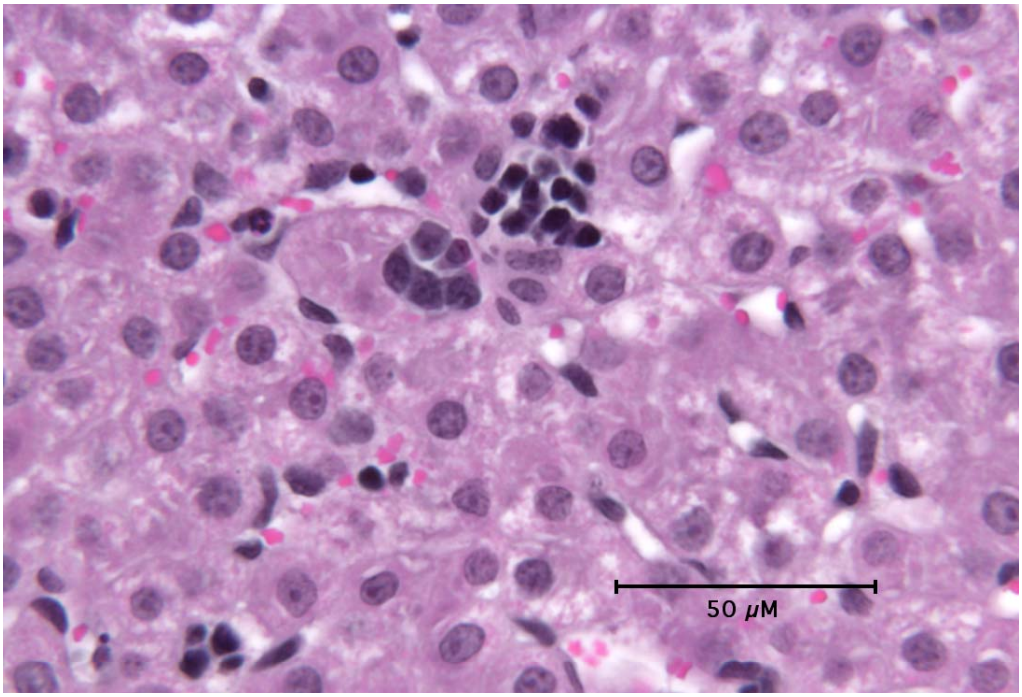
82. **Zhan, Y., A. Kelso, and C. Cheers.** 1995. Differential activation of *Brucella*-reactive CD4+ T cells by *Brucella* infection or immunization with antigenic extracts. Infect Immun **63:**969-975.

APPENDIX

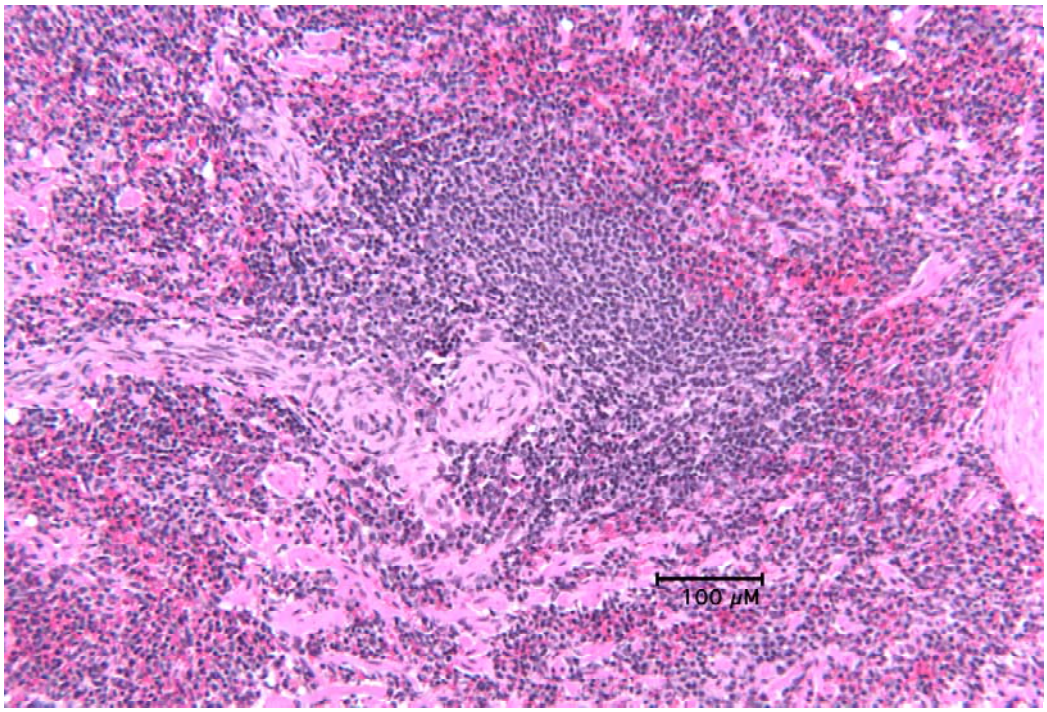
Histology slides from the goat safety study



