EVALUATION OF MICROENCAPSULATION AS AN IMPROVED VACCINATION

STRATEGY AGAINST BRUCELLOSIS

A Dissertation

by

ANGELA MARIA ARENAS GAMBOA

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

May 2007

Major Subject: Veterinary Microbiology

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Approved by:

Co-Chairs of Committee,   Allison Rice Ficht
                                    Thomas A. Ficht
Committee Members,   C. Jane Welsh
                                    Waithaka Mwangi
Head of Department   Gerald Bratton

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ABSTRACT

Evaluation of Microencapsulation as an Improved Vaccination

Strategy against Brucellosis. (May 2007)

Angela Maria Arenas Gamboa, D.V.M., La Salle University, Colombia

Co-Chairs of Advisory Committee: Dr. Allison Rice Ficht
Dr. Thomas A. Ficht

Brucellosis is an important zoonotic disease of nearly worldwide distribution. Despite the availability of live vaccine strains for bovine (S19, RB51) and small ruminants (Rev 1), these vaccines have several drawbacks including residual virulence for animals and humans. Safe and efficacious immunization systems are therefore needed to overcome these disadvantages. *Brucella melitensis* and *Brucella abortus* mutants in the *luxR* gene were generated and investigated for theri potential use as improve vaccine candidates. Immunization with a sustained release vehicle to enhance vaccination efficacy was evaluated utilizing the live mutants in encapsulated alginate microspheres containing a non-immunogenic eggshell precursor protein of the parasite *Fasciola hepatica* (Vitelline protein B, VpB). BALB/c mice were immunized with either encapsulated or nonencapsulated vaccine candidates to evaluate immunogenicity, safety and protective efficacy. The results suggest that *luxR* mutants, are attenuated in the mouse and macrophage model and appear good and safe vaccine candidates when the immunogen is given in a microencapsulated format. We were also able to demonstrate the utility of microencapsulation in oral delivery by increasing vaccine performance of current licensed vaccine strains in a natural host, the Red Deer.

Together, these results suggest that microencapsulation of live *Brucella*

produces an enhanced delivery vaccine system against brucellosis increasing the

efficacy of poorly-performing nonencapsulated vaccine candidates.

DEDICATION

To my mom, to Carlos (finally!!)

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE   Page

FIGURE   Page

INTRODUCTION AND LITERATURE REVIEW

BACKGROUND

The organism. *Brucella* is a gram-negative, facultative intracellular coccobacillus that belongs to the alpha-2 subdivision of Proteobacteria. The traditional classification of *Brucella* species is largely based on its preferred hosts: *B. melitensis* (goats), *B. abortus* (cattle), *B. suis* (swine), *B. canis* (dogs), *B. ovis* (sheep) and *B. neotomae* (desert mice). Two new *Brucella* species, called *Brucella pinnipediae* and *B. cetaceae,* have been isolated from marine hosts within the past few years (24, 48). The *Brucella* genome consists of two circular chromosomes of approximately 2.1 MB and 1.2 MB and has a GC content of approximately 57%. The DNA sequences among different *Brucella* spp. share greater than 90% homology (38)

Among the six different *Brucella* species, *B. melitensis, B.suis* and *B. abortus* are pathogenic and virulent, not only for sheep, goats, swine and cattle, respectively, but also for humans. Infection in animals most commonly occurs through ingestion or inhalation of contaminated tissues or in many cases may be sexually transmitted (38). Typical manifestations of the disease are abortions and infertility. Human brucellosis or "Malta Fever" is characterized by the development of undulant fever, osteoarthritis, spondylitis and endocarditis (56). Manifestations are usually non-specific including lymphadenopathy, hepatomegaly, or splenomegaly. The disease is transmitted to

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This dissertation will follow the style of Infection and Immunity.

humans by consumption of unpasteurized milk and dairy products or by direct contact with infected animals. Airborne transmission of brucellosis has been studied in the context of using *Brucella* as a biologic weapon. In fact, *B. suis* was the first agent contemplated by the U.S. Army as a potential biologic weapon (56).

Pathogenesis. The key aspect of *Brucella* virulence is its ability to proliferate within macrophages and non professional phagocytes (18). The bacteria successfully bypasses the bactericidal effects of phagocytes and its virulence is thought to be due to their ability to avoid the killing mechanisms of the host cells. Typically, *Brucella* intracellular survival relies upon avoiding fusion of its membrane- bound compartment with lysosomes, evading bacterial killing (9, 59).

Recent research has allowed the identification of genes implicated in *Brucella* virulence. One virulence factor is the *VirB* system. It consists of a type IV secretion system homologous to the one encoded by A. *tumefaciens virB* and *B. pertussis ptl* genes (51). This system originally identified from attenuated transposon mutants in vitro, is responsible for the injection of toxins into the cytoplasm of infected cells. Previous reports using signature tag mutagenesis have demonstrated that *virB* mutants of B. *abortus* are unable to establish chronic infections in the mouse model (34). The exact role of the *Brucella* VirB system in virulence is still under investigation (8). Different studies have shown that *virB* mutants of both B. *abortus* and B. *melitensis* have lost their ability to modulate intracellular trafficking. *virB* mutants cannot transit to the replication compartment and are eventually degraded by the cell. Recent data indicates that *VjbR*, a quorum sensing-related transcriptional regulator controls the expression of the *virB* operon during vegetative growth or during intracellular infection,

although the mechanism of action is still unclear, it does hold an important role in VirB regulation (17).

In addition to the type IV secretion system, bacterial outer membrane components especially lipopolysaccharides (LPS) have also been described as virulence factors that are important for survival and replication in the host (44). *Brucella* has two forms, smooth and rough. The presence of O-polysaccharide is the basic difference between these two forms. In general, rough strains are less virulent than smooth strains. Observations includes a decrease survival rate of rough mutants from macrophages and rapid clearance from animal models (42, 43, 58). Although it is well accepted that rough variants exhibit reduced virulence in the host, the exact role of O antigen specifically in intracellular survival remains controversial (58).

Brucella vaccines. Due to the serious consequences of the disease, efforts have been made to prevent brucellosis through the use of vaccines. The vaccine strain that has been most widely used to prevent the disease in cattle is the B. a*bortus* strain 19 (64). This vaccine is a smooth organism that naturally became attenuated after being kept in the laboratory at room temperature for over a year. The disadvantages of this immunogen includes, poorly genetically defined, vaccination of pregnant animals can result in abortion and interference with serological assays used for the diagnosis of brucellosis, all of these factors are totally undesirable effects for human vaccination.

Rev. 1 vaccine is a live smooth attenuated B. *melitensis* strain for use in sheep, goats and rams (11). Although this vaccine is attenuated when compared with field strains it retains some virulence and can cause abortion with variable frequency. Because it is a smooth organism it induces the production of antibodies that interfere

with the diagnosis.

More recently, the development of a rifampicin-resistant rough mutant of B. *abortus* strain 2308 was developed called Strain RB51 (63). Due to its rough characteristic, it does not induce O- antibodies being an advantage for *Brucella* diagnosis. However, this vaccine confers less protection than S19, does not protect against infection or abortion in wildlife including bison and elk, possesses an antibiotic resistant gene and although rarely, it has been associated with infection in humans (7).

The use of recombinant antigens or killed vaccines to induce a protective immune response to animals has proven ineffective at this time (82). The strategy relies upon the identification of those *Brucella* antigens that are responsible for the induction of a protective response, and although some levels of protection have been achieved the vaccine efficacy is reduced when compared to the one elicited by the live vaccines (11).

Immunity towards *Brucella* infections. It is well established that *Brucella*-specific IgG levels become elevated during the early stages of infection in animals and humans, but there is no correlation between the appearance of these serologic responses and resolution of the infection. For *Brucella*, cellular immunity is understood to be critical for resolution of primary infection and for establishment of immunity to reinfection (79).

Considerable amount of experimental evidence suggests that T cells are the key players against brucellosis. The major T cell responses towards *Brucella* immunity can be divided into three principal functions: secretion of IFNγ for the activation of bactericidal function in macrophages, cytotoxic T-lymphocyte activity and contribution in IgG2a and IgG3 isotype switching (38). It has been clearly demonstrated that a Th1

response is essential for resolution of the primary infection caused by *Brucella*, and the essential aspect of this response appears to be IFNγ production (47). The role of IFNγ in the control of *Brucella* infections was initially demonstrated by supplementing BALB/c mice with recombinant IFNγ. The treatment resulted in a 10-fold decrease in the number of bacteria at 1 week after infection (68). In accordance with these observations, depletion of IFN-γ by the administration of monoclonal antibodies which neutralize IFN-γ, resulted in the increase in the bacterial burden 1 week post-infection (81). The importance of IFNγ in resolution of Brucellosis was recently supported by studies using BALB/c and C57BL/6 mice. C57BL/6 mice with gene deletions or disruptions in the interferon-gamma (IFN-gamma), perforin or beta(2)-microglobulin, genes had a decreased ability to control intracellular infections with B. *abortus* strain 2308 during the first week post-infection. However, only the IFN-gamma knock-out mice had a sustained inability to control the infection and this resulted in death of the mice at approximately 6 weeks post-infection (49). *In vitro* studies have suggested that the mechanism by which IFN-γ enhances resistance is through activation of macrophages for anti-*Brucella* activity, largely by promoting production of reactive oxygen intermediates (36).

Different studies have demonstrated a role for both CD4+ or CD8+ T cells in the control of brucellosis. Adoptive transfer in BALB/c mice with either CD4+, CD8+ from mice immunized for 6 weeks resulted in reduction of bacterial burden in the spleen compared to naive mice after B. *abortus* infection, suggesting that both T-cell populations are important (5). Clear involvement of CD8+ T cells in brucellosis was demonstrated in MHC class I and II knockout mice that were infected with B. *abortus* S19. MHC class I-deficient mice, which have no CD8+T cells, controlled the infection

more slowly than MHC II and wild-type (52). Additional evidence to support the CD8^{+} T

cell importance in brucellosis came from experiments where mice were immunized with

B. abortus conjugated to a peptide derived from the HIV-1 envelope. Following a series

of immunizations, these mice developed peptide-specific antibodies that neutralized the

virus. In addition, they developed MHC class I restricted CD8^{+}T cells capable of killing

target cell expressing HIV(31, 80). Most importantly, mice deficient in CD4^{+} T cells and

immunized using the same preparation elicited the same response, suggesting that the

conjugates were being taken up by DCs that stimulated CD8^{+} T cells to differentiate into

memory cells in the absence of CD4^{+} T cells. A more recent study regarding this aspect

was performed in BALB/c. Mice were immunized with *Escherichia coli* expressing

Brucella abortus Cu/Zn superoxide-dismutase. Animals that received the vaccine, were

able to elicit protective immunity against B. a*bortus* wild-type challenge and when

splenocytes were separated into CD4^{+} and CD8^{+} populations, CD8^{+} T cells were able to

elicit lytic capacities, while CD4^{+} cells induced IFNγ production (4).

Murine brucellosis is not lethal in mice, unless IFNγ effects are eliminated (49).

Extensive data demonstrates that the CD4^{+} T cells are the major producer of IFNγ in

Brucella (82). Experimental evidence obtained from infected CBA mice vaccinated with

S19 indicated that CD4^{+} T cell enriched population produced statistically significantly

higher amounts of IFNγ than CD8^{+} T cell populations. The strongest evidence to date,

comes from Araya and Winters. They reported that CD4^{+}T cells from mice immunized

intraperitoneally with the vaccine strain RB51, when stimulated with RB51 infected

macrophages, produced IFNγ in greater amounts than CD8^{+}T cells. The same authors

also confirmed that CD8^{+}T cells displayed highly significant lysis of macrophages

infected with *Brucella*. However, similarly stimulated CD4$^+$ T cells also were cytotoxic suggesting the possibility that CD4$^+$ also may function as cytotoxic cells(79). Previous studies have reported that exogenous IL-10 increases and lack of IL-10, reduces bacterial burden in BALB/c mice (27). Among T cells, the CD4$^+$ T cell appears to be the producer of this cytokine (28). Taking all of these data into account, it appears that CD4$^+$ T cells function as effector cells by producing IFNγ, which is beneficial for the control of the infection, but their role can be deleterious when secreting IL-10. (79).

Limited studies have investigated T cell responses in species other than mice. In humans, diminished percentage of peripheral CD4+ T lymphocytes expressing IL-2Rα has been associated with chronic relapsing brucellosis(65). In cattle, it is known that CD4+ cells proliferate and produce INFγ in a MHC-II restricted fashion, after immunization (76). A major difference between ruminants and other species such as mice and humans is the large number of $\gamma\delta$ T cells. In cattle, limited information regarding this aspect is available but the findings suggest that there is proliferation of the $\gamma\delta$ T cells to *Brucella* antigens, although relative numbers of all subsets appear to remain constant throughout infection (76).

MICROENCAPSULATION AND EVALUATION OF *Brucella* VACCINE CANDIDATES

INTRODUCTION

The development of safer and more efficacious vaccination strategies to prevent Brucellosis is needed to overcome the disadvantages of the currently licensed vaccine strains. One of the main conditions for an ideal anti *Brucella* vaccine is the lack of virulence for the target host in order to avoid virulence related sequelae. Recent research has focused in the development of such vaccines, however they have proven to be less effective in preventing the disease. These strategies include the use of killed organisms, cell extracts, or recombinant proteins. In general, vaccination using these methods induce lower levels of protection compared to live attenuated *Brucella* vaccines.

We hypothesized that in order to generate a good immune response from the vaccine, controlled release of live and highly attenuated vaccine strains can be used to potentiate their vaccine efficacy.

Controlled release. Years of investigation have been focused on a variety of both natural and synthetic systems for establishment of a controlled release methodology of different products (21, 78). The most widely used microparticle vaccine delivery systems used to date are liposomes and poly lactide-co-glycolide microcapsules (19, 20). In the case of controlled release formulations, a product is typically encapsulated and released from the capsule by erosion, diffusion or solvent activation and transport (46, 72). This process will create the means for a sustained release of the product. The desired polymer characteristics include low toxicity, biocompatibility, lack of immunogenicity and capacity of breakdown inside the body.

Many of the processes used to entrap substances involve the use of harsh conditions including organic solvents, shear stress and ultrasound homogenization all of these being bactericidal and capable of denaturing proteins (45).

Alginate microspheres. Alginate, a naturally occurring biopolymer is especially well suited for entrapment of living bacteria mainly due to its characteristics including relatively inert aqueous environment within the matrix, a mild room temperature encapsulation process free of organic solvents, a high gel porosity which allows for high diffusion rates of molecules, an ability to control its porosity with coating procedures, dissolution and biodegradation under normal physiological conditions (75). Commercial alginates are extracted primarily from three species of brown algae. These include *Laminaria hyperborea, Ascophyllum nodosum*, and *Macrocystis pyrifera*. Other sources include *Laminaria japonica, Eclonia maxima, Lesonia negrescens* and *Sargassum* species (66). In nature, Alginate is found in the intracellular matrix where it exists as a mixed salt of various cations found in sea water such as Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, and Na^+. The native alginate is mainly present as an insoluble Ca^{2+} crosslinked gel (75).

Alginates are a family of linear unbranched polysaccharides which contain varying amounts of $1,4'$-linked β--mannuronic acid and α--guluronic acid residues. The residues may vary widely in composition and sequence and are arranged in a pattern of blocks along the chain. The composition and extent of the sequences and the molecular weight determine the physical properties of the alginates (75).

Alginate microbeads can be prepared by extruding a solution of sodium alginate containing the desired material to be encapsulated, as droplets, into a divalent crosslinking solution such as Ca^{2+}, Sr^{2+}, or Ba^{2+}. Monovalent cations and Mg^{2+} ions do

not induce gelation. Depending on the divalent solution used such as Ba^{2+} and Sr^{2+} ions alginate gels can be stronger than Ca^{2+} crosslinked gels (66). Other divalent cations such as Pb^{2+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} and Mn^{2+} can also crosslink alginate gels but their use is limited due to their toxicity (75). The gelation and crosslinking of the polymers are mainly achieved by the exchange of sodium ions from the guluronic acids with the divalent cations (Fig 1). The physical properties of crosslinked alginate microspheres are dependent on the composition, sequential structure, and molecular size of the polymers.

Materials encapsulated in alginate hydrogels are released from the capsule by diffusion of the material though the pores of the polymer network and or by degradation of the polymer network (75). Electron microscopic analysis of calcium alginate microbeads has shown that the pore size ranges from 5 nm to 200 nm in diameter. Diffusion of small molecules such as glucose is unaffected by the alginate matrix while diffusion of larger materials from the gels has been shown to be dependent on their molecular weight. It has been clearly demonstrated that by increasing the concentration of alginate in the beads, the rate of diffusion of the materials is decreased from the gel (75). The gelation and crosslinking of the polymers are mainly achieved by the exchange of sodium ions from the guluronic acids with the divalent cations(75). Gels made from high α--guluronic acid alginates have the most open pore structure and exhibit the highest diffusion rates for encapsulants which may be related to the lower shrinkage of this type of gel or to a difference in the diffusion barrier at the surface of the spheres.
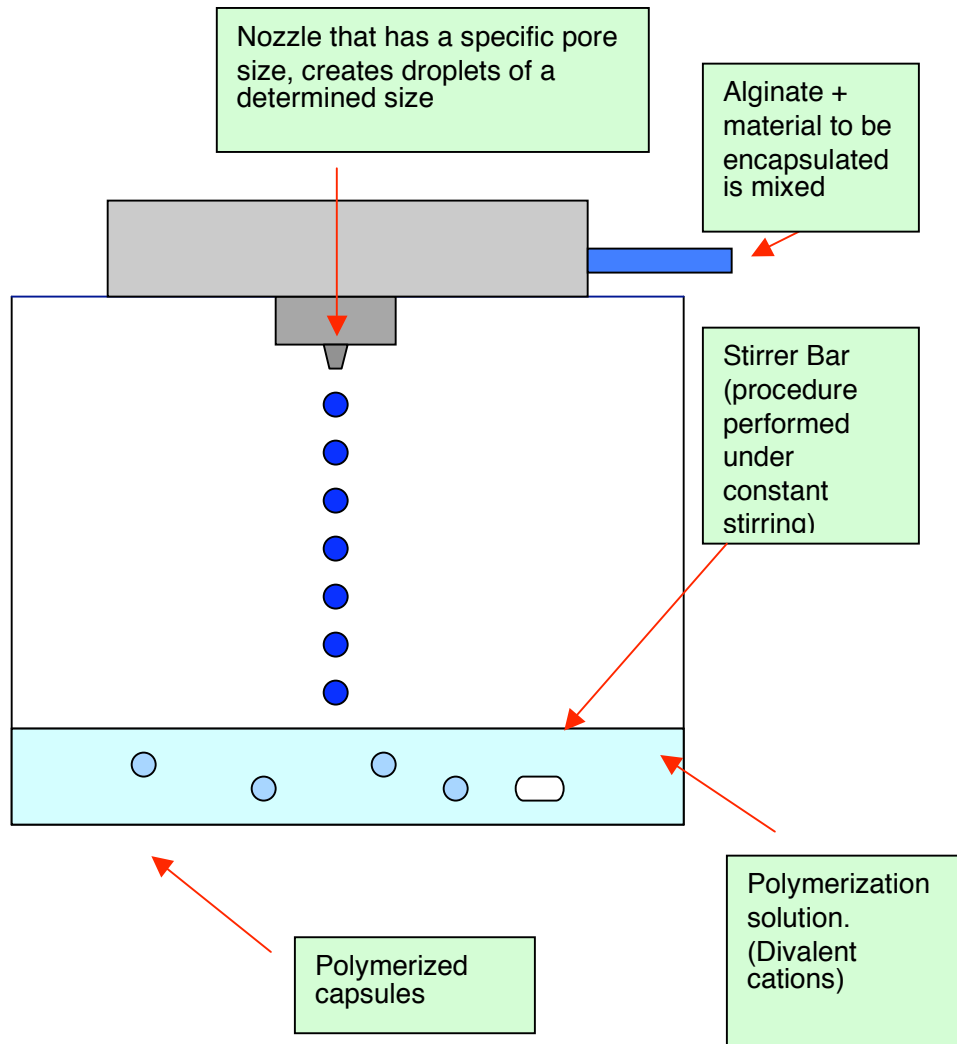
FIG. 1. Schematic representation of microencapsulation using alginate as the polymer. Microbeads can be prepared by extruding a solution of sodium alginate containing the desired material to be encapsulated, as droplets, into a divalent crosslinking solution such as Ca^{2+}

Another aspect that needs further attention for encapsulation is the charge of the material to be entrapped into the alginate bead. It has been demonstrated that the charge of the material can influence the diffusion rate from the alginate matrix. A protein, microorganism or drug with a potentially net positive charge can interact with the negatively charge alginate polymer, inhibiting diffusion from the gel (66). Conversely a protein with a low pI or low molecular weight drugs may be released more rapidly from the matrix than would be expected from free molecular diffusion (75). The porosity of an alginate gel can be reduced significantly by partially drying the microbeads (75). It has been shown that spheres made from a high α--guluronic acid alginate will reswell only slightly upon rehydration resulting in an increased alginate concentration in the bead and a reduced pore size (66). Complete dehydration can result in surface cracking which can facilitate the surface erosion of the beads upon rehydration.

Reduction in pore size of an alginate matrix can also be achieved by exposing the gel to low pH. Release of macromolecules from alginate beads in low pH solutions is also significantly reduced which could be advantageous in the development of an oral delivery system.

Modification of capsule formulation. Several modifications can be performed to change the capsule performance in the host. To test further increases in the efficacy of the capsular delivery, a novel recombinant form of the Vitelline protein B (VpB) derived from the eggshell precursor of the parasite *Fasciola hepatica* was incorporated into the capsules (61). VpB possesses an unusual resistance to enzymatic and chemical breakdown and this is expected to extend the time frame of erosion and release of the capsule content (73).

Vaccine candidates. Recently, a new technology for in vivo screening of virulence genes has been applied in several pathogenic bacteria and fungi. The method is described as signature-tagged mutagenesis (STM). STM allows the screening for loss of virulence in an animal model of infection (32). STM has identified several *Brucella* genes whose activity contributes to intracellular survival (3, 77). Mutants identified by this approach have differences in the degree of attenuation. Mutants exhibiting the greatest level of attenuation are the safest for the host and were the focus of this study. Among these, we have identified the *luxR gene* (BMEI1116), encoding the *luxR*-like quorum sensing-related transcriptional regulator that is required for virB expression (17). *luxR* mutants of *B. melitensis* in initial screenings appeared highly attenuated for virulence in macrophage screening, making this mutant ideal vaccine candidates for microencapsulation.