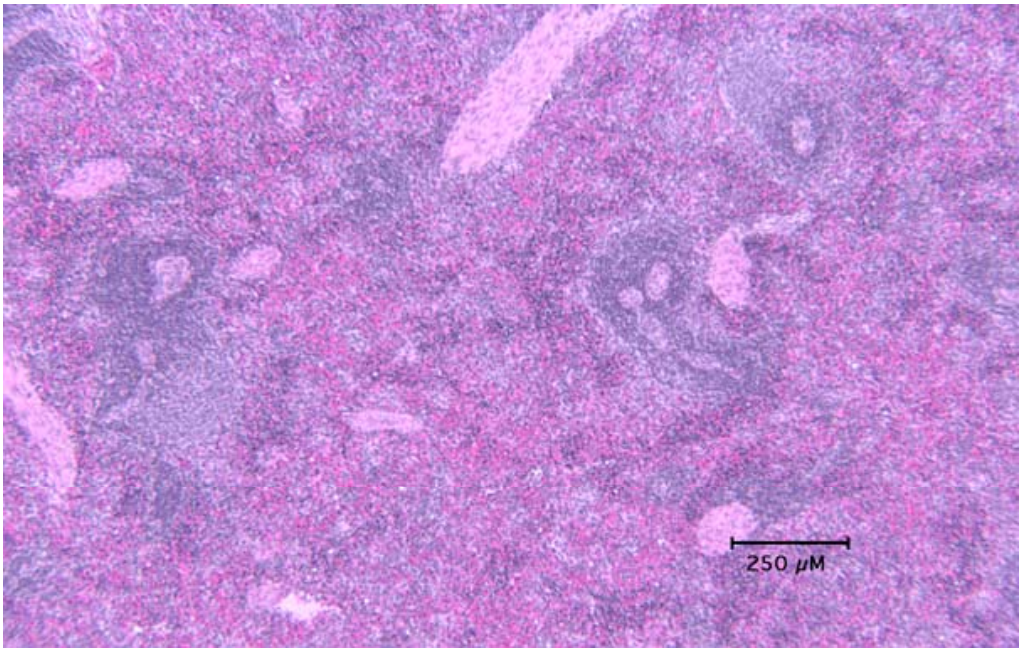
A-1. H&E stain of liver sample from fetal goat #28-A, 16M vaccinated group at 20X magnification. The tissue exhibits mild sinusoidal and marked portal triad mononuclear inflammation.