Using microencapsulation systems we conducted a study to develop safer and more efficacious anti *Brucella* vaccines. Previous research has shown that the only effective vaccines for Brucellosis are live bacteria that stimulate the immune system through infection (11). Non desired effects using this approach include abortion and development of arthropathy associated with *Brucella* antigen-containing immune complexes (11, 13). Previous studies had suggested a relationship between persistence of the vaccine and its efficacy (37). The use of an attenuated *Brucella* mutant which is not capable of producing these side effects has also the problem of low persistence in the host because of the reduced virulence, and such mutants are not capable of surviving in the host for the necessary amount of time to develop immunity. By encapsulating the organism we studied the possibility of providing the means for an

extended exposure time to the host immune system and thereby increase the vaccine efficacy.

MATERIALS AND METHODS

Mice. One hundred and twenty 4 to 6-week old female BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All experimental procedures and animal care were performed in compliance with institutional animal care regulations.

Bacterial strains. Bacteria used in these experiments include *B. melitensis luxR*::Tn5 used as the vaccine candidate and *B. melitensis* virulent strain 16M. For assessment of bacterial distribution throughout the capsule 16M transformed with the pBBR1mcs 6-y plasmid and expressing the reporter protein GFP was kindly provided by Dr Marty Roop. Bacteria were grown on tryptic soy agar (TSA) at 37°C with 5% (v/v) CO_2. For *luxR::Tn5*, the medium was supplemented with kanamycin (100μg/ml). Following 3 days of incubation, bacteria were harvested from the surface of plates into phosphate buffered saline (PBS). The bacteria were pelleted, and washed twice by resuspension in buffer containing MOPS (10mM MOPS, 0.85% NaCl, pH 7.4), and resuspended to a final concentration of $1x10^6$ CFU /ml for encapsulation, or $1x10^5$ CFU/ml nonencapsulated based upon optical density (OD) readings using a Klett meter and a standardized curve. The actual viable counts were confirmed by serial dilution and plating of portions onto TSA plates with or without antibiotic.

Evaluation of *B. melitensis luxR*::Tn5 in J774A.1 macrophages. Murine

macrophage-like J774.A1 (ATCC TIB-67) were used to assess *luxR*::Tn5 mutant

survival compared to the parental *B. melitensis* wild-type 16M. Macrophage survival

assays were performed as previously described with some modifications (58). Briefly,

macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10%

(v/v) fetal bovine serum, 1mM L-glutamine, and 1mM nonessential amino acids.

Monolayers of macrophages containing 2.5 x10^5 cells per well were infected at a

multiplicity of infection of 1:100, at 37°C with either *luxR::Tn5* or wild-type 16M. Thirty

minutes post infection, cells were washed twice with medium without antibiotics and

then incubated with 50µg/ml of gentamicin (Invitrogen,USA) for 30 min to kill any

extracellular bacteria. One and 48 hours post-infection, macrophages were lysed using

0.05% (v/v) Tween-20, and bacteria collected. *Brucella* entry and survival was

determined by performing serial dilutions and plating onto TSA plates with or without

antibiotic. All assays were performed in triplicate and repeated at least three times.

Evaluation of *B. melitensis luxR*::Tn5 attenuation in mice. Female BALB/c

mice were used to evaluate survival of the *luxR*::Tn5 mutant. Briefly, 4-to 6-week-old

mice were intraperitoneally inoculated with a total of 2x10^6 CFU of either mutant or wild

type organism. Groups of 5 mice were euthanized via carbon dioxide asphyxiation at

1,2,3 and 4 weeks post inoculation. At each time point, spleens were collected, weighed

and homogenized in 1ml of peptone saline. Serial dilutions were prepared, and 100µl

aliquots of the different dilutions were plated in duplicate onto TSA or TSA/kan. Levels

of infection were expressed as the mean ± standard error of the mean (SEM) of

individual log CFU/ spleen.

Preparation of *B. melitensis* antigen-loaded microspheres. Alginate beads were prepared as previously described with some modifications(1). Briefly, $6x10^6$ CFU of the live *B. melitensis* mutant *luxR*::Tn5 was re-suspended in 1ml of MOPS buffer (10mM MOPS, 0.85% NaCl, pH 7.4) and mixed with 5ml of alginate solution (1.5% sodium alginate, 10mM MOPS, 0.85% NaCl, pH 7.3). Spheres were obtained by extruding the suspension through a 200 micron nozzle into a 100mM calcium chloride solution and stirred for 15 minutes using the Inotech encapsulator I-50 (Inotech Biosystems International, Rockville USA). After extrusion of the bacteria-alginate mixture into the CaCl$_2$, the capsules were washed twice with MOPS for 5 minutes and further crosslinked with 0.05% poly-L-lysine (PLL, MW 22,000 Sigma, USA) for 10 minutes. Following two successive washes, the beads were stirred in a solution of 0.03% (w/v) alginate for 5 min to apply a final outer shell and washed twice with MOPS before storage at 4°C.

In a second formulation, VpB was added as a component of the alginate core (*luxR*::Tn5/VpBcore) by the addition of 1mg of VpB to the bacteria-alginate suspension described above. Extrusion and capsule formation used the same preparation conditions.

VpB was alternatively added to the shell of the capsule (*luxR*::Tn5/VpBshell) as a cross-linking agent by the addition of VpB in an equimolar ratio of poly-L-lysine/ VpB as the third capsule formulation used.

Bacterial viability. To determine the bacterial content per ml of alginate particles, 1 ml of capsules was removed from the encapsulator prior to permanent crosslinking with poly-L-lysine or VpB with poly-L-lysine. The capsules were allowed to

settle and washed twice with MOPS buffer and particles were dissolved using 1 ml of depolymerization solution (50 mM Na_3-Citrate, 0.45% NaCl 10mM MOPS, pH 7.2) with stirring for 10 min. Bacterial number (CFU/ml) per ml of capsules was determined by plating onto TSA plates.

Bacterial loading and viability was also assessed after the permanent crosslinking with poly-L-Lysine. The capsules were allowed to settle and washed twice with MOPS buffer. Particles were treated for 10 min with depolymerization solution to dissolve the interior of the capsule. Following the depolymerization treatment 0.02% of trypsin (Invitrogen, USA) was added for 2 hours at 37°C to cleave the lysine bonds. Bacterial number (CFU/ml) per ml of capsules was determined by plating onto TSA plates.

Characterization of microspheres. The bacterial distribution throughout the capsule, sphere morphology and size were assessed via fluorescence microscopy (Nikon, TMS). Three size determinations were made for each formulation and the mean value reported.

Determination of kinetics of bacterial clearance from mice vaccinated with encapsulated *luxR::Tn5* To further understand the kinetics of bacterial release from alginate microspheres in vivo, BALB/c mice were vaccinated with either encapsulated or nonencapsulated *luxR::Tn5* at a dose of $1x10^5$ CFU. Mice were euthanized at several time-points, spleens were harvested, weighed and homogenized in 1ml of peptone saline. Bacterial suspension was serially diluted and plated onto TSA/kan plates. Bacterial release from the beads was indirectly measured by determination of CFU encountered in the spleen.

In vitro **Bacterial release from the capsules.**   Bacterial release from the

capsules was assessed using 1ml of capsules resuspended in 10 mls of peptone

saline). The release study was conducted at 4°C for 37 days. At 24hr time intervals,

1ml peptone saline was removed and plated onto TSA to determine CFU/ml. The buffer

was replaced completely after the withdrawal of each aliquot. Nonencapsulated bacteria

was used as a control and subjected to the same procedure. Release profiles were

expressed in terms of daily release, and plotted versus time.

Immunization of mice. Sixty 4-6 week-old female BALB/c mice were randomly

distributed into groups of 10 for intraperitoneally (IP) vaccination. Animals were given a

single dose of microcapsules containing $1x10^5$ CFU of either (a) encapsulated

B.melitensis mutant *luxR*::Tn5 in alginate (*luxR*::Tn5/alginate), (b) encapsulated

luxR::Tn5 in alginate with VpB inside the capsule (*luxR::*Tn5/VpB core), (c)

encapsulated *luxR*::Tn5 in alginate with VpB in the shell of the sphere (*luxR*::Tn5/VpB

shell). Control groups received $1x10^5$ CFU of either (a) nonencapsulated *luxR*::Tn5, (b)

Empty capsules (no bacteria entrapped) or (c) two hundred microliters of MOPS buffer.

Evaluation of *Brucella* specific antibody. To determine anti-*Brucella*-specific

antibody in sera from inoculated mice, 100µl of blood was taken from each mouse after

0, 2, 4, 8,10,12,14,18 and 24 weeks post vaccination and used for IgG determination by

ELISA. Heat killed and sonicated *B. melitensis* whole cell antigen was used to coat 96

well plates (Nunc-Immuno plates) at a concentration of 25µg total protein/well.

Following overnight incubation at 4°C, plates were washed, blocked with 0.5 ml of

blocking buffer [0.25% (w/v) BSA] then incubated with mouse sera diluted 1:100 in the

same blocking buffer for 1 hr at room temperature. Following extensive washing to

remove unbound antibody, rabbit anti-mouse IgG horseradish peroxidase (HRP)

conjugate (KPL, USA) was added at a dilution of 1:1000 and incubation continued for

an additional hour. Following this incubation, the plates were washed again and HRP

substrate was added, and incubated for 18 minutes. The reaction was stopped by

addition of 50 μl of 0.5M NaOH and the absorbance measured at 450 nm (A_{450}). All

assays were performed in triplicate and repeated at least three times.

Determination of splenocyte cytokine production. At selected times post-

vaccination, individual spleens from 5 animals per group were extracted and single cell

suspensions were obtained by grinding the spleen using glass tissue grinders. After

lysis of erythrocytes using red blood cell lysing buffer (8.3 g of NH_4Cl in 0.01 M Tris-HCl,

pH 7.5) the cell suspension was washed in complete RPMI 1640 medium containing

10% (v/v) fetal bovine serum and 50 μg/ml of gentamycin. The cell number was

adjusted and added to 24 well plates at a concentration of to 2.5 x 10^5 cells/well and

cultured following the addition of 25 μg/well of *Brucella melitensis* lysate prepared as

described above. Cells in control wells received medium alone. After 72 h post

incubation at 37°C, 5% (v/v) CO_2, culture supernatants were collected and stored at -

20°C. A panel of Th1 vs T helper 2 (Th2) [(Interleukin 2 (IL-2), Interleukin 4 (IL-4),

Interleukin 12 [IL-12-(p70)] and gamma Interferon (IFNγ)] cytokines was determined in

triplicate using the Bioplex array system (BIO-RAD, USA) according to the

manufacturer's instructions. For each cytokine, eight standards ranging from 2 to 32,000

pg/ml were performed.

Determination of cytokine production *In vivo.* At 0, 10, 30 weeks post-

vaccination and 2 days post-challenge, the mice animals were bleed and serum was

obtained from each individual mouse for cytokine determination. Quantification of IFNγ and IL-12p70 was determined by ELISA (Ebioscience, USA) following manufacturer's instructions. For each cytokine, standards ranging from 0.2 to 1000 pg/ml were performed. Background levels obtained for t=0 were subtracted from the samples and the differences between the encapsulated and nonencapsulated formulations at each timepoint were determined by ANOVA. Results are expressed as the mean pg/ml of each cytokine.

Efficacy of vaccination. At selected times post vaccination, mice (n=5 per group) were challenged IP using $1x10^5$ CFU/mouse of *B.melitensis* wild-type 16M. One week post challenge, mice were euthanized by CO_2 asphyxiation and spleens were removed, weighed and homogenized in 1ml of peptone saline. Serial dilutions were prepared and 100 μl portions were plated onto TSA plates. In some instances, 200-1000μl of spleen homogenate was plated to determine organism recovery. To differentiate between vaccine candidate and the challenge strain, each dilution was also plated on TSA with kanamycin to identify any residual kanamycin resistant vaccine strain present. Levels of infection were expressed as the mean ± SEM of individual log_{10} CFU/spleen. The efficacy of the vaccine compared to naïve animals was determined by subtracting the mean CFU/spleen recovered from mice vaccinated with the nonencapsulated or encapsulated (*luxR::Tn5*/alginate, *luxR::Tn5*/VpBcore or *luxR::Tn5*/VpB shell) vaccine from the mean CFU/spleen recovered from naïve non-vaccinated but challenged mice. The efficacy of the encapsulated vaccine compared to the nonencapsulated vaccine was determined by subtracting the mean CFU/spleen

recovered from mice vaccinated with the capsules from the mean CFU/spleen recovered from mice immunized with the nonencapsulated vaccine.

Statistical Procedures. Macrophage infection and survival was expressed as the mean log CFU ± SD for each group. Intensity of infection (bacterial clearance and efficacy of vaccine) at each time point was expressed as mean log CFU±SEM for each group. Cytokine production *in vitro* was expressed as mean cytokine concentration ± SD for each group of five mice. The significance of differences between groups was determined by analysis of variance (ANOVA). *P* values <0.05 were considered statistically significant.

RESULTS

Verification of attenuation of vaccine candidate *in vivo* and *in vitro*.   To assess the *luxR*::Tn5 attenuation, J774A.1 macrophages were infected with the mutant and compared to the wild-type *B. melitensis* 16M. Using an MOI of 100, the ability of the bacteria to enter and replicate within macrophage was evaluated. Shortly after infection (t=0 hours) there was no difference ($p>$ 0.05) between the wild type and mutant in the number of bacteria (3-4 per cell) infecting the macrophage (Fig. 2). In contrast, by 48 h post infection there was a 2.5 log difference ($p<$ 0.005) in the number of organisms infecting the macrophage (100 per cell for 16M and less than 1 per cell for the *luxR*::Tn5). These results demonstrate that the *luxR::Tn5* mutant has a severely limited capacity to replicate within J774A.1 macrophages.

To evaluate survival in vivo, BALB/c mice were inoculated intraperitoneally (IP) with 2×10^6 CFU of *B. melitensis luxR::Tn5* or the 16M parental wild-type strain.

Compared to the wild-type strain 16M, the *luxR::Tn5* mutant was significantly reduced at

all time points between 1.5 and 3.6 logs. There appears to be a brief period between

week one ($p < 0.001$) and two ($p > 0.05$) during which the organism replicates and the

numbers of *B. melitensis luxR*::Tn5 in the spleen increase, but by three weeks post-

inoculation the number of organisms in the spleen exhibits a drastic decline ($p < 0.001$)

and drops below the level of detection by four weeks post-infection ($p < 0.001$) (Fig. 3).

In addition, disruption of *luxR* significantly reduced ($p < 0.001$) splenomegaly at all time

points consistent with the reduction in colonization of the spleen (Fig. 4).

Evaluation of capsule preparation and formulation using *B. melitensis.*

Three different types of capsules were prepared using the same basic alginate

formulation. Variations on the formulations include the addition of VpB to the core or to

the shell of the capsule to slow the degradation of the capsules and the release of the

organism. In general, the results revealed that despite the differences in formulations,

all of the capsules prepared appeared spherical and uniform with a mean diameter of

300μm. To characterize the bacterial distribution throughout the capsule, *B. melitensis*

16M transformed with a GFP expressing plasmid was encapsulated. The results shown

in figure 5 reveal uniform bacterial distribution throughout the capsule. Furthermore,

bacterial viability following encapsulation exceeded 95% as demonstrated by recovery

of the organism following dissolution of the capsules (Fig. 6).
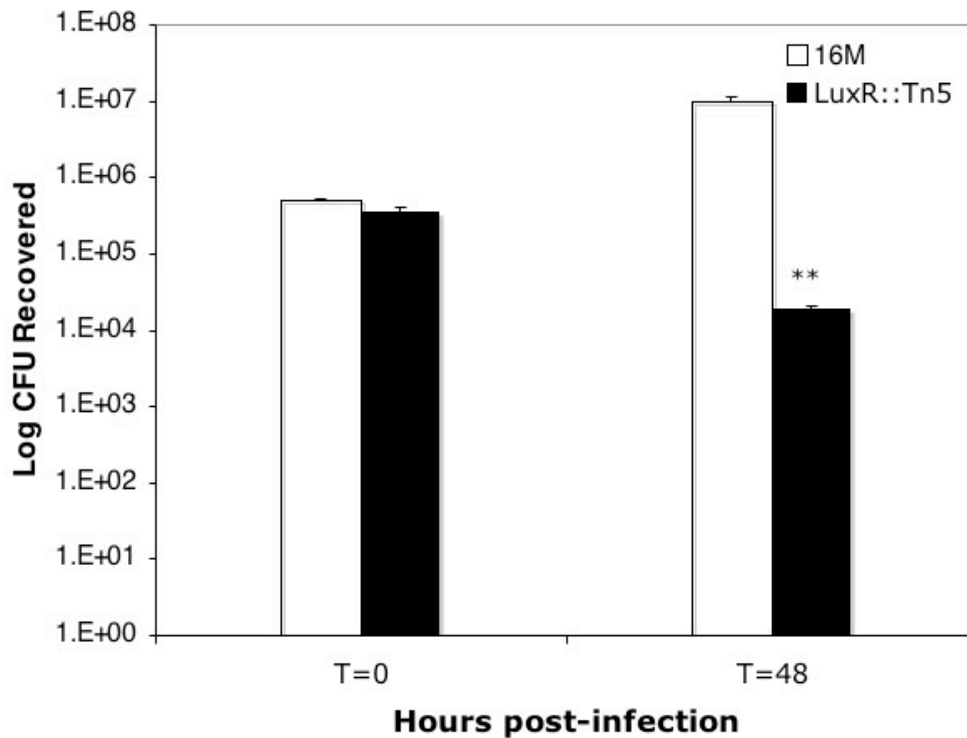
FIG. 2. Survival of the *B. melitensis luxR*::Tn5 mutant in J774A.1 macrophages.

Wild-type strain 16M and the *B. melitensis* mutant *luxR*::Tn5 were used to infect

J774A.1 macrophages at a multiplicity of infection of 1:100. After 30 min of incubation

followed by 1 hr of treatment with gentamycin, infected macrophages were further

incubated for 0 or 48h. Treated cells were lysed, serially diluted and plated on TSA or

TSA/kanamycin plates for CFU determination. Values are the mean of three

independent experiments ± standard deviation. Differences in macrophage colonization

by wild-type 16M and *luxR::Tn5* are indicated (**P<0.005)
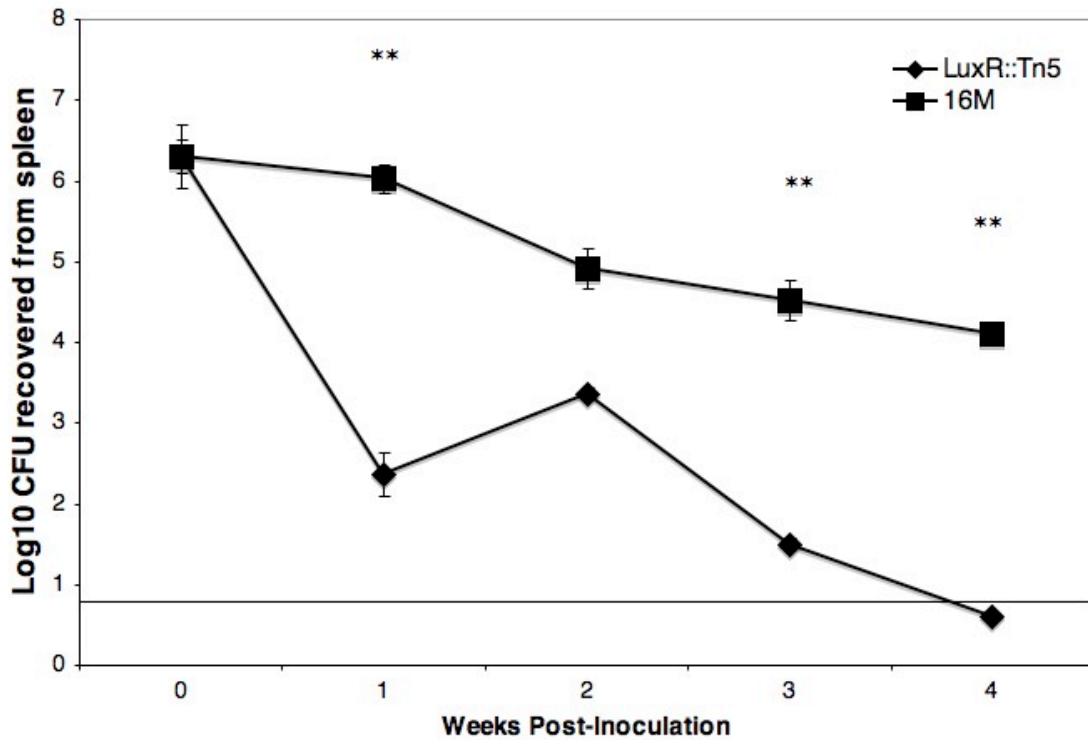
FIG. 3. Kinetics of clearance of *B. melitensis luxR::Tn5* after infection. BALB/c

mice (n=5/ time point) were infected with 2 x10^6 CFU/mouse of wild-type 16M or

luxR::Tn5. At 1,2,3 and 4 weeks post-infection, mice were euthanized and the spleens

were assessed for bacterial colonization. Values are the means of individual mice ±

standard error of the mean. Differences in colonization were determined by ANOVA

comparing the wild-type to the mutant (**P<0.005). The solid line represents the limit of

detection which is ≥5 CFU.
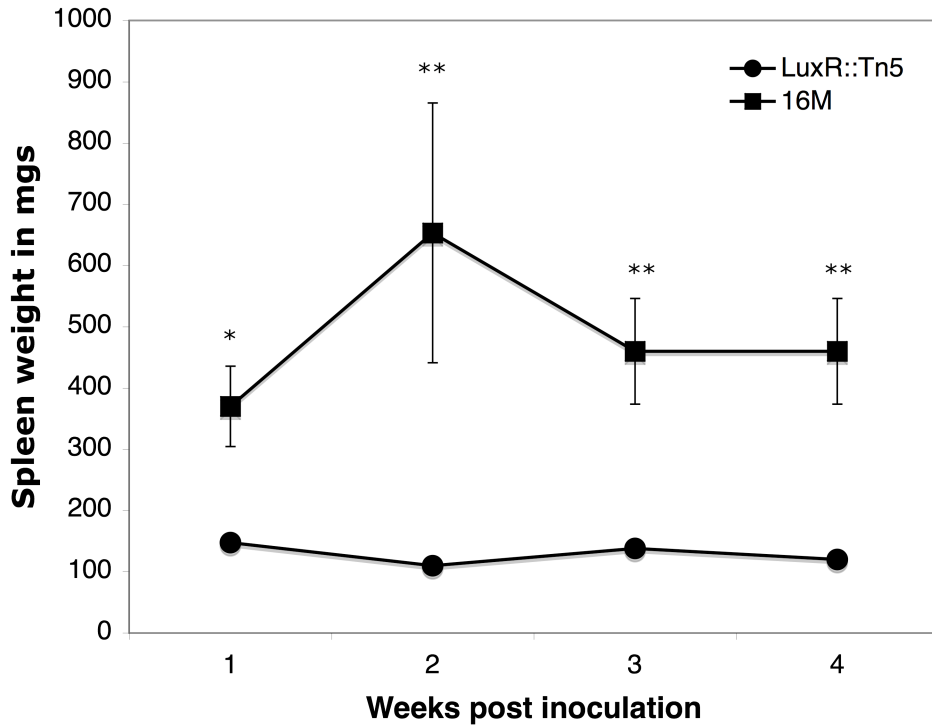
FIG. 4. Spleen weights after *B. melitensis luxR::Tn5* infection. BALB/c mice were infected with 2 x10^{6} CFU/mouse of wild-type 16M or *luxR::Tn5*. At 1,2,3 and 4 weeks post-infection, mice were euthanized and the spleens were weighed to determine the degree of inflammation conferred by the mutant. Results are expressed as the mean ± standard error of the mean. Differences in weight were determined by ANOVA comparing the wild-type to the mutant (*P<0.05) (**P<0.005).
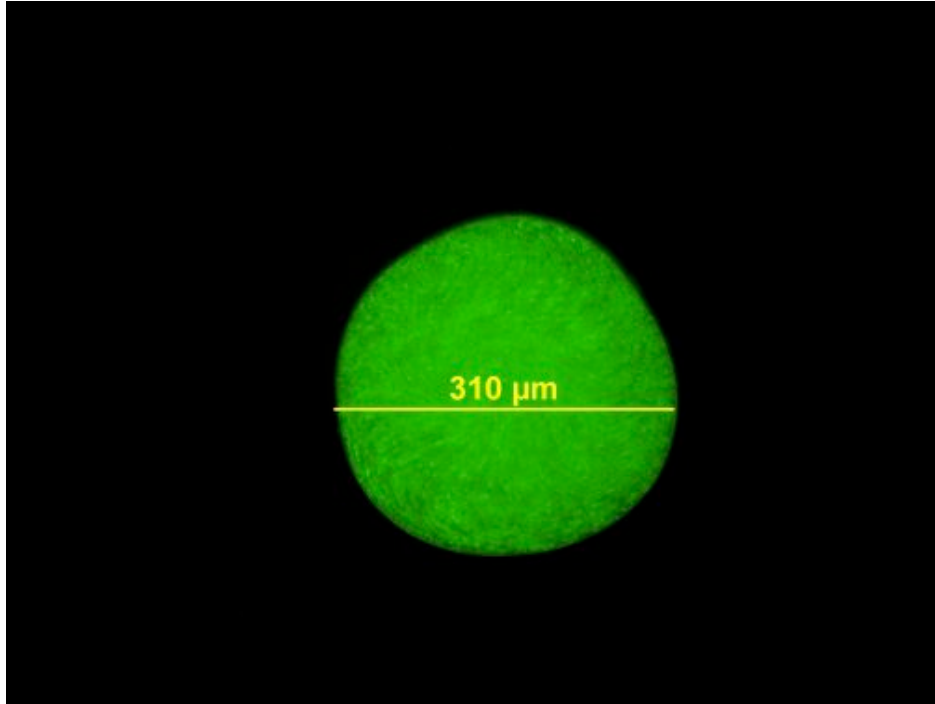
FIG. 5. Fluorescent microscopy image of alginate microspheres loaded with wild-type 16M. *B. melitensis* wild-type 16M was fused with a GFP plasmid and further encapsulated into alginate microspheres. A uniform bacterial distribution was observed throughout the capsule. Microsphere size was measured indicating an average mean size of 300μm.
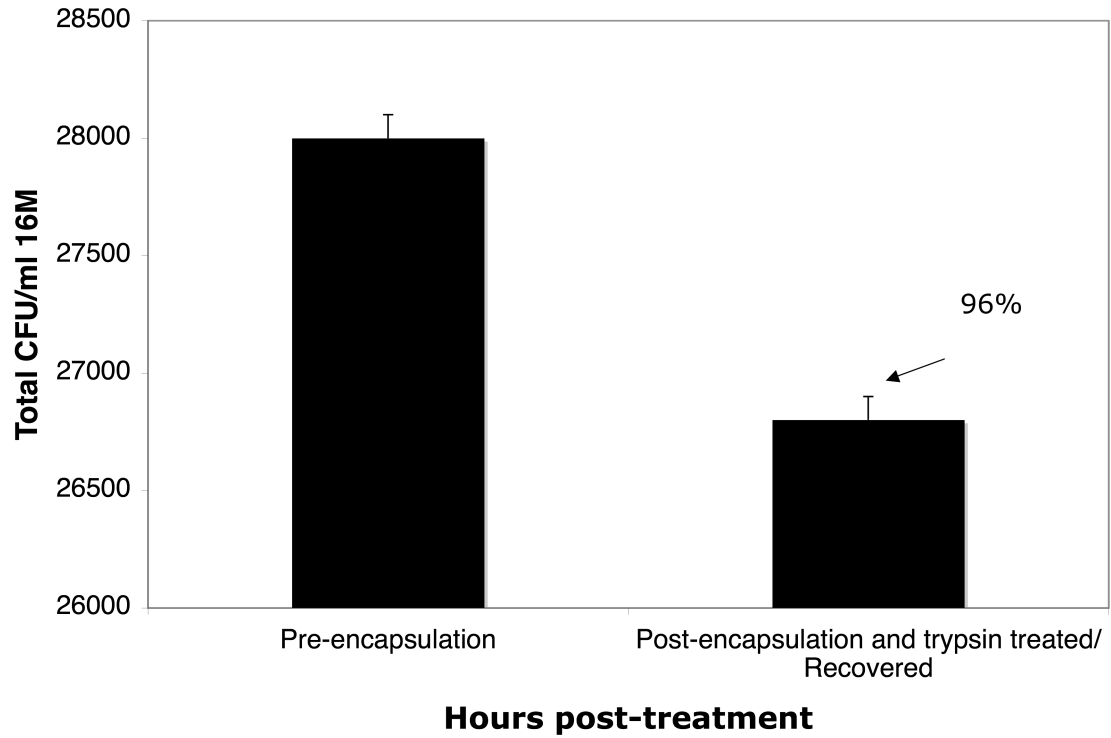
FIG. 6. Total CFU recovered after the encapsulation procedure. Wild-type 16M was encapsulated into alginate microspheres at a concentration of $2x10^4$ CFU/ml. Immediately after encapsulation 1ml of capsules was treated with a depolymerization solution followed by an enzymatic treatment that dissolved the beads. Total CFU recovery was determined by plating the recovered bacterial suspension onto TSA plates.

As an indirect method of measurement of the release of the capsule content in vivo, mice were inoculated with either encapsulated or nonencapsulated vaccine at the same doses and euthanized at selected time-points described in materials and methods. Spleens were homogenized and plated to determine the number of CFU. Surprisingly, the mutant *luxR::Tn5* did not persist in the tissue for a prolonged period of time as predicted, by four weeks the mutant was cleared as observed with the nonencapsulated vaccine (Fig. 7). Results in vitro demonstrated that capsules were completely dissolved and bacteria released from the capsules by 36 days of treatment (maintained in peptone saline) (Fig. 8).

In order to determine the effect of encapsulation on immunization, the level of protection provided by equal numbers of encapsulated and nonencapsulated *B. melitensis luxR::Tn5* was evaluated against wild-type challenge at 31 weeks post vaccination. At this time point, nonspecific activation due to innate immune response to persistent infection or delayed release of the encapsulated organism had passed based upon the decrease observed in humoral immunity and the absence of recovery of the vaccine strain (Fig 9). At 32 weeks post-vaccination (one week after challenge), all mice including the nonencapsulated group had a statistically significant decrease in bacterial load in the spleen relative to naïve mice with a 3.14 log reduction ($p < 0.05$) for nonencapsulated mutant, 4.14 log reduction ($p < 0.005$) for BM *luxR::*Tn5 in alginate, a 4.45 log reduction ($p < 0.001$) for BM *luxR::*Tn5 in VpB core, and a 4.98 log reduction ($p < 0.001$) for BM *luxR::*Tn5 in VpB shell compared to MOPS vaccinated mice. All groups of mice receiving encapsulated vaccination exhibited a statistically significant degree of protection against infection compared to the non-encapsulated mutant
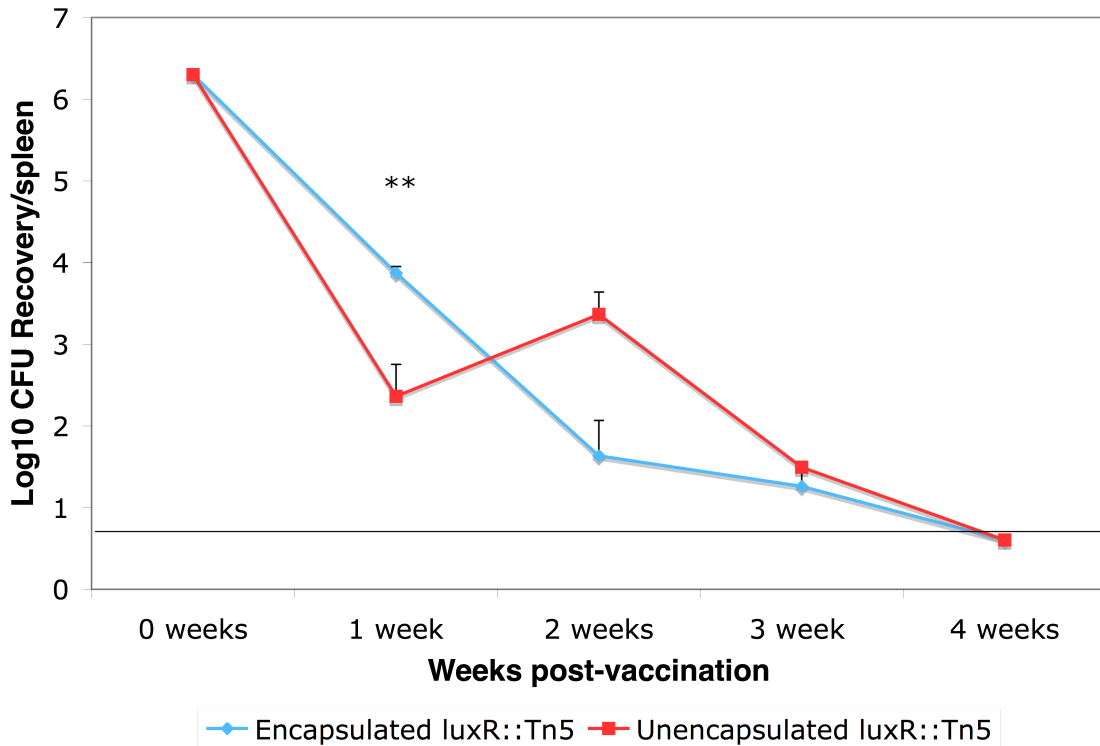
FIG. 7. Kinetics of clearance of encapsulated *B. melitensis luxR::Tn5* after infection. BALB/c mice (n=5/ time point) were infected with 2 x10^6 CFU/mouse of encapsulated or nonencapsulated *luxR::Tn5*. At 1,2,3 and 4 weeks post-infection, mice were euthanized and the spleens were assessed for bacterial colonization. Values are the means of individual mice ± standard error of the mean. Differences in colonization were determined by ANOVA comparing the nonencapsulated to the encapsulated vaccine (**$P<0.005$). The solid line represents the limit of detection which is ≥5 CFU.
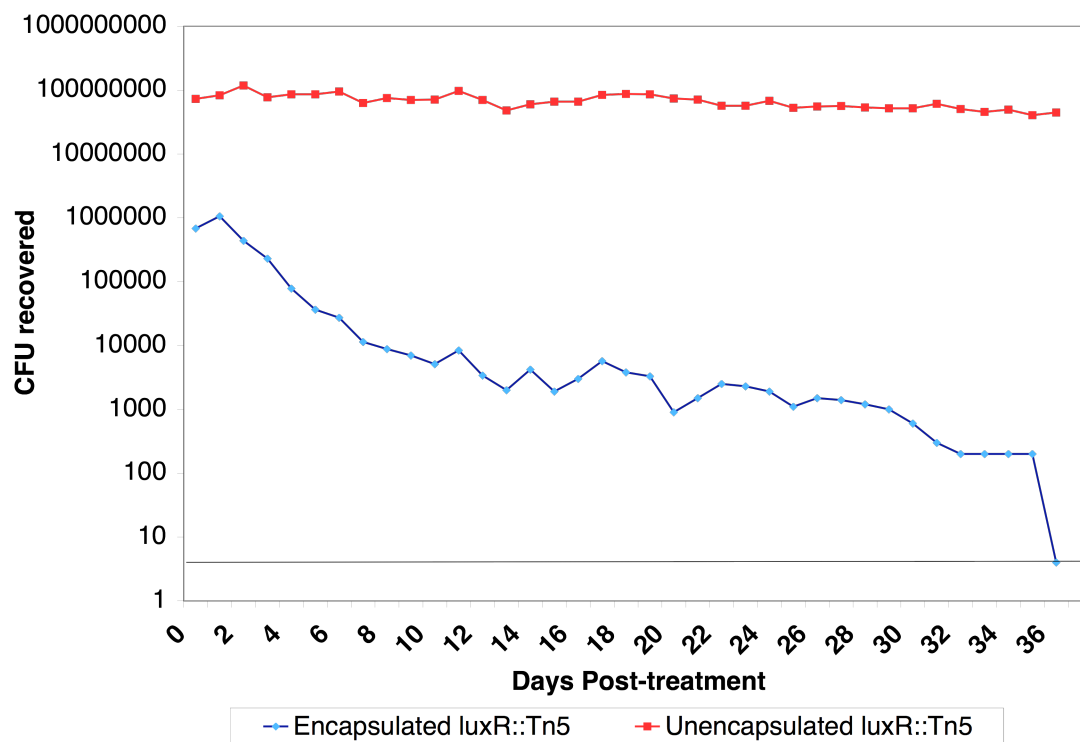
FIG. 8. Kinetics of bacterial release *in vitro* from alginate microcapsules. Alginate

microbeads were prepared containing 1×10^{9} CFU/ml of *luxR::Tn5* and 1ml of capsules

was resuspended in peptone saline and stored at 4°C for the duration of the

experiment. Every day, buffer was completely removed and new peptone saline was

added. 100μl aliquots of the buffer was serially diluted and plated onto TSA/kan plates

to determine the number of bacteria released into the buffer. The solid line represents

the limit of detection which is ≥5 CFU.
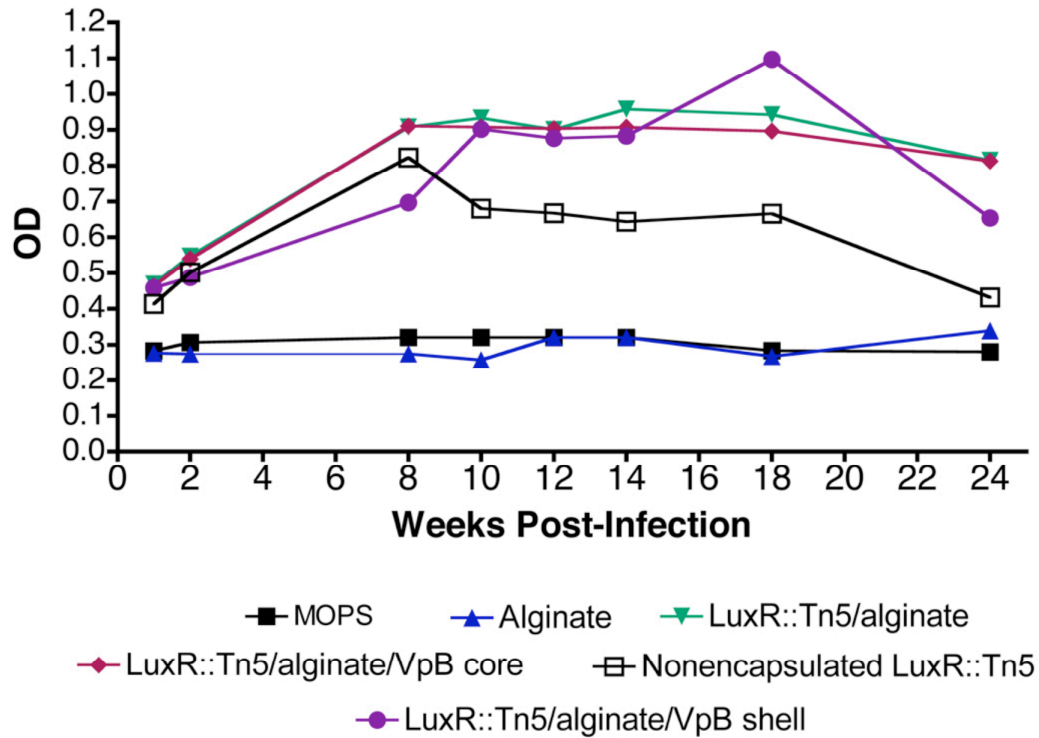
FIG. 9. IgG anti-*Brucella* antibodies in serum from mice immunized with

***luxR*::Tn5.** BALB/c mice (n=10) were inoculated IP with 1x 10^5 CFU of either

nonencapsulated *B. melitensis luxR::Tn5*, encapsulated *B. melitensis*

(*luxR::Tn5*/alginate), encapsulated *B. melitensis luxR::Tn5* with VpB in the core

(*luxR::Tn5*/alginate/VpBcore) or encapsulated *B. melitensis luxR::Tn5* with VpB in the

shell (*luxR::Tn5*/alginate/VpBshell). Control groups received empty capsules or MOPS.

At 0,2,4,8,10,12,14,18 and 24 weeks post vaccination, serum samples were collected

and used for IgG determination by ELISA. Results are shown as the mean ± standard

deviations of absorbance at A_{450}.

(p<0.001). Among the three encapsulated vaccines, the BM *luxR::Tn5* in VpB shell

provided the best level of protection, increasing the vaccine efficacy by 1.84 logs

(p<0.05) compared to the nonencapsulated vaccine. Moreover, the challenge organism

was undetectable from 60% of the mice, which appeared to exhibit sterile immunity (Fig

10).

Encapsulated BM *luxR*::Tn5 elicits vigorous humoral and cellular immune

responses. Serum collected at 0, 1, 2, 8,10,12,14,18 and 24 weeks post vaccination

was assayed for the presence of *Brucella*-specific antibodies by ELISA. Immunization

with *BM luxR*::Tn5 elicited a IgG response that was clearly detectable after 2 weeks

post-vaccination for either encapsulated or nonencapsulated vaccines (Fig. 9).

However, the three capsule formulations elicited similar IgG responses, but over a

prolonged period compared with the nonencapsulated vaccine. In mice vaccinated with

the nonencapsulated BM *luxR*::Tn5 vaccine, IgG levels peaked at 8 weeks post-

immunization, while BM *luxR*::Tn5 in VpB shell IgG levels increased steadily and

reached a maximum after 18 weeks post immunization (p>0.05). In this case, an

induction of higher and sustained antibody levels coincides with increased protection for

both BM *luxR*::Tn5 in alginate and *luxR*::Tn5 in VpB core formulations.

To obtain additional information on the type of immune response induced by the

different BM *luxR*:Tn5 capsule formulations at the time of bacterial challenge, the

cellular immune response was characterized based on cytokine secretion in culture

supernatants of spleen cells from immunized mice. All three capsule formulations

stimulated elevated levels of IFNγ (p< 0.005), IL-12p/70 (p< 0.05), GM-CSF (p< 0.001)

(Fig. 11A-C) compared to the nonencapsulated group and only capsules with VpB in the
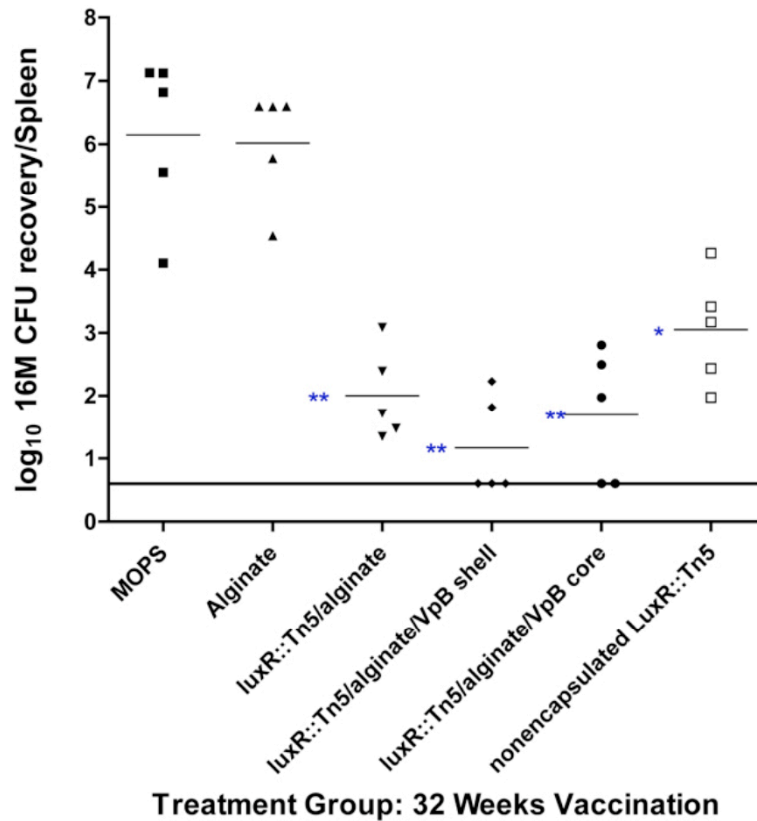
FIG. 10. Immunization efficacy of *B. melitensis luxR::Tn5* vaccine formulations. BALB/c mice were immunized i.p. with 1x 10^5 of either nonencapsulated *B. melitensis luxR::Tn5*, encapsulated *B. melitensis luxR::Tn5*/alginate, encapsulated *B. melitensis luxR::Tn5* with VpB in the core (*luxR::Tn5*/alginate/VpBcore) or encapsulated *B. melitensis luxR::Tn5* with VpB in the shell (*luxR::Tn5*/alginate/VpBshell). Control groups received empty capsules or MOPS. After 31 weeks, mice were challenged i.p. with 1 x 10^5 CFU wild-type 16M. One week post challenge, mice were euthanized, spleens harvested and the bacterial load was determined. Values are reported as the $\log_{10}$ recovery of 16M from spleens, with individual mice in each treatment group represented. The solid line represents the limit of detection which is ≥5 CFU. Differences in colonization between the nonencapsulated and encapsulated groups was determined by ANOVA (*P<0.05) (**P<0.005).

formulation stimulated higher IL-2 ($p<$ 0.005) production compared to the

nonencapsulated vaccine (Fig. 11D). All cytokine levels were statistically significant

compared to naïve non-vaccinated animals ($p<$ 0.005). In general, induction of higher

Th1 cytokine levels coincided with the elevated protection exhibited in the encapsulated

vaccine groups. Spleen cells from all immunized mice failed to produce IL-4 when

stimulated with antigen (Fig. 11E). No statistical significance was observed either

between the vaccinated and non-vaccinated animals, or between encapsulated and

nonencapsulated formulations with this cytokine. Empty capsules failed to elicit

production of any of the cytokines measured.