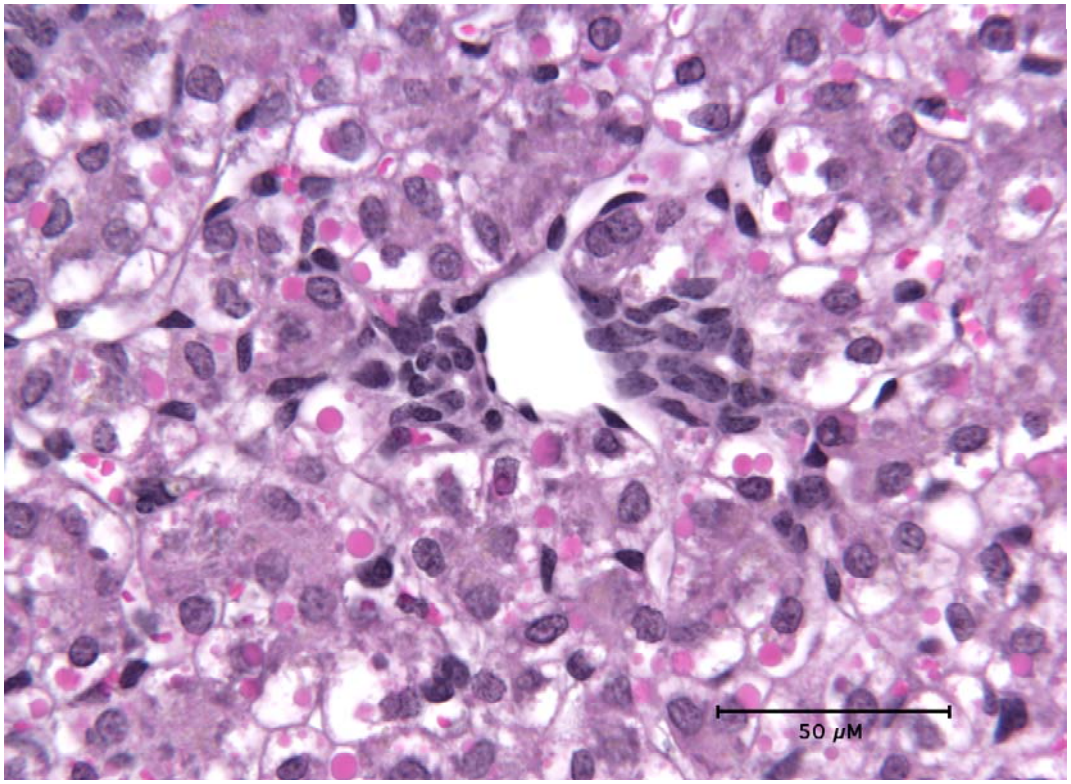
A-2. H&E stain of liver sample from fetal goat #48-b from 16M vaccinated group at 40X magnification. The tissue sample shows early sinusoidal microgranuloma formation.