To correlate cytokine expression in vivo, animals were bleed at 0, 10, 30 weeks

and 2 days post-vaccination to determine IFNγ and IL12 concentrations in serum.

Interestingly, results correlated with the observations obtained in vitro. Animals

receiving the encapsulated vaccine were able to elicit higher levels of IFNγ at all

timepoints in mice vaccinated with either alginate/*luxR::Tn5* or alginate/*luxR::Tn5*/VpB

shell ($p<$ 0.05) (Fig. 12A). IL-12 was only significant at 10 and 30 weeks post-

vaccination in animals vaccinated with alginate/*luxR*::Tn5 ($P<$ 0.005, $P<$ 0.05,

respectively) and at 10 weeks in animals that received the other two capsule

formulations ($P <$ 0.05) (Fig 12B).

A.

Treatment Groups: at 31 weeks
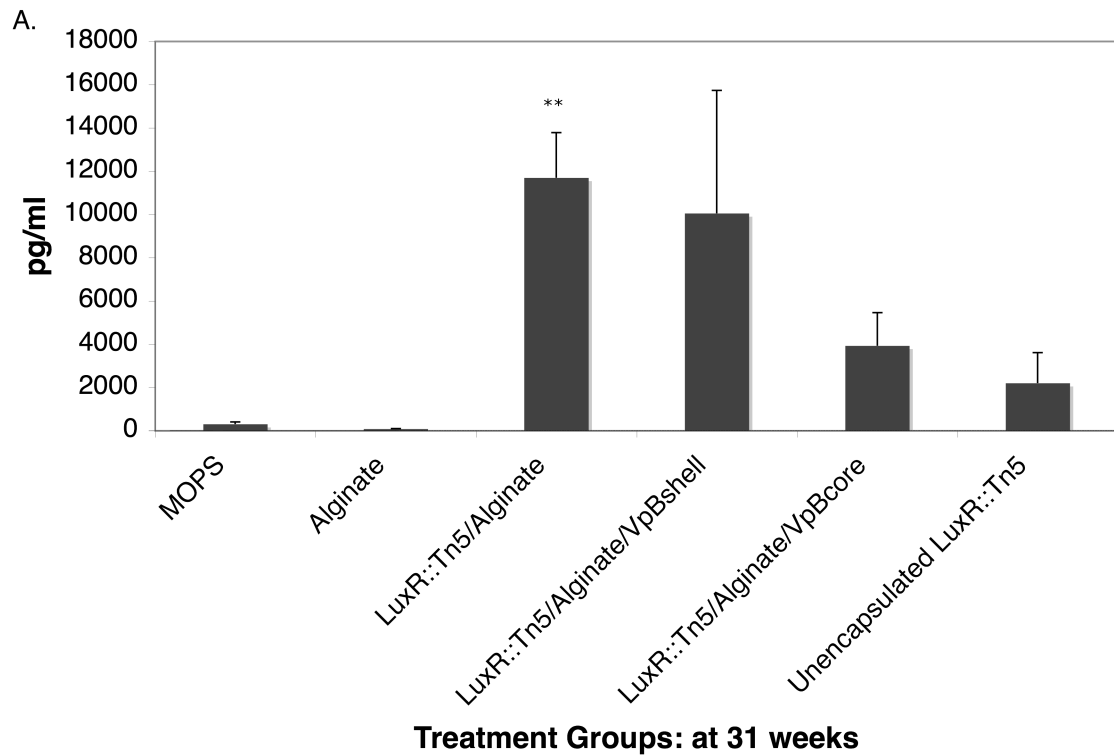
FIG. 11. Production of cytokines in stimulated spleen cells from *luxR::Tn5* vaccinated BALB/c mice.

Mice were vaccinated with 1 x 10^5 CFU of either nonencapsulated *B. melitensis luxR::Tn5*, encapsulated

B.*melitensis luxR::Tn5*/alginate, encapsulated *B. melitensis luxR::Tn5* with VpB in the core

(*luxR::Tn5*/alginate/VpBcore) or encapsulated *B. melitensis luxR::Tn5* with VpB in the shell

(*luxR::Tn5*/alginate/VpBshell). Control groups received empty capsules or MOPS. Thirty one weeks post

vaccination, mice were euthanized, splenocytes harvested and stimulated with heat inactivated *B.*

melitensis 16M or medium alone as a control. IFNγ (A), IL-12p70 (B), GM-CSF (C), IL-2 (D) and IL-4 (E)

production (pg/ml) were detected after 72 h of stimulation using a multiplex suspension array system

(Bioplex). Cytokine production was expressed as mean cytokine concentration ± SD for each group of five

mice. The significance of differences between nonencapsulated and encapsulated formulations was

determined by ANOVA. (*P<0.05), (**P<0.005).

B.

Treatment Groups: at 31 weeks

C.

Treatment Groups: at 31 weeks

FIG. 11 (continued)

Treatment Groups: at 31 weeks
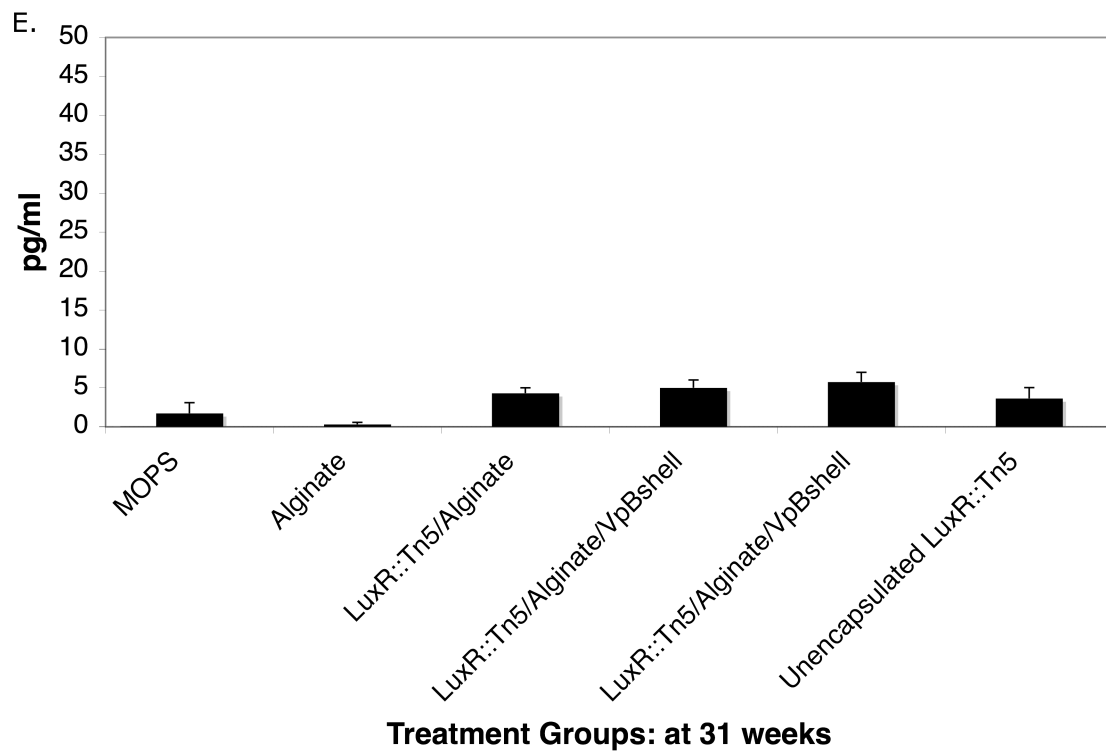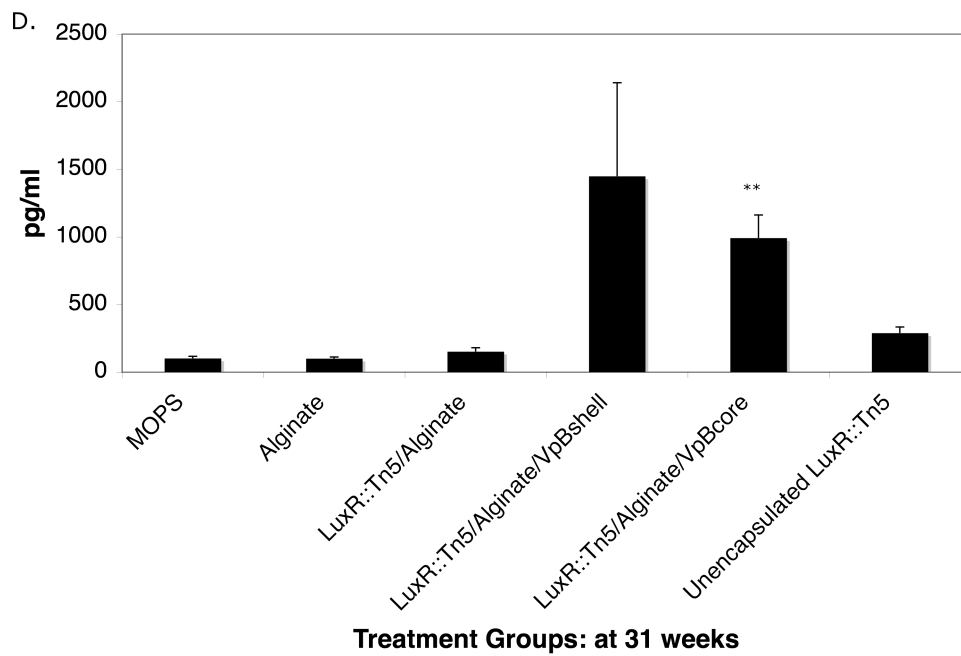
FIG 11. (Continued)
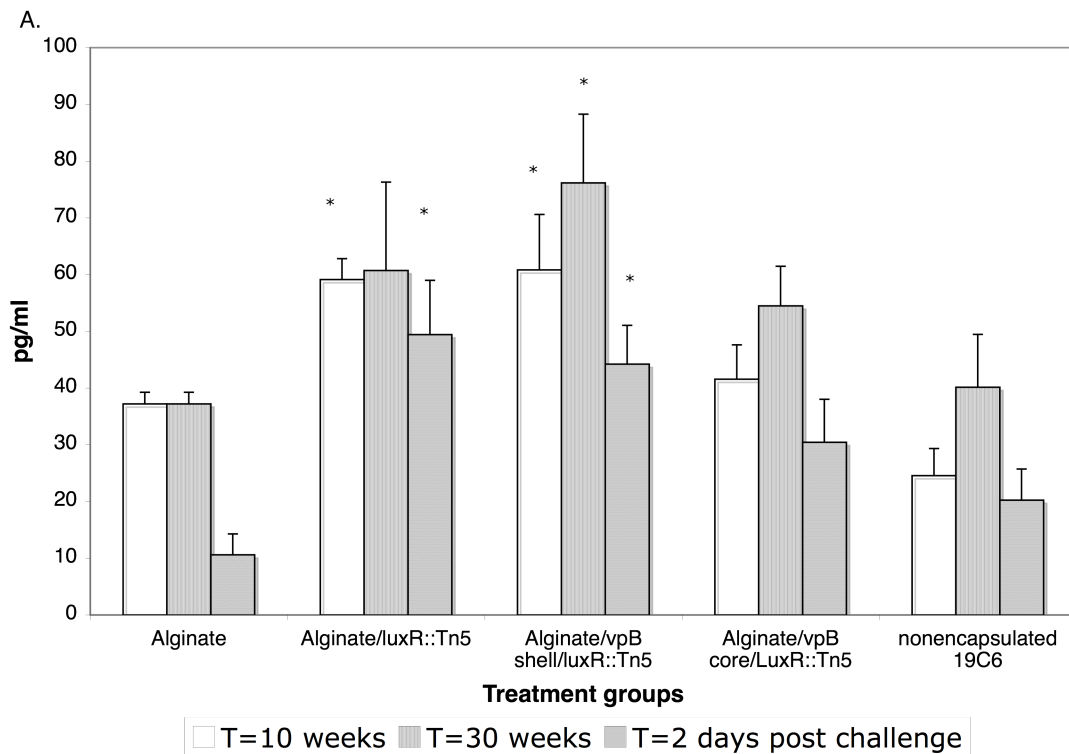
FIG. 12. Cytokine production in BALB/c mice immunized with *luxR::*Tn5. Mice were vaccinated with 1 x 10^5 CFU of either nonencapsulated *B. melitensis luxR::Tn5*, encapsulated B.*melitensis luxR::Tn5*/alginate, encapsulated *B. melitensis luxR::Tn5* with VpB in the core (*luxR::Tn5*/alginate/VpBcore) or encapsulated *B. melitensis luxR::Tn5* with VpB in the shell (*luxR::Tn5*/alginate/VpBshell). Control groups received empty capsules or MOPS. Mice were bled at 0,10,30 weeks post-vaccination and 2 days post-challenge and IFNγ (A) or IL-12 (B) determination from the serum was performed by ELISA. Results are represented as the mean cytokine concentration ± SEM for each group of ten mice. The significance of differences between nonencapsulated and encapsulated formulations at each timepoint was determined by ANOVA. (*P<0.05), (**P<0.005).
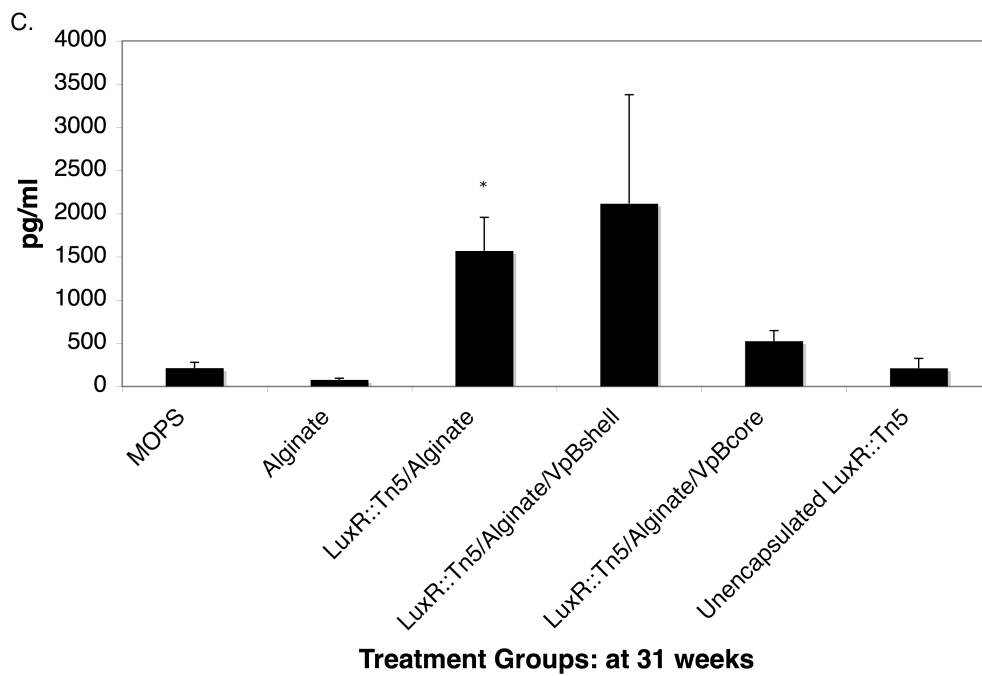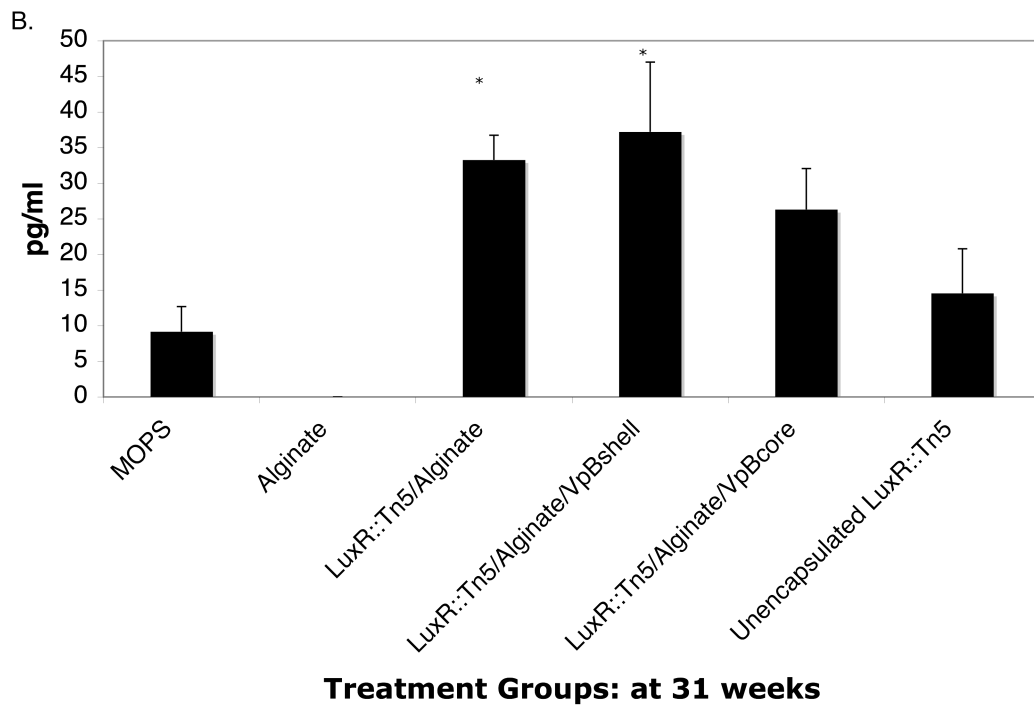
B.
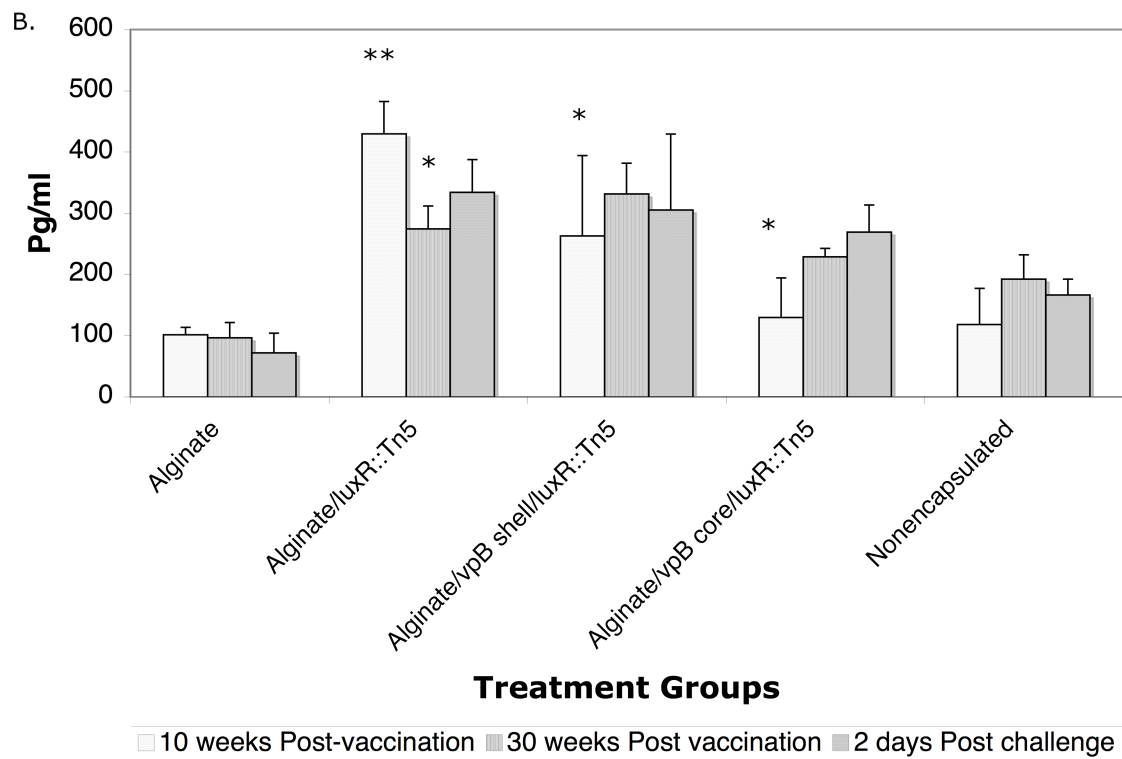
FIG. 12 (Continued)

DISCUSSION

Efficacious immunization systems to protect against brucellosis are needed to overcome the disadvantages of the currently available vaccines with regard to efficacy in a wide variety of hosts. Second only to immune protection, safety is of critical importance for ideal live *Brucella* vaccines. The absence of virulence in the target host and in humans will help to avoid virulence related sequellae. Increasing knowledge of virulence factors and *Brucella* pathogenesis mechanisms provide us with the tools to construct new attenuated strains. Previous studies in our lab have identified genes related to survival and virulence using transposon-based mutagenesis strategies (77). Among the genes identified, *luxR*::Tn5 mutants were evaluated for survival in macrophages and the mouse model to confirm attenuation and immune potential. As shown in this study, BM *luxR*::Tn5 was defective for survival within macrophages, and rapidly cleared from the spleen in BALB/c mice. In mice, the mutant was significantly reduced in number by 1 week post infection and was completely cleared by 4 weeks; in contrast, 16M clearance from the spleen takes more than 16 weeks (37). *luxR*::Tn5 safety was further revealed by the absence of splenomegaly in inoculated mice. Even at 2 weeks, when the bacterial load in the spleen was high, the mean spleen weights in BM *luxR*::Tn5 inoculated mice was 110 mg vs. 653 mg for 16M (Fig. 4). In contrast, currently available vaccine strains S19 and Rev-1 induce splenomegaly in mice (67). Differences in survival and inflammatory response exhibited by *luxR*::Tn5 provide an opportunity to evaluate its use as an improved vaccine candidate.

Recent reports demonstrate that persistence of the vaccine strain in the host is needed for the development of a suitable and long term immunity (37). Consistent with this, currently used protective strains exhibit only modest attenuation, meaning they survive longer in the host and produce unwanted side effects (67). To overcome this problem, we developed a controlled release strategy using highly attenuated mutants to increase the exposure of host to the organism. Alginate-VpB microspheres permit a constant or pulsed release of encapsulated organism, simulating continuous exposure, that potentiates vaccine efficacy. Release from alginate matrices generally occurs by diffusion through pores of the capsule or by erosion of the polymer network. In the case of standard bacterial entrapment methods used for Mycobacterium *bovis* BCG or bifidobacteria, cells escape through erosion rather than diffusion due to bacterial size and surface charge (2, 14). VpB was added to the formulation, in an attempt to extend the time frame over which the capsule dissolution occurs, due to the characteristic resistance of the protein to enzymatic breakdown.