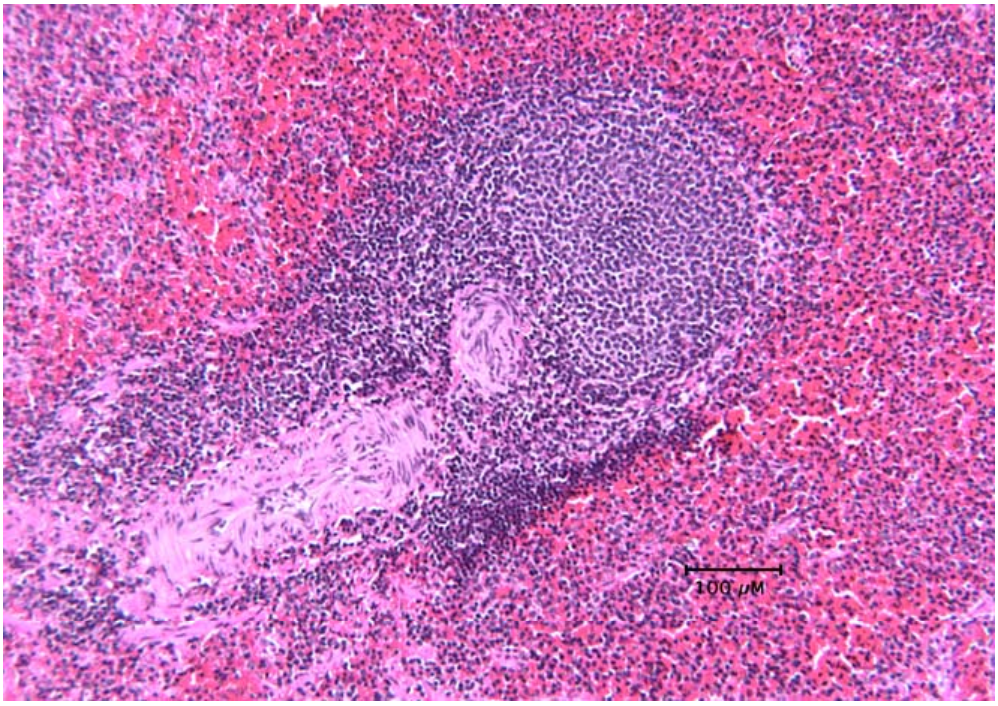
A-3. H&E stain of spleen from goat #29 of the 16M vaccinated group at 10X magnification. The tissue shows excessive amounts of polymorphonuclear cells and mild hemorrhage.



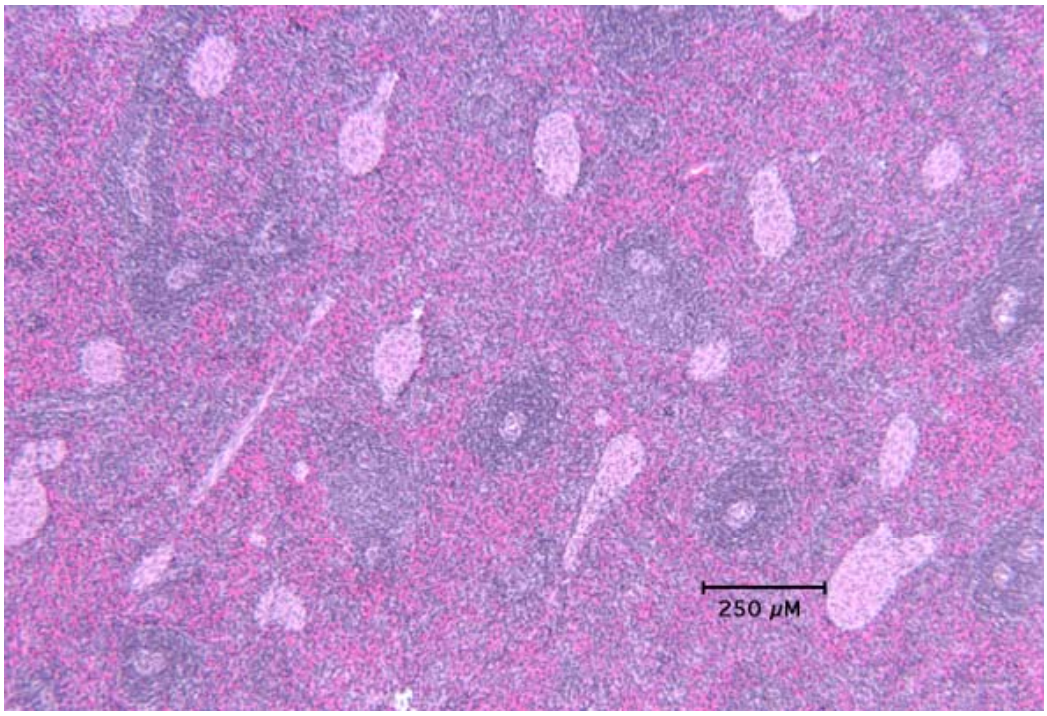
A-4. H&E stain of spleen from fetal goat #48-A of the 16M vaccinated group at 4X magnification. The tissue shows marked B and T cell zone expansion with mild congestion.