During microsphere formulation microorganisms were exposed to relatively mild conditions that did not cause significant bacterial death; this approach permits the development of live encapsulated vaccines in contrast to previously published encapsulation procedures in which the viability of the bacteria is compromised due to the extreme conditions including ultrasound homogenization, direct exposure to organic solvents and shear stress (45). The use of GFP plasmid transformed organism helped to confirm bacterial viability and distribution inside the capsules and demonstrated uniform spherical capsule formation. This morphology was maintained even with the

inclusion of VpB, which enhances capsule stability via crosslinking due to its high lysine and arginine content when substituted for poly-L-lysine (61, 73).

Protection studies against wild-type challenge with both nonencapsulated and encapsulated organisms protected mice significantly, but the vaccine efficacy was increased substantially when the mutant was delivered in an encapsulated form. Addition of VpB in the core or shell of the capsule further enhanced protection , corroborating the observation of protection as a function of vaccine persistence. Encapsulation and incorporation of VpB in vaccine formulations is an interesting approach to improve immunization efficacy not only for *Brucella*, but potentially for other organisms as well.

The degree of protection conferred by the different vaccine formulations was compared to the humoral profiles. Immunization with *B. melitensis luxR::Tn5* in microcapsules induced prolonged elevation of IgG levels when compared with the nonencapsulated vaccine. Persistent levels of IgG even at 18 weeks post-vaccination might reflect the extended exposure time of the host to the organism. In the case of *luxR::Tn5*/alginate/VpB core we were able to correlate elevated and persistent levels at late time points post-vaccination with a sterile immunity provided by the vaccine in 3 of 5 animals.

Published reports have shown that spleen cells from mice previously inoculated with live vaccines induced a Th1 type response to *Brucella* antigens in vitro, while splenocytes from mice immunized with killed vaccines induced Th2 responses (82). Moreover, it has been clearly established that IFNγ is an important component of the Th1 response, activating macrophages for increased microbial killing. Studies

performed with this mutant indicate that both *luxR*::Tn5 and encapsulated *luxR*::Tn5 induced higher IFNγ levels relative to naïve mice, but the levels of IFNγ induced by the nonencapsulated vaccine were lower compared to the three capsule formulations. Higher cytokine concentrations coincided with the increased levels of protection generated by the capsule formulations.

Although IFNγ is critical for activating macrophages, other cytokines also play an important role in controlling *Brucella*. IL-12, a Th-1 type cytokine mainly produced by macrophages, promotes IFNγ production by T cells that induce murine B cells to switch to IgG2a isotype (29). It also induces differentiation of CD4 Th1 cells. This data was also correlated with the results obtained from the serum at the different timepoints post-immunization. In our vaccination trials, IL-12 was also produced in significant levels compared to naïve mice. Two of the three groups of animals that received microcapsules (*luxR::Tn5*/alginate and *luxR*::Tn5/alginate/VpBcore) produced higher statistically significant levels compared to the nonencapsulated vaccine suggesting that IL-12 has also a protective role. An alternative explanation could be that the higher IgG levels in serum produced by the capsule formulations might be caused by the elevated IL-12 concentrations observed here. Further analysis to determine which type of IgG is being produced should be assessed in order to correlate IgG2a antibody production with an IL-12 response.

Finally, IL-2 was also analyzed. The only formulation that had a significant difference compared to the nonencapsulated vaccine was the *luxR::Tn5*/vpBcore. It has been previously demonstrated that IL-2 not only promotes secretion of Natural Killer cells (NK) derived cytokines such as GM-CSF and INFγ but is also diminished in

patients with chronic brucellosis (65). In this case, presence of high levels of this cytokine might be a reflection of an improved memory response with subsequent protection against wild-type challenge. The levels of GM-CSF observed in spleen supernatants could also be explained by the high IL-2 levels exhibited here.

Cytokines described above are all associated with a Th1 response. Significant quantities of Th2-type cytokines such as IL-4 were not detected in these studies. It is important to mention that the microcapsule by itself is not sufficient to provoke the secretion of cytokines in culture supernatants. Statistically significant concentrations of any cytokine were not detected in mice vaccinated with empty capsules, suggesting that the booster effect generated by the three capsule formulations was due to a continuous presentation of antigen to the host cells. Investigation to further define the basis for this response are under way.

In this study, mice were vaccinated with a single dose of an attenuated *Brucella* mutant for 31 weeks. Previous studies have not analyzed vaccine candidate performance at such late time points. We chose the 6 month time point for two reasons, to rule out the action of the innate immunity due to presence of the mutant (because of the pulsatile release of the mutant from the capsule) and also to better assess the quality of the memory response generated by the different vaccine formulations delivered as a single dose.

In summary, our findings indicate that encapsulation of live *Brucella* demonstrates enhancement of vaccine efficacy. The *luxR::Tn5* mutant is a suitable vaccine candidate that needs further investigation based on its reduced virulence in macrophages and BALB/c mice and ability to generate protection in the mouse model.

We were able to demonstrate a specific Th1 response that did improve when the vaccine was given using a controlled release vehicle. Further studies are planned to generate an unmarked knockout of the gene to avoid the use of vaccine strains with antibiotic resistance genes in the field, and to rule out the possibility of a polar effect due to the transposon insertion.

EVALUATION OF MICROENCAPSULATED RB51 VACCINE AGAINST

REINFECTION IN A NATURAL HOST: THE RED DEER MODEL

INTRODUCTION

Brucellosis eradication programs in North America have been successful in controlling the pathogen in domestic livestock, but not in wildlife populations (60). Currently, elk (Cervus *elaphus nelsoni*) and bison (Bison *bison*) are the wildlife species that remain problematic in the Greater Yellowstone Area (GYA). Within the past few years, a big concern has been *B. abortus* persistence and spread in wildlife reservoirs and the threat of disease to cattle (70).

The increasing prevalence of brucellosis can be mainly attributed to the lack of highly effective vaccines for wildlife species. Commercially available vaccine strains used for eradication of brucellosis in cattle have been studied for use in different wildlife species (15). Currently, all vaccination trials have revealed reduced vaccine efficacy in elk compared with cattle and several associated drawbacks including interference with diagnosis (64), resistance to antibiotics and residual virulence for animals and humans do not favor extensive use (6, 7). *B. abortus* S19 vaccine strain appears to be safe for use in adult elk, but has been shown to reduce abortion rates by only 30% (Thorne et al., 1981). Biosafety results in other species including pronghorn antelope (Antilocapr*a americana*) (22), bison (Biso*n bison*) (16) and coyotes (Canis *latrans*) indicates that the vaccine does not cause morbidity or mortality, thus favoring its use to protect elk. The other currently used vaccine strain RB51, has been shown to be safe in a wider range of non-target species including ravens (Corvus *corax*), Richardson ground squirrels

(Spermophilus *richardsonii*), deer mice (Peromyscus *maniculatus*) (35), bighorn sheep (Ovis canadensis), pronghorn (Antilocapra *americana*), mule deer (Odocoileus *hemionus*), moose (Alces alces *shirasi*) (40), black bears (Ursus *americanus*) (55). However, when RB51 vaccine is administered parenterally it does not protect against abortion in elk (39) (12).

The distribution of the disease appears to correlate with elevated animal densities associated with winter feeding (23). Enhancing the probability of transmission, Infected and susceptible elk gather on feed grounds assuring exposure of a great number of animals to *B. abortus*. Control of brucellosis should focus on these sites to prevent or reduce the transmission of the pathogen from infected animals. Difficulties with *Brucella* vaccines arise not only because of the reduced efficacy of vaccine strains (S19 and RB51), but also resulting from the delivery method used to immunize the animals. Currently, elk vaccination utilizes biobullets of S19 to ballistically vaccinate animals from distances of 30 meters (53). Although this represents an improvement over methods requiring rounding up the animals, problems associated with this methodology include excessive time, labor, logistics and elevated cost.

In failure of currently available vaccines to provide safe and efficacious immunity in elk, suggests the need for a different approach to vaccinate wildlife. As one alternative, we have investigated the ability to combine the use of live attenuated vaccines delivered via a controlled microencapsulated release vehicle. For this purpose, alginate a naturally occurring biopolymer offers several advantages including biocompatibility, low toxicity and relatively mild conditions required to encapsulate live organisms (75). In an attempt to enhance the efficacy of the capsule, we have also

incorporated a novel protein from the eggshell precursor of the parasite *Fasciola hepatica*, Vitelline protein B (VpB) a recombinant protein of 31KDa, which possess an unusual enzymatic resistance of the protein to breakdown (61) that could extend the time frame of erosion and release of the capsule content. Oral delivery of microencapsulated vaccine was also investigated, since this represents the most cost-effective way to deliver a vaccine in wildlife populations.

MATERIALS AND METHODS

Animals. Fifty four, 1- to 2- year- old Red Deer (*Cervus elaphus elaphus*) females were bought from a privately owned tuberculosis and brucellosis commercial free herd as an animal model for Rocky Mountain elk (Cervus *elaphus nelsoni*) due to their close genetic relationship, ease of handling and animal husbandry. Upon arrival, animals were re-tested for anti *Brucella* IgG levels (total IgG) by ELISA, dewormed using Moxidectin and Cydectin and allowed to acclimate for 3 months. For experimental purposes Red Deer were randomly assigned into 6 different treatment groups (n=9). All animal care and experimental procedures were performed in compliance with institutional animal care and guidelines.

Bacterial strains. Bacterial strains used in these experiments include the *B. abortus* vaccine strains RB51 and S19. *Brucella* was grown on tryptic soy agar (TSA) plates incubated at 37°C in atmosphere containing 5% (w/v) CO_2 for 72 hours. Bacteria were harvested from the plates by scraping them off the plate surface into phosphate buffered saline (PBS). The harvested bacteria were pelleted by centrifugation at 3000 x g and the pellet resuspended in fresh PBS. This step was

repeated two more times and the concentration of bacteria determined by optical density using a standardized curve of optical density (Klett units) vs. colony forming units (CFU). The concentration of viable organisms was verified retrospectively by plating serial dilutions of cell suspensions.

Preparation of *B. abortus* RB51-loaded microspheres. Alginate beads loaded with $1.5x10^{10}$ CFU/ml of the vaccine strain RB51 were prepared as previously described for live bacteria (1) with several modifications. Briefly, enumerated live RB51 vaccine strain (total $1.5x10^{11}$ for 10 doses) was re-suspended in a total of 100μl of MOPS (3-[N-Morpholino] propanesulfonic acid) buffer (10mM MOPS, 0.85% NaCl, pH 7.4) and mixed with 10 mls of alginate solution (1.5% sodium alginate, 10mM MOPS, 0.85% NaCl, pH 7.3). Three hundred μm spheres were obtained by extruding the suspension through a 200 micron nozzle into a 100mM calcium chloride solution and stirred for 15 minutes using the Inotech encapsulator I-50 (Inotech Biosystems International, Rockville USA). In order to permanently crosslink the capsule, the spheres were resuspended in MOPS buffer supplemented with 0.05% (w/v) poly-L-lysine (PLL, MW 22,000) for 15 minutes. Following two successive washes, a final outer coat was applied to the beads (0.03% (w/v) alginate for 5 min). All capsules were stored at 4°C in MOPS buffer until use. To determine the number of bacteria per 1ml of capsule suspension (or one ml of sedimented capsules), spheres were removed from the encapsulator prior to permanent crosslinking, washed three times with 50 mls of MOPS buffer and were dissolved using 10ml of depolymerization solution (50 mM Na_3– citrate, 0.45% NaCl 10mM MOPS, pH 7.2). Enumeration of bacteria was determined by plating serial dilutions onto TSA plates.

The addition of VpB as a component of the alginate core was achieved by the supplementing the mixtures described above with 1 mg of VpB to the bacteria-alginate suspension. Extrusion and capsule formation was performed exactly as described above.

Immunization of Red Deer. Fifty four 1-2 year-old female Red Deer were randomly distributed into 6 different treatment groups (n=9). Three groups were inoculated subcutaneously with a total dose of 1.5×10^{10} CFU of either nonencapsulated RB51, encapsulated RB51 with alginate or encapsulated RB51 with alginate and VpB. One group was vaccinated orally with 1.5×10^{10} CFU of alginate encapsulated RB51 and another group with RB51 encapsulated with alginate and VpB. A control group received a subcutaneous injection of 1 ml of empty capsules (no bacteria entrapped). A single vaccination dose was given to each animal in all groups.

Detection of *Brucella*-specific antibody levels. To determine the level of anti-*Brucella*-specific antibody in serum, blood samples were collected by jugular venipuncture immediately prior to vaccination, and 6, 12, 17 and 28 weeks post-vaccination. Serum samples were evaluated for total IgG determination by ELISA. Heat killed *B. abortus* RB51 was used as antigen to coat 96 well plates (Nunc-Immuno plates, high binding protein) at a concentration of 25μg/well. After overnight incubation at 4°C, plates were washed with PBS containing 0.05% (w/v) Tween-20, and blocked in the same solution supplemented with 0.25% (w/v) BSA to prevent non-specific binding. The blocking buffer was removed and deer serum samples diluted 1:100 in blocking buffer were incubated for 2 hrs at room temperature with rocking. Following three more washes, horseradish peroxidase conjugated goat anti-deer IgG (KPL, USA) was added

to the wells at a dilution 1:1000 and incubated at room temperature for 1 hr. After

incubation, plates were washed as describe above and the peroxidase substrate o-

phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, USA) was added following

manufacturer's instructions and incubated for no more than 20 min. The reaction was

stopped by the addition of 50μl of 0.5M NaOH and the absorbance measured at 450nm

(A_{450}). All assays were performed in triplicate and repeated at least two times.

Lymphocyte proliferation Assay from peripheral blood mononuclear cells.

At 12 weeks post-vaccination, mononuclear cells were isolated from the buffy coats of

peripheral blood as previously described with some modifications (74). Briefly, 2 x 10^5

cells/well were seeded in 96 well plates (Falcon, Becton Dickinson, USA) in RPMI

medium containing 10% (v/v) fetal bovine serum, 1mM L-glutamine, and 1mM non-

essential amino acids. Cells were stimulated with either *B. abortus* S2308 (wild-type)

lysate at a concentration of 12.5μg/ml, Concanavalin A (5μg/ml) or medium alone during

incubation for 6 days at 37°C in atmosphere containing 5% (v/v) CO_2. Following

incubation, 1μCi of methyl-[^{3}H] thymine was added to each well and incubation was

continued for an additional 18 hours. The cells were harvested onto glass fiber filters

using a 96 well plate cell harvester and the incorporated [3H]thymine was determined

using a Beckman liquid Scintillation counter. Data is represented as mean counts per

minute (CPM) ± standard deviation.

Host response against infection by intraocular S19 exposure. Seven

months post- vaccination three- to four animals from each vaccination group (except

RB51/alginate SC n=2) were exposed intraocullary as previously described (41) using a

challenge dose of 1 x 10^9 CFU/deer of *B. abortus* strain S19 as confirmed by plating

serial dilutions onto TSA plates. Two weeks post-challenge, animals were euthanized and spleens harvested, weighed and homogenized in 1 ml of peptone saline per gram of tissue for 5-10 minutes using a stomacher. One hundred microliter portions of each sample were serially diluted and plated in duplicate onto Farrell's media. Three to five days post-inoculation bacterial counts were enumerated. Results are represented as the mean CFU per gram of tissue ± SEM.

Statistical procedures. IgG levels elicited by vaccination are expressed as the mean A_{450}±SD for each group. For blastogenesis, counts per minute (CPM) from each group are expressed as the mean CPM±SD. Intensity of infection from S19 infection was expressed as mean log CFU±SEM for each group. The significance of differences between groups was determined by analysis of variance (ANOVA) using Prism software (GraphPad). A P value <0.05 is considered statistically significant.

RESULTS

Encapsulation of _B. abortus_ RB51 in alginate microspheres. Two different capsule formulations were prepared using the same basic alginate formulation. Variation on the formulation included the addition of VpB to the capsule core to modify degradation kinetics and release of the organism. When capsules were analyzed using light microscopy, all of the capsules formulations appeared spherical and uniform with a mean diameter of 310μm (Fig. 13). Furthermore, bacterial viability following encapsulation exceeded 95% as demonstrated by recovery of the organism following dissolution of the capsules (_data not shown_).

Encapsulated RB51 enhances humoral immune responses. Serum collected at different times post-vaccination was assayed for the presence of *Brucella*-specific antibodies by ELISA. Immunization with RB51 elicited an IgG response in oral and SC-vaccinates that was significantly different from naïve animals by 6 weeks post-vaccination (Fig. 14). Over the first 17 weeks, IgG levels were consistently higher in animals that received the injected vaccine compared to the groups that were immunized orally (p <0.05). Between 17 to 28 weeks, IgG levels in animals that were vaccinated orally had an increase in the IgG compared to deer vaccinated via SC (p <0.05).

Encapsulated RB51 enhances cellular immune responses. To determine the possible effect of encapsulation in the cellular response elicited in Red Deer, a cellular proliferation assay was performed. Animals were bled twelve weeks post-vaccination, and blastogenesis was performed using cells isolated from the buffy coat. Stimulation was performed in vitro as described in the materials and methods section and only animals receiving the encapsulated vaccine in which VpB was added to the core formulation exhibited statistically significant proliferative responses (P<0.0005 oral vaccinates, P<0.005 SC group) regardless of the immunization route (Fig. 15). Interestingly, animals receiving encapsulated RB51 with VpB orally, exhibited greater
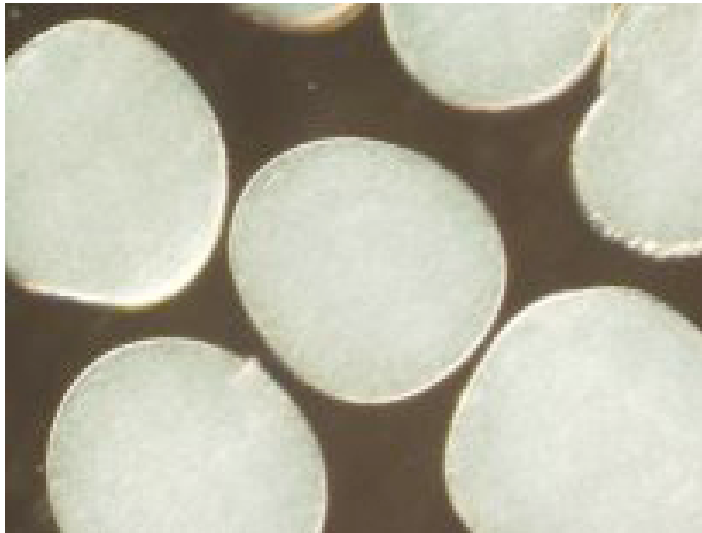
FIG. 13. Light microscopy image of alginate-VpB microspheres loaded with the vaccine strain RB51. *Brucella abortus* RB51 strain was encapsulated into alginate microspheres at a concentration of 1.5 x10^{10} CFU/ml of capsules. Uniform size and shape was observed. Microsphere size was measured indicating an average mean size of 310μm.
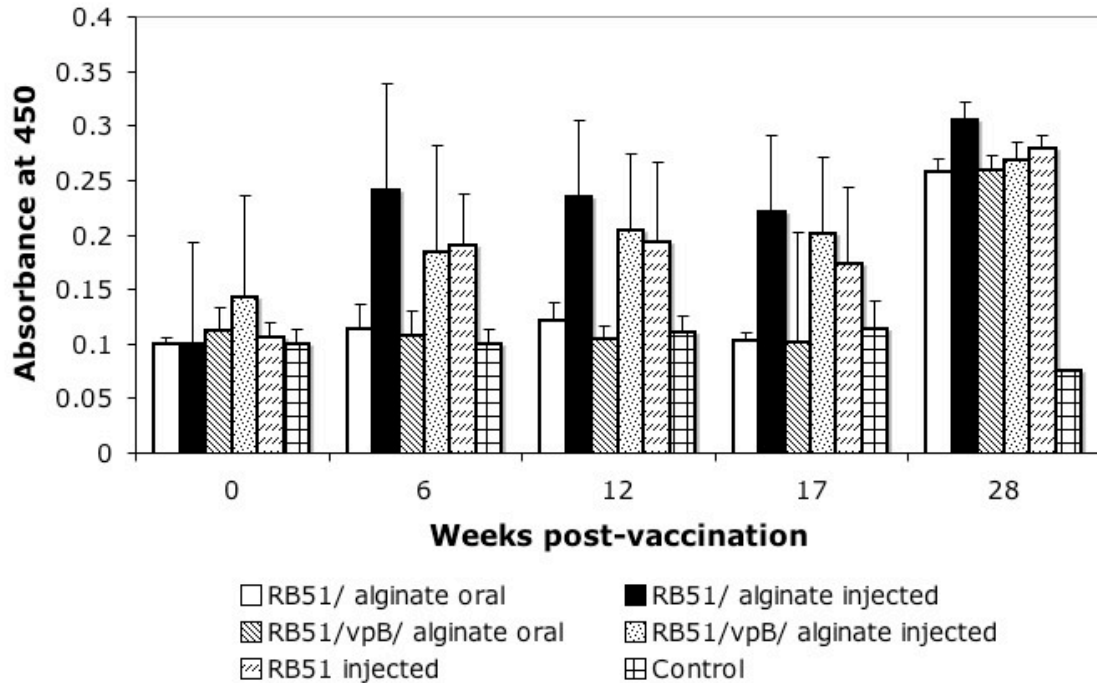
FIG. 14. IgG anti-*Brucella* antibodies in serum from deer vaccinated with RB51.

Red Deer (n=9) were inoculated SC with 1.5×10^{10} CFU of either non encapsulated B.

abortus RB51, encapsulated B. *abortus* RB51, encapsulated B. *abortus* with VpB.

Control group received empty capsules. Two groups received encapsulated oral

vaccine (RB51 with alginate) or (RB51 with VpB) at the same dose. 0,6,12,17 and 28

weeks post- vaccination, serum samples were collected and analyzed for IgG

determination by ELISA. Results are shown as the means ± standard deviations of

absorbance at 450. Significant differences were determined by ANOVA **($P<0.005$)
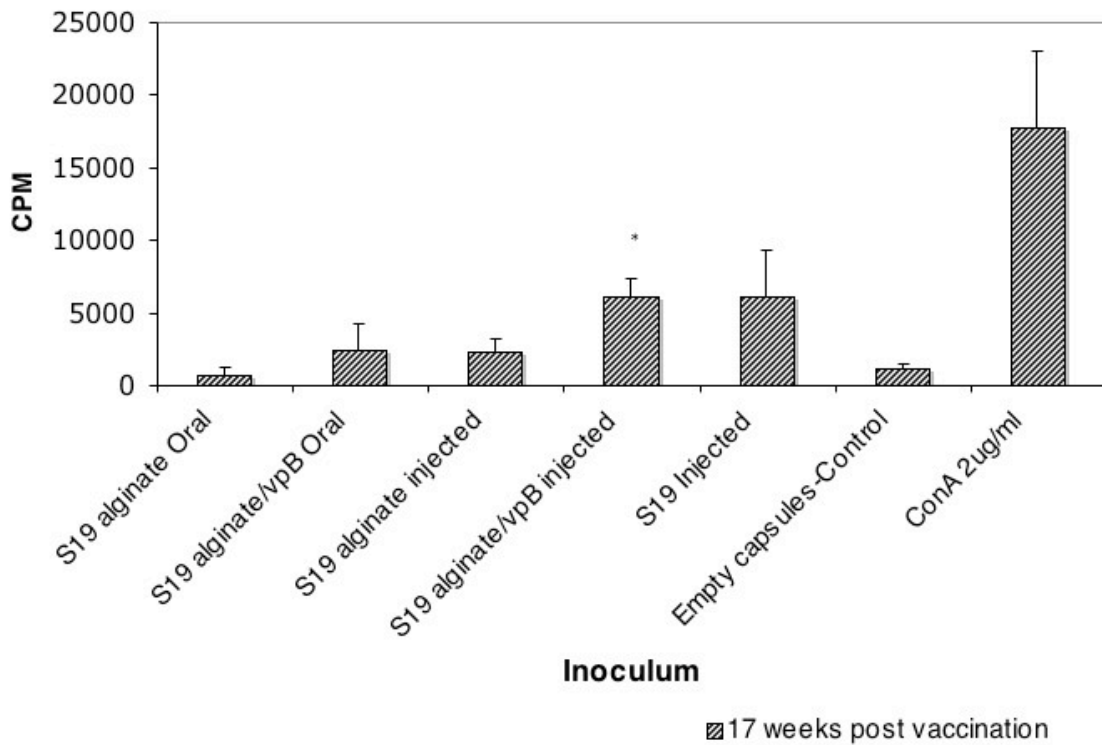
FIG 15. Lymphocyte proliferative responses of peripheral blood mononuclear cells from deer immunized with RB51. Red Deer (n=9) were vaccinated SC with 1.5x10^10 CFU of either nonencapsulated B. *abortus* RB51, encapsulated B. *abortus* RB51 (RB51 with alginate) , encapsulated B. *abortus* with VpB. Two groups received encapsulated oral vaccine (RB51 with alginate) or (RB51 with VpB) at the same dose. Control group received empty capsules. Twelve weeks post-vaccination, peripheral blood mononuclear cells were isolated, cultured at 37°C for 6 days and pulsed for 18h with 1μCi of methyl-[^{3}H] thymine. Results are expressed as mean CPM± SD. *P<0.05 are statistically different from the control by ANOVA analysis.

proliferation than Red Deer receiving the same formulation via SC inoculation (p <0.3).

Animals receiving nonencapsulated vaccine failed to mount significant cellular

responses.

Encapsulated RB51 provides enhanced protection against S19 challenge.

To determine the level of immune protection to infection against *B. abortus*, deer that

had been vaccinated with RB51 using either oral or SC routes were challenged

conjunctivally with a dose of $1x10^9$ CFU of *B. abortus* S19. Although not fully virulent in

the bovine, S19 is virulent in humans and virulence in deer is suggested. In 10 studies

utilizing over 150 elk, S19 caused abortions in up to 27% of the animals. Furthermore,

the fact that this strain is not a select agent, permitted field testing. The protective

immune response was determined by subtracting the mean number of CFU of S19

recovered per gram of spleen from deer vaccinated with the nonencapsulated or

encapsulated vaccine from the mean CFU recovered per gram of spleen from naïve

non-vaccinated deer. Seven months post-vaccination (two weeks post-challenge), only

animals that received encapsulated RB51 with VpB had a significant decrease in

bacterial load in the spleen (Fig. 16). Red Deer that received the vaccine orally were

the only group that was statistically significant compared to the nonencapsulated

injected RB51 (p <0.04). Animals that were immunized with the VpB capsules given

orally had a 1.27 log reduction in spleen counts compared to animals vaccinated with

nonencapsulated RB51 and 1.68 log reduction compared to naïve non-vaccinated

animals. S19 spleen counts in deer that received the VpB capsules via SC was also

diminished by 1.21 log compared to the nonencapsulated RB51 and 1.62 logs reduction

compared to non-RB51 vaccinated controls (p <0.2).
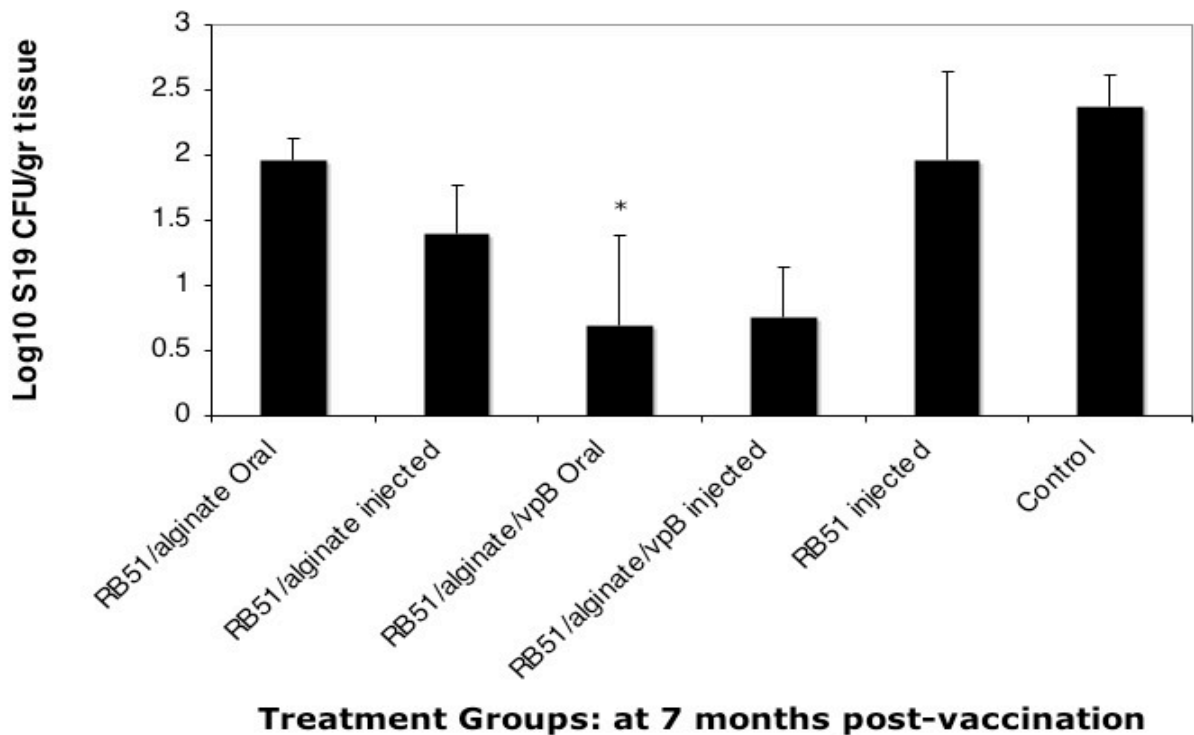
FIG. 16 Host response against infection by intraocular S19 exposure. Red Deer were vaccinated SC with 1.5×10^{10} CFU of either nonencapsulated B. *abortus* RB51, encapsulated B. *abortus* RB51 (RB51 with alginate), encapsulated B. *abortus* with VpB. Two groups received encapsulated oral vaccine (RB51 with alginate) or (RB51 with VpB) at the same dose. Control group received empty capsules. Seven months post-vaccination animals were intraocullary infected with 1×10^9 CFU of S19. Two weeks post-infection, animals were euthanized, spleens harvested and the bacterial count per gram of spleen was determined. Values are reported as the mean $\log_{10}$ recovery of S19 from spleens. Difference in colonization between the vaccinated and control was determined by ANOVA *$P < 0.05$.

DISCUSSION

The ultimate goal of vaccination is to control disease prevalence within and originating from reservoirs. To accomplish this goal in the elk population with current *Brucella* vaccines, development of new delivery mechanisms are needed to enhance vaccine efficacy.

Recent data suggests that the manner in which antigen reaches the lymphoid organs and how it is delivered to antigen presenting cells is fundamental to induction of a good immune response (69). There is experimental evidence to support the observation that microencapsulation serves to modify antigen uptake and processing (25, 69). It has also been suggested that prolonged persistence of the vaccine strain in the host is necessary for development of suitable anti-*Brucella* immunity (37). In an effort to develop a more efficient way to present live attenuated vaccines to the lymphoid tissue and to increase exposure time of the organism to the host cells, a controlled release strategy was developed in which RB51 was encapsulated into alginate-VpB microspheres. RB51 was successfully entrapped developing uniform spherical batches of capsules with or without VpB. The addition of this supplement to the capsules effectively modified vaccine efficacy as demonstrated by differences in cellular and humoral responses.

Live vaccines are more efficacious than vaccination with heat-killed *Brucella* or cellular extracts in providing a significant level of immunity for protection (64, 82). During microsphere formulation, RB51 was exposed to relatively mild conditions that did not cause a significant bacterial death (95% viability). This approach permitted the development of a live, encapsulated vaccine in contrast to other encapsulation

procedures in which the viability of the bacteria is seriously compromised mainly resulting from extreme encapsulation conditions needed during the polymerization steps. Harsh conditions include, direct exposure of the bacteria to organic solvents, shear stress and ultrasound homogenization have been avoided here (45).

Oral bait administration of vaccines to wildlife is the most practical and cost effective approach. Since many pathogens access the body via the mucosal surfaces, neutralization of the microorganism at the mucosal site would be an ideal method to prevent *Brucella* from infecting the host. Successful oral immunization of foxes against rabies has been extensively demonstrated using similar techniques and strategies (57, 62). Increased efficacy of current *Brucella* vaccine strains is also expected if the antigen is presented orally, generating an enhanced host response. By using alginate-VpB microspheres, capsules are expected to serve as a shield to protect the vaccine from degradation in the stomach resulting from exposure to the harsh conditions encountered here. The capsule might also provide a "vaccine package" that could be combined with baits for easy delivery.

Correlates of humoral immunity were assessed with all RB51 vaccine formulations and routes of vaccination. Immunization with RB51 induced remarkable IgG levels (total IgG), and these levels were enhanced by microencapsulation techniques reported here. For example, oral vaccinates exhibited increasing levels of IgG between 17 to 28 weeks, which may reflect the protective benefits of the capsule during exposure to the digestive tract.

Induction of specific cellular mediated immunity (CMI) responses following immunization is a hallmark for the establishment of a protective immune response.

Published results suggest that in elk a cellular and a humoral response might be needed in order to generate a good immunity towards *Brucella* infections (39, 53). Studies performed with the encapsulated RB51, indicated that by 12 weeks alginate/VpB capsules administered subcutaneously or orally, were able to stimulate a significantly elevated cellular response compared to nonencapsulated RB51. This data suggests that by incorporating the RB51 vaccine into a delivery vehicle, conditions necessary to trigger a cellular response confer enhanced protection. Moreover, the reduced cellular response elicited by nonencapsulated RB51, corroborated previous reports by other researchers (12, 39).

Following assessment of the initial cellular and humoral responses, three to four animals from each group were used to determine the degree of protection against challenge exposure to S19. This strain was utilized mainly because it has been previously shown to cause prolonged infection in deer (Thorne, 1980). Among animals exposed to an intraocular dose of $1\text{x}10^9$ CFU, those immunized with the capsules especially those containing VpB exhibited a significant (p <0.04) level of protection. The results corroborate the observed cellular and humoral responses, and support the idea that capsules serve as a vehicle for enhanced immunogenicity indicating that oral delivery is promising and relevant to the current needs of a practical vaccination strategy. Future experiments will utilize greater number of animals and challenge with fully virulent organisms.

The results observed using nonencapsulated RB51 were similar to those obtained in previous studies (54) where RB51 by itself is not sufficient to induce a good cellular response or reduce infection (39) in significant levels in elk. In contrast, our

findings indicate that alginate-VpB encapsulation of live *Brucella* may be used to

enhance vaccine efficacy in elk. Furthermore, the observation that oral vaccination

invoked humoral and cell mediated responsiveness and a reduction in S19 burden via

supports the hypothesis of enhanced and prolonged immunity due to a mucosal

immune stimulation.

EVALUATION OF ORAL VACCINATION WITH MICROENCAPSULATED S19

VACCINE STRAIN AGAINST *B. abortus* WILD-TYPE CHALLENGE IN RED DEER

INTRODUCTION

The prevalence of brucellosis within Red Deer populations in the Greater Yellowstone area constitutes a serious public health problem mainly because of the threat of free-ranging animals transmitting the infection to cattle. Within the last years, a number of *Brucella*-infected cattle herds have been identified in Wyoming and Idaho, in which the source of infection was presumed to be Bison and Elk (33). The seroprevalence of the disease can be attributed to the management practices implemented during winter feeding. Artificial winter feeding in the Greater Yellowstone area has resulted in significant congregations of elk in feeding grounds increasing the risk of exposure to the bacterium (30).

Current vaccine strains used to control the disease in cattle have proven to be less effective in wildlife populations. Experimental data suggest that S19 prevents abortion in only 30% of the population, while RB51 when given in a conventional format is ineffective against the disease (12, 39, 41, 71). Difficulties with vaccination arise not only because of the reduced efficacy of vaccine strains, but also resulting from the delivery method used to immunize the animals. Although its efficacy in elk is not comparable to its performance in cattle, vaccination with *Brucella abortus* S19 it is the best alternative and has been used since 1985 for elk vaccination. The methodology implemented to immunize these animals includes the use of biobullets containing live S19 to ballistically vaccinate Red Deer from distances of 30 meters (53).

Inconveniencies associated with this methodology include excessive time, labor, logistics and elevated cost.

It is acknowledged that the removal of winter feeding grounds is not a viable strategy for the short-term control of brucellosis in the Yellowstone area wildlife population. Alternative control measures such as improvement of vaccine efficacy and a more feasible methodology to immunize animals needs further investigation, not only to reduce the prevalence of the disease, but also to prevent the transmission of infected elk herds to bison and cattle, and more importantly to humans.

We have investigated the possibility of delivering the current licensed vaccine strain S19 in a controlled microencapsulated format. We have previously demonstrated an increased efficacy when live *Brucella* vaccines candidates are delivered in microspheres containing alginate, a naturally occurring biopolymer combined with a novel protein of the parasite *Fasciola hepatica*, Vitelline protein B (VpB) which possess an unusual enzymatic resistance to protein breakdown, modifying the erosion time of the capsule content (61). In the present study, we evaluated the vaccine efficacy when delivered by either oral or subcutaneous route, since the former represents the most feasible way to deliver vaccines in wildlife populations.

MATERIALS AND METHODS

Animals. Fifty- four, 1- to 2- year- old Red Deer (*Cervus elaphus elaphus*) females were bought from a privately owned tuberculosis and brucellosis commercial free herd as an animal model for Rocky Mountain elk (Cervus *elaphus nelsoni*) due to their close genetic relationship ease of handling and animal husbandry. Upon arrival,

animals were re-tested for anti *Brucella* IgG levels (total IgG) by ELISA, dewormed using Moxidectin and Cydectin and allowed to acclimate for 3 months. For experimental purposes Red Deer were randomly assigned into 6 different treatment groups (n=9). All animal care and experimental procedures were performed in compliance with institutional animal care and guidelines.

B. abortus **S19 and wild-type 2308 cultures.** Bacteria used in these experiments include B. *abortus* S19 used as the vaccine candidate and B. *abortus* virulent strain 2308. *Brucella* was grown on tryptic soy agar (TSA) plates incubated at 37°C in atmosphere containing 5% (w/v) CO_2 for 72 hours. Bacteria were harvested from the plates by scraping them off the plate surface into phosphate buffered saline (PBS). The harvested bacteria were pelleted by centrifugation at 3000 x g and the pellet resuspended in fresh PBS. This step was repeated two more times and the concentration of bacteria determined by optical density using a standardized curve of optical density (Klett units) vs. colony forming units (CFU). The concentration of viable organisms was verified retrospectively by plating serial dilutions of cell suspensions.

Preparation of *B. abortus S19*-loaded microspheres. Alginate beads loaded with 1.5×10^{10} CFU/ml of the vaccine strain S19 were prepared as previously described for live bacteria (1) with several modifications. Briefly, enumerated live S19 vaccine strain (total 1.5×10^{11} for 10 doses) was re-suspended in a total of 100µl of MOPS (3-[N-Morpholino] propanesulfonic acid) buffer (10mM MOPS, 0.85% NaCl, pH 7.4) and mixed with 10 mls of alginate solution (1.5% sodium alginate, 10mM MOPS, 0.85% NaCl, pH 7.3). Three hundred µm spheres were obtained by extruding the suspension through a 200 micron nozzle into a 100mM calcium chloride solution and stirred for 15

minutes using the Inotech encapsulator I-50 (Inotech Biosystems International, Rockville USA). In order to permanently crosslink the capsule, microspheres were resuspended in MOPS buffer supplemented with 0.05% (w/v) poly-L-lysine (PLL, MW 22,000) for 15 minutes. Following two successive washes, the beads were washed with stirring to a MOPS buffer supplemented with 0.03% (w/v) alginate for 5 min to apply a final outer coating. The addition of VpB as a component of the alginate core was achieved by the supplementing the mixtures described above with 1 mg of VpB to the bacteria-alginate suspension. Extrusion and capsule formation was performed exactly as described above. All capsules were stored at 4°C in MOPS buffer until use.

Bacterial enumeration and viability. To determine the bacterial content per ml of alginate particles, 1 ml of capsules was removed from the encapsulator prior to permanent crosslinking with poly-L-lysine or VpB with poly-L-lysine. The capsules were allowed to settle and washed twice with MOPS buffer and particles were dissolved using 1 ml of depolymerization solution (50 mM Na_3-Citrate, 0.45% NaCl 10mM MOPS, pH 7.2) with stirring for 10 min. Bacterial number (CFU/ml) per ml of capsules was determined by plating onto TSA plates.

Immunization of Red Deer. Red Deer were randomly distributed into 6 different treatment groups (n=9). Animals were given a single dose of vaccine containing either (a) encapsulated S19 (SC), (b) encapsulated S19 with alginate and VpB (SC), (c) encapsulated S19 (oral), (d) encapsulated S19 with alginate and VpB (oral). Control groups received 1.5x10^{10} CFU of either (a) Nonencapsulated S19 (SC) or (b) Empty capsules (no bacteria entrapped) (SC).

Detection of *Brucella* -specific antibody levels. To determine the level of anti-*Brucella*-specific antibody in serum, blood samples were collected by jugular venipuncture immediately prior to vaccination, and at 6, 12, and 17 post-vaccination. Serum samples were evaluated for total IgG determination by ELISA. Heat killed and sonicated *B. abortus* 2308 lysate was used as antigen to coat 96 well plates (Nunc-Immuno plates, high binding protein) at a protein concentration of 25μg/well. After overnight incubation at 4°C, plates were washed with PBS containing 0.05% (w/v) Tween-20, and blocked in the same solution supplemented with 0.25% (w/v) BSA to prevent non-specific binding. The blocking buffer was removed and deer serum samples diluted 1:100 in blocking buffer were incubated for 2 hrs at room temperature with rocking. Following three more washes, horseradish peroxidase conjugated goat anti-deer IgG (KPL, USA) was added to the wells at a dilution 1:1000 and incubated at room temperature for 1 hr. After incubation, plates were washed as describe above and the peroxidase substrate o-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, USA) was added following manufacturer's instructions and incubated for no more than 20 min. The reaction was stopped by the addition of 50μl of 0.5M NaOH and the absorbance measured at 450nm (A_{450}). All assays were performed in triplicate and repeated at least two times.

Lymphocyte proliferation assay from peripheral blood mononuclear cells. At 17 weeks post-vaccination, mononuclear cells were isolated from the buffy coats of peripheral blood as previously described with some modifications(74). Briefly, 2×10^5 cells/well were seeded in 96 well plates (Falcon, Becton Dickinson, USA) in RPMI medium containing 10% (v/v) fetal bovine serum, 1mM L-glutamine, and 1mM non-

essential amino acids. Cells were stimulated with either (a) *B. abortus* S2308 (wild-type) lysate at a concentration of 12.5μg/ml, (b) Concanavalin A (5μg/ml) or (c) medium alone during incubation for 6 days at 37°C in atmosphere containing 5% (v/v) CO_2. Following incubation, 1μCi of methyl-[^{3}H] thymine was added to each well and incubation was continued for an additional 18 hours. The cells were harvested onto glass fiber filters using a 96 well plate cell harvester and the incorporated [3H]thymidine was determined using a Beckman liquid Scintillation counter. Data is represented as mean counts per minute (CPM) ± standard deviation.

Efficacy of vaccination. One year post-vaccination, two to three animals from each group were housed into BL3- facility buildings (5 to 6 animals per building) and acclimated for two weeks prior to challenge. Animals were exposed intraocullary using a challenge dose of 1x 10^9 / deer of *B. abortus* wild-type 2308 as confirmed by plating serial dilutions onto TSA plates. Two weeks post-challenge, animals were euthanized and spleens, lungs and liver were harvested, weighed and homogenized in 1 ml of peptone saline per gram of tissue for 5-10 minutes using a stomacher. Additionally parotid, mesenteric and mammary lymph nodes were extracted and each lymph node was homogenized in 1ml of peptone saline. One hundred microliter portions of each sample were serially diluted and plated in duplicate onto Farrell's media (TSA supplemented with 10% (v/v) horse serum, 2% (w/v) dextrose and Oxoid *Brucella* supplement). Three to five days post-inoculation bacterial counts were enumerated. Results are represented as the mean CFU per gram of tissue ± SEM. For lymph nodes results are represented as the mean CFU per lymph node ± SEM.

Statistical procedures. IgG levels elicited by vaccination are expressed as the mean $A_{450}\pm SD$ for each group. For determination of cellular responses, counts per minute (CPM) from each group are expressed as the mean CPM±SD. Efficacy of vaccination was expressed as mean log CFU±SEM for each group. The significance of differences between groups was determined by analysis of variance (ANOVA) using Prism software (GraphPad). A P value <0.05 is considered statistically significant.

RESULTS

Encapsulated S19 elicited *Brucella*-specific IgG responses. Serum collected at different times post-vaccination was assayed for the presence of *Brucella*-specific antibodies by ELISA. Immunization with S19 elicited an IgG response in SC-vaccinates that was significantly different from naïve animals by 6 weeks post-vaccination (p<0.005). Animals that received the oral vaccine elicited lower detectable IgG levels at all time points, but interestingly, only animals that received the oral vaccine with VpB in the formulation had increasing levels of IgG. The IgG peak in Red Deer that received this formulation was delayed up to 17 weeks post-vaccination compared to all of the other groups were IgG peaked at 6 weeks post-vaccination (Fig 17).