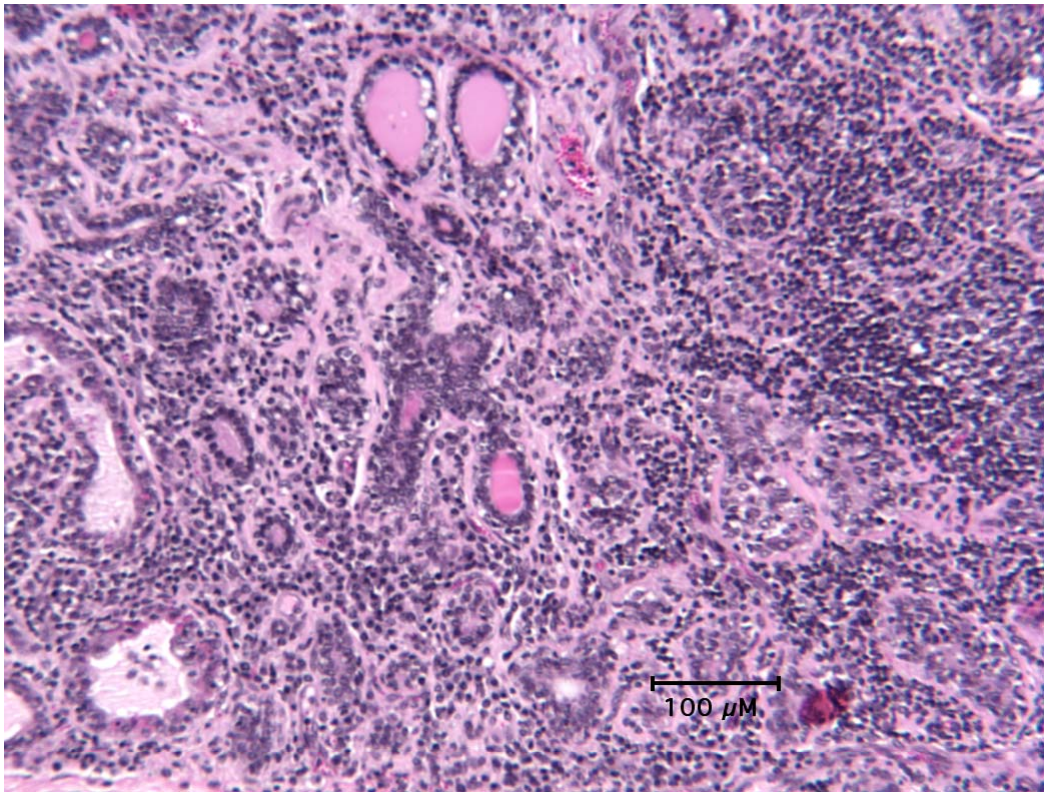
A-5. H&E stain of fetal goat #43-B from the BMΔ*cydBA* vaccine group at 40X magnification. The tissue shows a marked sinusoidal microgranuloma around the terminal hepatic venule.