Encapsulated S19 enhances cellular immune responses. To determine the possible effect of encapsulation in the cellular response elicited in Red Deer vaccinated with S19, a cellular proliferation assay was performed. Animals were bled 17 weeks post-vaccination, and blastogenesis was performed using cells isolated from the buffy coat.
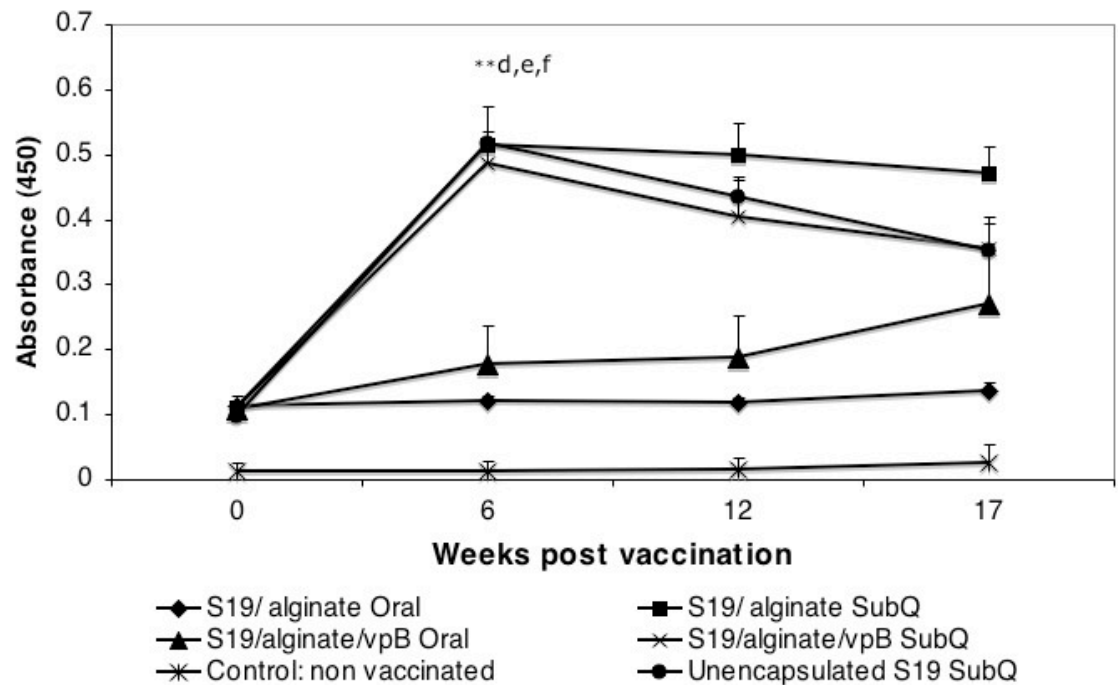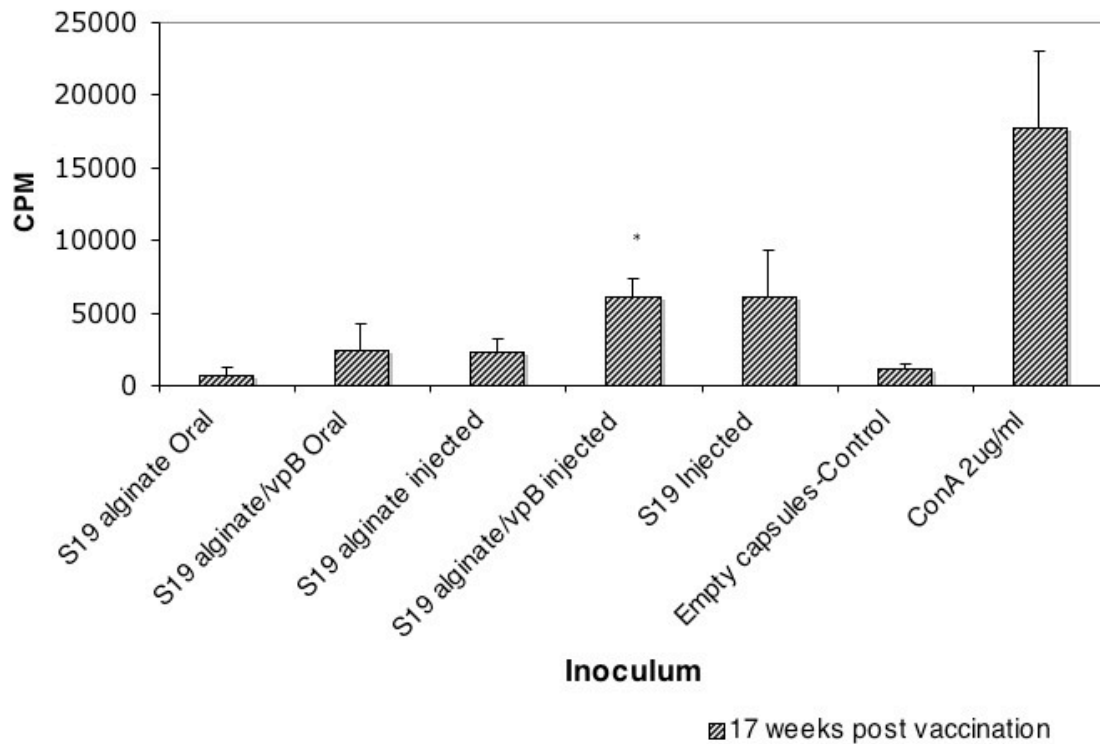
FIG 17. IgG anti-*Brucella* antibodies in serum from deer vaccinated with S19. Red Deer

(n=9) were inoculated SC with 1.5×10^{10} CFU of either non encapsulated B. *abortus* S19,

encapsulated B. *abortus* S19, encapsulated B. *abortus* with VpB. Control group received empty

capsules. Two groups received encapsulated oral vaccine (S19 with alginate) or (S19 with VpB)

at the same dose. 0,6,12 and 17 weeks post- vaccination, serum samples were collected and

analyzed for IgG determination by ELISA. Results are shown as the means ± standard

deviations of absorbance at 450. Significant differences were determined by ANOVA **(P<0.005)

[d] Encapsulated B. *abortus* S19 SC, [e] Encapsulated B. *abortus* with VpB SC, [f] Non encapsulated

B. *abortus* Stimulation was performed in vitro as described in the Materials and Methods section.

Only animals receiving the encapsulated vaccine in which VpB was added to S19 SC

(Differences were significant at all timepoints).

The core formulation via a subcutaneous route exhibited statistically significant proliferative responses compared to naïve animals. (p <0.05). Animals receiving nonencapsulated vaccine failed to mount significant cellular responses (Fig 18).

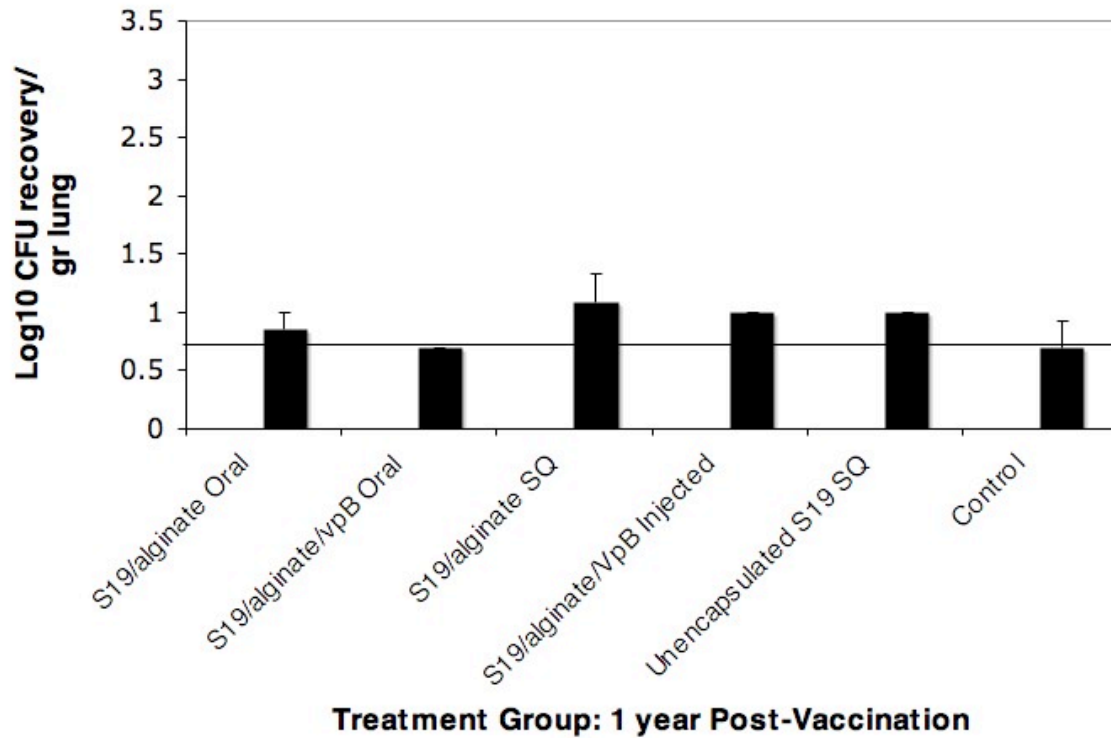**Evaluation of immune protection provided by encapsulated *B. abortus* S19.** In order to determine the effect of encapsulation on immunization, the level of protection provided by vaccination with either encapsulated and nonencapsulated S19 was evaluated against wild-type challenge at 1 year post vaccination. Recovery of challenge organism in spleen, lung, liver and parotid, mesenteric and mammary lymph nodes was evaluated. Two weeks post-challenge, only Red Deer vaccinated orally with the encapsulated vaccine containing VpB in the formulation had a statistically significant decrease in bacterial load in the spleen relative to naïve deer. Animals vaccinated using this formulation had a 1.85 logs reduction (p <0.0005) in bacterial burden (Fig 19) compared to naïve animals. Colonization of wild-type 2308 in lung and livers was not significant different between the controls and the immunized groups but animals that received the encapsulated oral vaccine with VpB had a reduced bacterial burden (p>0.05) (Fig 20, 21). Parotid lymph nodes that were closer to the site of inoculation exhibited the highest CFU counts (Fig 22, 23, 24). In accordance to the reduced recovery of wild-type organism in the spleen, only the oral vaccinees that received the encapsulated vaccine with VpB, had a significant reduction in bacterial burden in the parotid lymph nodes (p<0.005) (Fig 24).

FIG 18. Lymphocyte proliferative responses of peripheral blood mononuclear cells from deer immunized with S19. Red Deer (n=9) were vaccinated SC with 1.5×10^{10} CFU of either nonencapsulated B. *abortus* S19, encapsulated B. *abortus* S19 (S19 with alginate), encapsulated B. *abortus* with VpB. Two groups received encapsulated oral vaccine (S19 with alginate) or (S19 with VpB) at the same dose. Control group received empty capsules. Twelve weeks post-vaccination, peripheral blood mononuclear cells were isolated, cultured at 37°C for 6 days and pulsed for 18h with 1μCi of methyl-[^{3}H] thymine. Results are expressed as mean CPM± SD. *$P<0.05$ are statistically different from the control by ANOVA analysis.
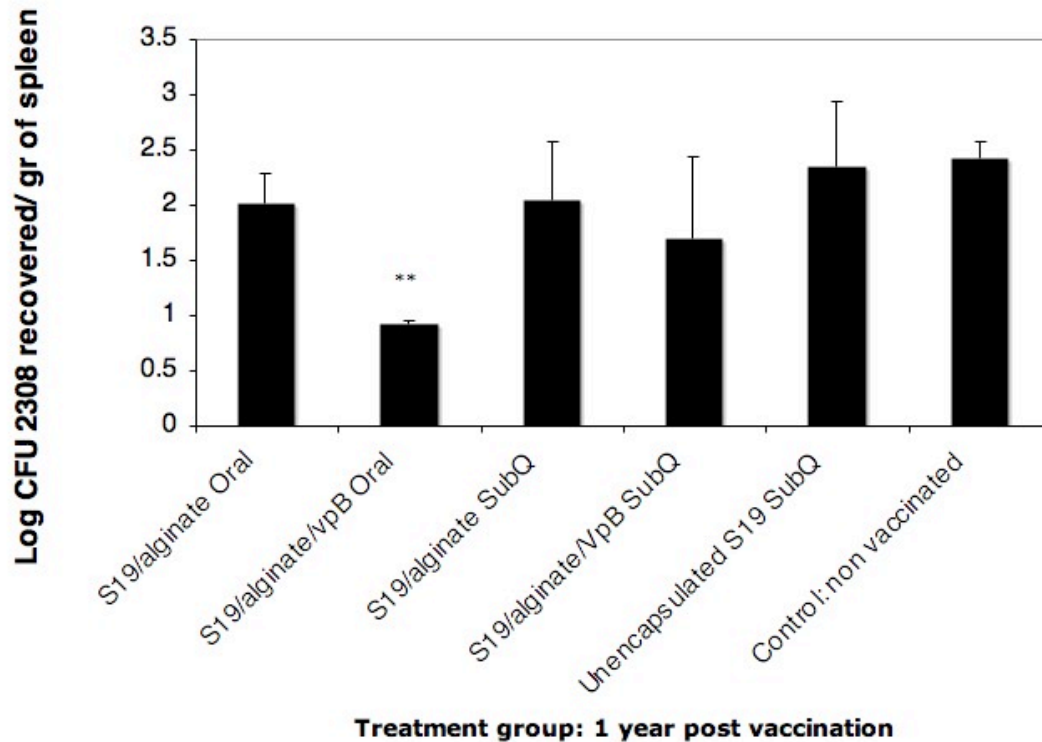
FIG 19. Vaccination efficacy against 2308 wild-type challenge. Red Deer

were vaccinated SC with 1.5×10^{10} CFU of either nonencapsulated B. *abortus*

S19, encapsulated B. *abortus* S19 (S19 with alginate), encapsulated B. *abortus*

with VpB. Two groups received encapsulated oral vaccine (S19 with alginate) or

(S19 with VpB) at the same dose. Control group received empty capsules. One

year post- vaccination animals were intraocullary infected with 1×10^9 CFU of wild-

type 2308. Two weeks post- infection, animals were euthanized, spleens

harvested and the bacterial count per gram of spleen was determined. Values

are reported as the mean $\log_{10}$ recovery of 2308 from spleens. Difference in

colonization between the vaccinated and control was determined by ANOVA

*$P<0.05$.

FIG 20. Bacterial recovery from lung tissue in Red Deer challenged with B.

abortus 2308. Red Deer were vaccinated SC with 1.5×10^{10} CFU of either

nonencapsulated B. _abortus_ S19, encapsulated B. _abortus_ S19 (S19 with alginate),

encapsulated B. _abortus_ with VpB. Two groups received encapsulated oral vaccine (S19

with alginate) or (S19 with VpB) at the same dose. Control group received empty

capsules. One year post- vaccination animals were intraocullary infected with 1×10^{9} CFU

of wild-type 2308. Two weeks post- infection, animals were euthanized, lungs harvested

and the bacterial count per gram of lung was determined. Values are reported as the

mean $\log_{10}$ recovery of 2308 from spleens. Difference in colonization between the

vaccinated and control was determined by ANOVA *P<0.05.
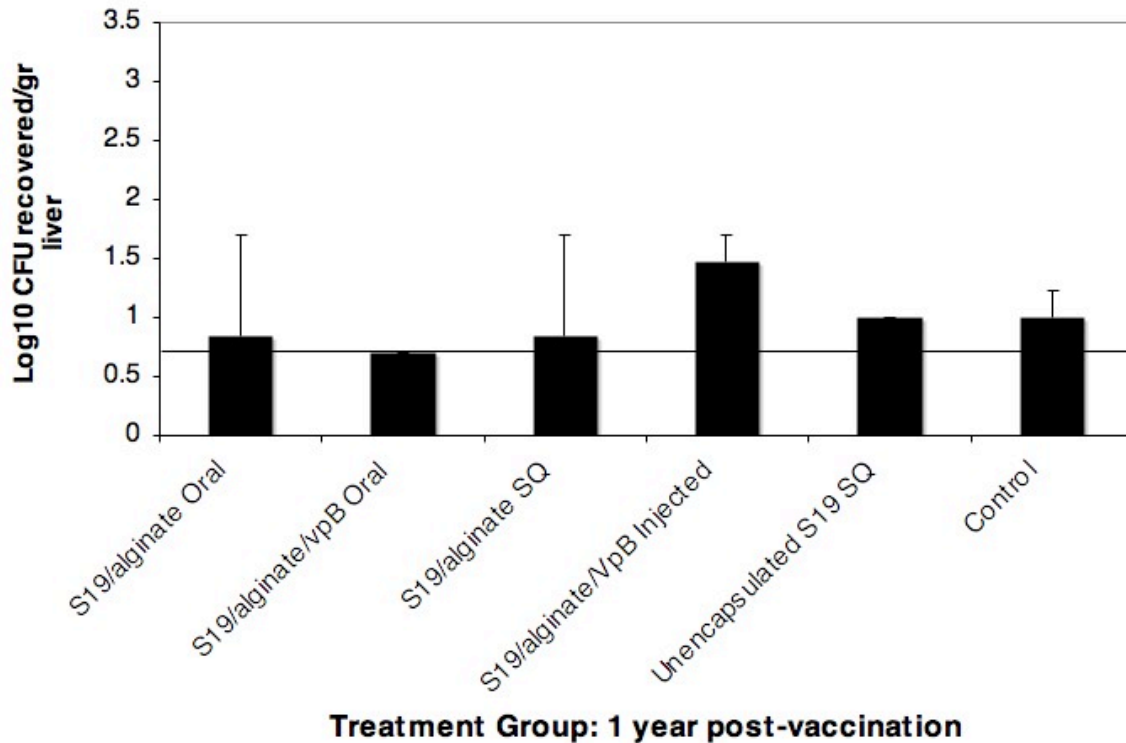
FIG 21. Bacterial recovery from liver tissue in Red Deer challenged with B.

***abortus* 2308.** Red Deer were vaccinated SC with 1.5×10^{10} CFU of either

nonencapsulated B. *abortus* S19, encapsulated B. *abortus* S19 (S19 with alginate),

encapsulated B. *abortus* with VpB. Two groups received encapsulated oral vaccine (S19

with alginate) or (S19 with VpB) at the same dose. Control group received empty

capsules. One year post- vaccination animals were intraocullary infected with 1×10^9 CFU

of wild-type 2308. Two weeks post- infection, animals were euthanized, livers harvested

and the bacterial count per gram of livers was determined. Values are reported as the

mean $\log_{10}$ recovery of 2308 from spleens. Difference in colonization between the
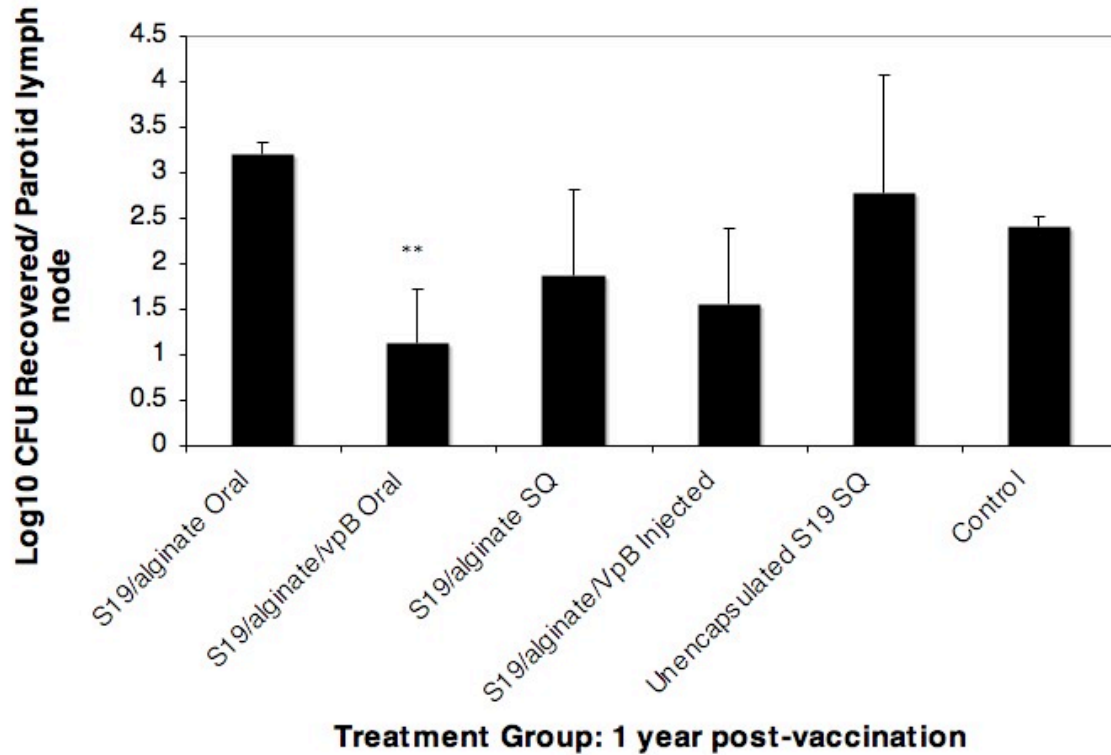
vaccinated and control was determined by ANOVA *P<0.05.

FIG 22. Bacterial recovery from parotid lymph nodes post-challenge with B.
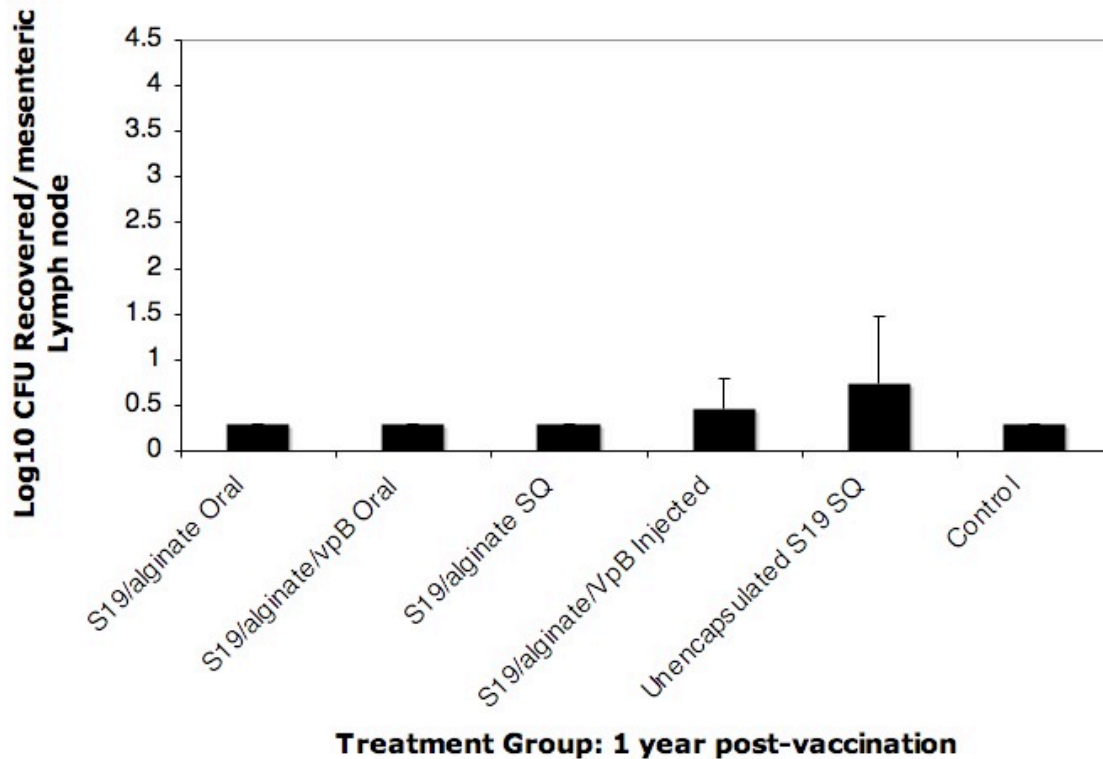
abortus 2308. Red Deer were vaccinated SC with 1.5×10^{10} CFU of either

nonencapsulated B. *abortus* S19, encapsulated B. *abortus* S19 (S19 with alginate),

encapsulated B. *abortus* with VpB. Two groups received encapsulated oral vaccine (S19

with alginate) or (S19 with VpB) at the same dose. Control group received empty

capsules.Two weeks post-challenge, animals were euthanized, parotid lymph nodes

were harvested, resuspended in 1ml of peptone saline, homogenized and plated for

bacterial enumeration. Results are represented as the mean CFU recovered per lymph

node. Difference in colonization between the vaccinated and control was determined by

ANOVA *P<0.05.