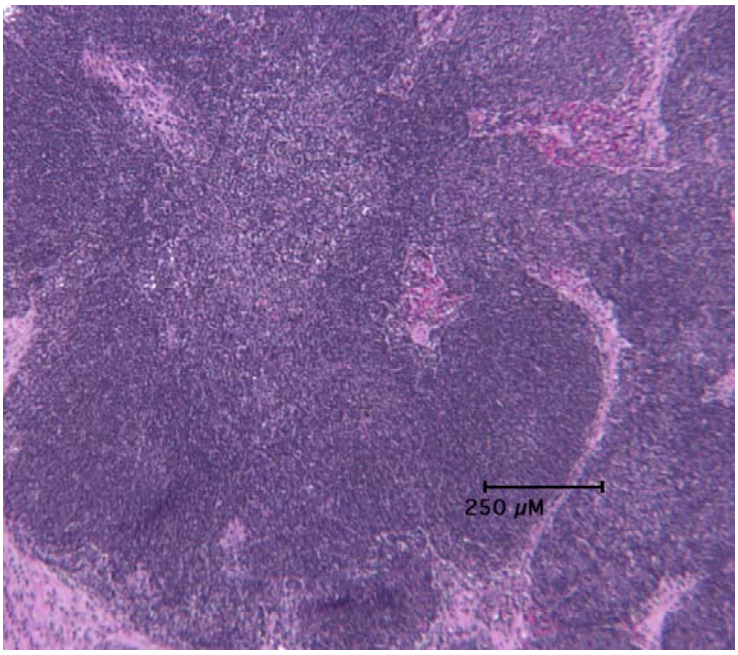
A-6. H&E stain of spleen from goat #32 of the BMΔ*cydBA* vaccinated group at 10X magnification. The tissue shows excessive amounts of polymorphonuclear cells and mild hemorrhage.



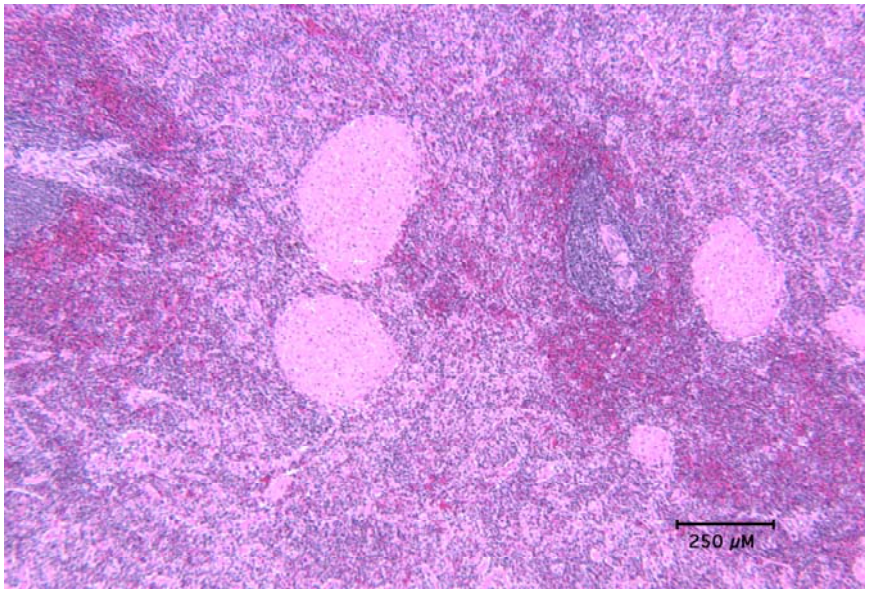
A-7. H&E stain of spleen from fetal goat #43-A of the BMΔ*cydBA* vaccinated group at 4X magnification. The tissue shows marked B and T cell zone expansion, moderate to high congestion, and mild sinusoidal hyperplasia.



A-8. H&E stain of mammary gland from goat # 47 of the BMΔ*asp24* vaccine group at 10X magnification. The tissue shows large, well-formed microgranulomas with a very high proportion of mononuclear infiltrate as well as polymorphonuclear cells.



A-9. H&E stain of supramammary lymph node of goat #12 from BMΔ*asp24* vaccinates at 4X magnification. The tissue shows very active B cell expansion.



A-10. H&E stain of spleen from goat # 44 from the BMΔ*virB2* vaccinates at 4X magnification. The tissue shows a marked lymphoid depletion and a moderate to high degree of hemorrhage.

VITA

MELISSA MARIE KAHL

ADDRESS
4467 TAMU
College Station, Texas 77843-4467

EDUCATION
Ph.D. Veterinary Microbiology, Texas A&M University- August 2005
B.S. Animal Science, University of Connecticut- December 1998