FIG 23. Bacterial recovery from mesenteric lymph nodes post-challenge with B.

***abortus* 2308.** Red Deer were vaccinated SC with 1.5×10^{10} CFU of either

nonencapsulated B. *abortus* S19, encapsulated B. *abortus* S19 (S19 with alginate),

encapsulated B. *abortus* with VpB. Two groups received encapsulated oral vaccine (S19

with alginate) or (S19 with VpB) at the same dose. Control group received empty

capsules.Two weeks post-challenge, animals were euthanized, parotid lymph nodes

were harvested, resuspended in 1ml of peptone saline, homogenized and plated for

bacterial enumeration. Results are represented as the mean CFU recovered per lymph

node. Difference in colonization between the vaccinated and control was determined by
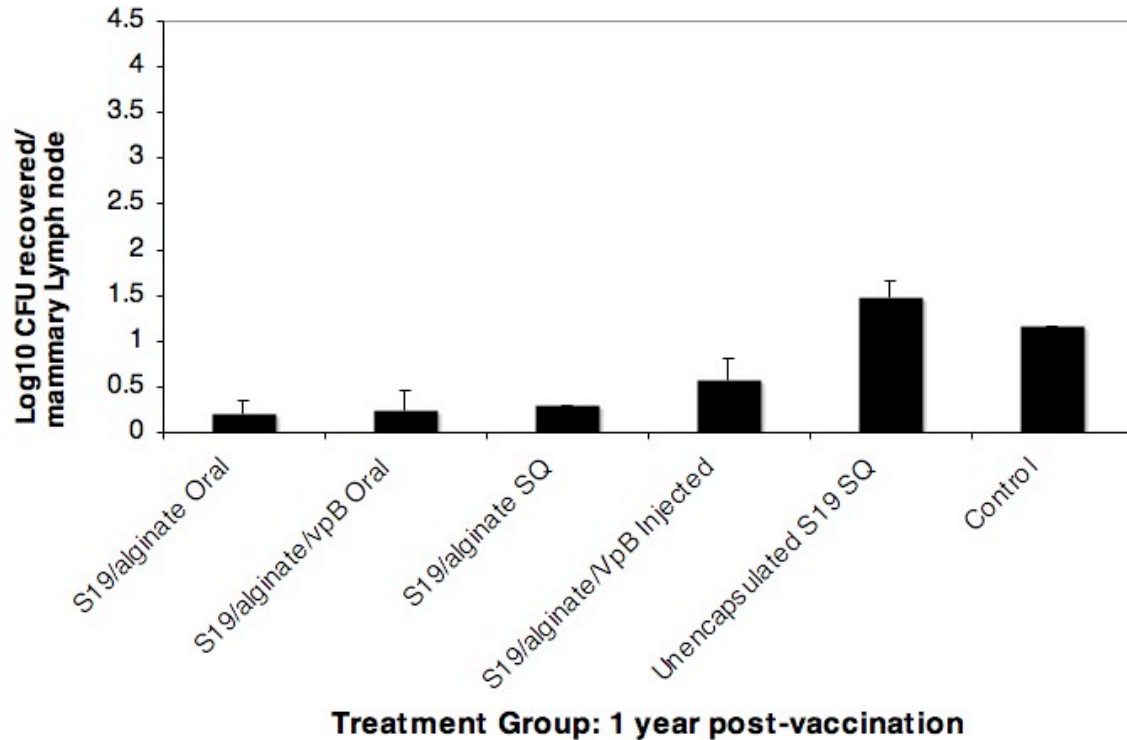
ANOVA *$P<0.05$.

FIG 24. Bacterial recovery from mammary lymph nodes post-challenge with B. *abortus* 2308. Red Deer were vaccinated SC with 1.5×10^{10} CFU of either nonencapsulated B. *abortus* S19, encapsulated B. *abortus* S19 (S19 with alginate), encapsulated B. *abortus* with VpB. Two groups received encapsulated oral vaccine (S19 with alginate) or (S19 with VpB) at the same dose. Control group received empty capsules.Two weeks post-challenge, animals were euthanized, parotid lymph nodes were harvested, resuspended in 1ml of peptone saline, homogenized and plated for bacterial enumeration. Results are represented as the mean CFU recovered per lymph node. Difference in colonization between the vaccinated and control was determined by ANOVA *P<0.05.

DISCUSSION

Wildlife vaccination represents a practical tool for the control of transmissible diseases that represent a risk to public health. Common issues with vaccines for this population includes: the need of a good protective immunity, minimization of side effects, ease of handling and administration, and a reduced cost of production and delivery. Current vaccination strategies used to control brucellosis in the Greater Yellowstone area do not fulfill these requirements: B. *abortus* S19 vaccine only confers protection in 30% of the population, can produce abortion in a low percentage of the vaccinates and it is ballistically delivered, increasing the cost and risk of administration. By using microencapsulation techniques we studied the ability to enhance S19 efficacy and provide an easier delivery method to administer the vaccine in Red Deer.

Experimental evidence suggests that live vaccines are more efficacious against brucellosis than killed vaccines (11, 38). Vaccination results using B. *abortus* S2308, S19 and RB51 suggests that the persistence of the *Brucella* vaccine strain is also critical to confer protection (37, 38). These two observations have to be taken into consideration when designing new vaccines or vaccination strategies. In this study, we were able to develop a method of vaccine delivery in which the viability of the bacteria is not compromised due to the mild procedure used to encapsulate the vaccine. Also, we tested the ability of the vaccine to protect against wild-type challenge when the immunogen is given by an oral route. Oral administration was analyzed because the most cost-effective method of vaccinating wildlife animals would be to incorporate the vaccine in bait.

Correlates of humoral immunity were monitored in all S19 vaccine formulations and routes of vaccination. Immunization with S19 induced remarkable IgG levels even when the vaccine was given in a microencapsulated format (Fig. 12). Animals that received VpB in the formulation orally, were able to mount increasing IgG levels that animals that did not receive VpB in the formulation fail to induce, which may reflect the increased resistance of the capsule to the stomach conditions due to the highly proteolitic resistance to breakdown of VpB.

Previous investigations suggests that induction of specific cell mediated immune responses following immunization is important for the establishment of a protective immune response in Red Deer (12, 53). Studies performed with the encapsulated S19, indicated that by 17 weeks only alginate/vpB capsules administered subcutaneously were able to stimulate a significant elevated cellular response compared to naïve animals. Red Deer that received the same formulation orally did show enhanced cellular responses but was not statistically significant (P<0.1). This data suggests that by incorporating the S19 vaccine into microcapsules, conditions necessary to trigger a cellular response are provided. Further studies adjusting the VpB concentration for the oral formulation are still needed to obtain the response observed with the subcutaneous vaccination.

To determine the degree of protection against wild-type challenge, two to three animals from each group were housed in large animal BL3 facility buildings. Because of federal regulations and difficulty in housing these animals, group sizes were very limited. Among animals exposed to an intraocular dose of 1×10^9 CFU of wild-type 2308, the highest bacterial burden was observed from spleens and parotid lymph nodes. This

finding was expected due to the proximity of these lymph nodes to the site of inoculation. When vaccination efficacy was analyzed based on CFU reduction in spleen of immunized vs. naïve but challenged deer, only animals that received the vaccine orally within capsules containing VpB in the formulation exhibited a significant (p<0.005) degree of protection compared to the control. In accordance with this observation, this group also exhibited the lowest numbers of recoverable bacteria in lungs and livers. Interestingly, bacterial counts in the parotid lymph nodes was also diminished (p<0.005). These results support the idea of induction of mucosal immunity by the oral vaccine.

Prevention of loss of S19 viability inside the stomach, is one of the most critical steps toward the development of good mucosal immunity by oral vaccination. Since the microencapsulated oral vaccine elicited stronger immune responses than nonencapsulated vaccine, we suggest that the alginate-VpB formulation protected the vaccine from the harsh enzymatic environment encountered in the stomach. This correlates with the observations made by other groups, in which alginate has be proven to resist low pH degradation (75). The exact mechanism of protection and delivery remains to be determined. These results are promising to the current needs for an oral bait vaccine.

It is also important to mention that this study was conducted over a year, in which animals were vaccinated only once. If the vaccine is given as an oral bait, probably animals will be naturally boosted and this might enhance even more the vaccine efficacy or create tolerance that would be detrimental to protection. Also, the intraocular dose used for challenge is probably more severe than natural exposure of

elk to B. *abortus* in the field, if indeed the challenge in the field is less severe, protection against field challenge could be even higher that observed in this experimental challenge study. Further studies to address the effect of multiple doses of vaccine and different challenge doses with higher animal numbers are currently under investigation.

In conclusion, a novel method of vaccination was examined using a controlled release mechanism. Collectively, our preliminary data demonstrate that oral vaccination of Red Deer via alginate-vpB encapsulation of S19 is a robust and efficient way to induce protective immune responses in Red Deer. We were able to observe that protection against 2308 wild-type challenge was sustained for a period of at least 1 year post-vaccination. This methodology of vaccination might be an alternative to enhance vaccines that are currently used for vaccination. This might be a safe and effective means of controlling brucellosis in the Greater Yellowstone area.

EVALUATION OF *luxR* DELETION IN S19 VACCINE AND DETERMINATION OF ITS POTENTIAL AS A VACCINE WHEN DELIVERED IN A MICROENCAPSULATED DELIVERY VEHICLE

INTRODUCTION

Brucella abortus S19 live vaccine has been extensively used to prevent the disease in cattle (50). This strain was first isolated from the milk of a Jersey cow in 1923, and after being kept in the laboratory at room temperature it became attenuated (50). Extensive efficacy studies conducted in cattle with this vaccine, have demonstrated that 70% of the vaccinates are protected from a wild-type exposure (50). Effectiveness depended on a series of variables including age of vaccination, prevalence of the disease in vaccinated herds, dose and route of vaccination (50, 64). Although S19 is of low virulence for cattle, there is an incidence between 1% up to 2.5% of abortion rates, when the vaccine is given to pregnant animals (64). A less frequent adverse effect of strain 19 vaccination is the development of an arthropathy associated with *Brucella* antigen-containing immune complexes (50, 64). In many developing nations immunizations derived from S19 vaccine have been evaluated in humans. In the former Soviet Union, administration of live S19 preparations were immunogenic and protection was achieved and considered to last 1 year but, caused a modest but notable incidence of clinical cases, as well as a hypersensitivity reaction (10, 50).

Previous research in our lab, has identified *Brucella melitensis* genes required for virulence and survival via transposon mutagenesis (3, 34). Among these, *vjbR* (BMEI1116), encoding the *luxR*-like quorum sensing-related transcriptional regulator is required for virB expression (17). *luxR* mutants of *B. melitensis* were highly attenuated for virulence in mice and macrophages. BALB/c mice immunized with the *luxR* mutant

were protected against wild-type challenge, did not provoke any local or adverse reactions in mice, making such mutants ideal vaccine candidates. In the present study, the effects of eliminating the *luxR* gene from B. *abortus* S19 were evaluated in vitro and in BALB/c mice. The capacity of B. *abortu*s S19Δ*luxR*::kan mutant to elicit *Brucella* specific immune responses was also evaluated. Furthermore, to try to enhance vaccination efficacy, the knockout was encapsulated into microspheres with the aim of producing vaccines that are safer and still very effective.

MATERIALS AND METHODS

Mice. One hundred and fifty 4 to 6-week old female BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All experimental procedures and animal care were performed in compliance with institutional animal care regulations.

Bacterial strains. Bacteria used in these experiments include *B. abortus* S19*, B. abortusΔluxR::*Kan and *B. abortus* virulent strain 2308. Bacteria were grown on tryptic soy agar (TSA) at 37°C with 5% (v/v) CO_2. For B. *abortusΔluxR::*Km, the medium was supplemented with kanamycin (100μg/ml). Following 3 days of incubation, bacteria were harvested from the surface of plates into phosphate buffered saline (PBS). The bacteria were pelleted, and washed twice by resuspension in buffer containing MOPS (10mM MOPS, 0.85% NaCl, pH 7.4), and resuspended to a final concentration of $1x10^6$ CFU /ml for encapsulation, or $1x10^5$ CFU in 100μl of PBS of nonencapsulated based upon optical density (OD) readings using a Klett meter and a standardized curve. The actual viable counts were confirmed by serial dilution and plating of portions onto TSA plates with or without antibiotic. To inoculate mice, bacteria was harvested the same day of challenge, and the density was determined using a Klett meter. Inoculum doses were plated retrospectively to determine the number of organisms inoculated.

Transfection of *Brucella* was performed via electroporation, using cultures prepared from a frozen stock and grown to confluence on TSA at 37°C for 3 days. Bacteria were harvested into PBS to yield a final suspension containing approximately 4 x 10^{11} CFU/ml, estimated using a Klett Meter. *Escherichia Coli* cultures were grown on Luria-Bertani (Difco, Laboratories) plates overnight at 37°C with or without kanamycin (100mg/liter), carbenicillin (100mg/liter), or chloramphenicol (50mg/liter).

Plasmid construction. Primers were designed to amplify sequences flanking the genes following a methodology previously described (37).

Construction of S19Δ*luxR*::kan deletion mutant. The marked deletion mutant S19Δ*luxR*::kan was constructed in S19 as previously described with some modifications (37). Briefly, the mutant was created in *B. abortus S19* via allelic exchange following electroporation of the marked plasmid into S19. Bacteria were grown as described above and pelleted via centrifugation at 1,700 x *g* for 15 min at 4°C. The cell pellet was washed three times with ice-cold sterile water. After the final wash, the cells were resuspended in 1 ml sterile water. The bacterial cell suspension was used in each electroporation with 1 *μ*g DNA in a prechilled 1-mm gap cuvette (Bio-Rad, California) and shocked in a BTX electroporation apparatus set at 2.2 to 2.5 kV. SOC-B (6% [wt/vol] tryptic soy broth, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) medium was immediately added to the cuvette, transferred to microcentrifuge tubes, and incubated overnight at 37°C with agitation. Following incubation, the entire culture was plated onto TSA containing kanamycin. Verification of mutant genotypes was obtained via PCR and Southern blot analysis *(data not shown).*

Evaluation of *B. abortus* S19Δ*luxR*::kan in J774A.1 macrophages. Murine macrophage-like J774.A1 (ATCC TIB-67) were used to assess S19Δ*luxR*::kan mutant survival compared to the parental *B. abortus S19*. Macrophage survival assays were performed as previously described with some modifications (58). Briefly, macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum, 1mM L-glutamine, and 1mM nonessential amino acids. Monolayers of macrophages containing 2.5×10^5 cells per well were infected at a multiplicity of infection of 1:100, at 37°C with either S19Δ*luxR*::kan, S19 or wild-type 2308. Thirty minutes post infection, cells were washed twice with medium without antibiotics and then incubated with 50μg/ml of gentamicin (Invitrogen,USA) for 30 min to kill any extracellular bacteria. One and 48 hours post-infection, macrophages were lysed using 0.05% (v/v) Tween-20, and bacteria collected. *Brucella* entry and survival was determined by performing serial dilutions and plating onto TSA plates with or without antibiotic. All assays were performed in triplicate and repeated at least three times.

Evaluation of *B. abortus* S19Δ*luxR*::kan attenuation in mice. Female BALB/c mice were used to evaluate survival of the S19Δ*luxR*::kan mutant. Briefly, 4-to 6-week-old mice were intraperitoneally inoculated with a total of 1×10^6 CFU of either mutant or the parental strain. Groups of 5 mice were euthanized via carbon dioxide asphyxiation at 1,3,5,7 and 9 weeks post inoculation. At each time point, spleens were collected, weighed and homogenized in 1ml of peptone saline. Serial dilutions were prepared, and 100μl aliquots of the different dilutions were plated in duplicate onto TSA or TSA/kan. Levels of infection were expressed as the mean ± standard error of the mean (SEM) of individual log CFU/ spleen.

Histopathology. Twelve 4-6 week-old female BALB/c mice were distributed into groups of 3 mice and inoculated with $1x10^5$ CFU per mice of either (a) S19, (b) S19Δ*luxR*::kan, (c) B. *abortus* 2308, (d) PBS. Two weeks post inoculation, which corresponded to the peak of splenomegaly, animals were euthanized by CO_2 asphixiation, and spleen, lungs, liver, kidneys and heart were harvested, fixed in 10% buffered formalin, paraffin embedded and stained with hematoxylin and eosin following a standard procedure. Histological changes were assessed between treatment groups.

Preparation of *B*. *abortus* -loaded microspheres. Alginate beads were prepared as previously described with some modifications(1). Briefly, $6x10^6$ CFU of the live *B. abortus* S19Δ*luxR*::kan or B. *abortus* S19 was re-suspended in 1ml of MOPS buffer (10mM MOPS, 0.85% NaCl, pH 7.4) and mixed with 5ml of alginate solution (1.5% sodium alginate, 10mM MOPS, 0.85% NaCl, pH 7.3). VpB was added as a component of the alginate core (S19Δ*luxR*::kan/core) by the addition of 1mg of VpB to the bacteria-alginate suspension described above. Spheres were obtained by extruding the suspension through a 200 micron nozzle into a 100mM calcium chloride solution and stirred for 15 minutes using the Inotech encapsulator I-50 (Inotech Biosystems International, Rockville USA). After extrusion of the bacteria-alginate mixture into the $CaCl_2$, the capsules were washed twice with MOPS for 5 minutes and further crosslinked with 0.05% poly-L-lysine (PLL, MW 22,000 Sigma, USA) for 10 minutes. Following two successive washes, the beads were stirred in a solution of 0.03% (w/v) alginate for 5 min to apply a final outer shell and washed twice with MOPS before storage at 4°C. To determine the bacterial viability post- encapsulation, 1 ml of capsules was removed from the encapsulator prior to permanent crosslinking with poly-L-lysine. The capsules were allowed to settle and washed twice with MOPS buffer and particles were dissolved using

1 ml of depolymerization solution (50 mM Na$_3$-Citrate, 0.45% NaCl 10mM MOPS, pH

7.2) with stirring for 10 min. Bacterial number (CFU/ml) per ml of capsules was

determined by plating onto TSA plates.

Immunization of mice. Twenty 4-6 week-old female BALB/c mice were

randomly distributed into groups of 5 mice for intraperitoneally (IP) vaccination. Animals

were given a single dose of vaccine containing 1x10^5 CFU of either (a) encapsulated *B.*

abortus S19Δ*luxR*::kan in alginate with VpB inside the capsule (S19Δ*luxR*::kan /VpB

core), (b) Nonecapsulated *B. abortus* S19Δ*luxR*::kan, Control groups received 1x10^5

CFU of either (a) nonencapsulated *S19,* (b) Empty capsules (no bacteria entrapped)

resuspended in 100μl of MOPS.

Evaluation of *Brucella* specific antibody. To determine the effect of

encapsulation in the production of anti-*Brucella*-specific antibody in sera from inoculated

mice, 100μl of blood was taken from each mouse after 0, 3, 7 weeks post vaccination

and 1 week post-challenge. Serum was separated and used for IgG1 and IgG2a

determination by ELISA. Heat killed and sonicated *B. abortus* whole cell antigen was

used to coat 96 well plates (Nunc-Immuno plates) at a concentration of 25μg total

protein/well. Following overnight incubation at 4°C, plates were washed, blocked with

0.5 ml of blocking buffer [0.25% (w/v) BSA] then incubated with mouse sera diluted

1:100 in the same blocking buffer for 1 hr at room temperature. Following extensive

washing to remove unbound antibody, goat anti-mouse IgG1 or IgG2a horseradish

peroxidase (HRP) conjugate (Serotec, USA) was added at a concentration of 500ng/ml

of antibody and incubation continued for an additional hour. Following this incubation,

the plates were washed again and HRP substrate was added, and incubated for 18

minutes. The reaction was stopped by addition of 50 μl of 0.5M NaOH and the

absorbance measured at 450 nm (A_{450}). All assays were performed in triplicate and repeated at least three times.

Efficacy of vaccination. At selected times post vaccination, mice (n=5 per group) were challenged IP using 1×10^5 CFU/mouse of *B. abortus* wild-type 2308. One week post challenge, mice were euthanized by CO_2 asphyxiation and spleens were removed, weighed and homogenized in 1ml of peptone saline. Serial dilutions were prepared and 100 μl portions were plated onto TSA plates. In some instances, 200-1000μl of spleen homogenate was plated to determine organism recovery. To differentiate between vaccine candidate and the challenge strain, each dilution was also plated on TSA with kanamycin to identify any residual kanamycin resistant vaccine strain present. Levels of infection were expressed as the mean ± SEM of individual $\log_{10}$ CFU/spleen. The efficacy of the vaccine compared to naïve animals was determined by subtracting the mean CFU/spleen recovered from mice vaccinated with the nonencapsulated or encapsulated vaccine from the mean CFU/spleen recovered from naïve non-vaccinated but challenged mice. The efficacy of the encapsulated vaccine compared to the nonencapsulated vaccine was determined by subtracting the mean CFU/spleen recovered from mice vaccinated with the capsules from the mean CFU/spleen recovered from mice immunized with the nonencapsulated vaccine.

Determination of TNFα cytokine production *In vivo.* At 0, 3, 7 weeks post-vaccination and 1 week post-challenge, BALB/c mice animals were bleed and serum was obtained from each individual mouse for cytokine determination. Quantification of TNFα was determined by ELISA (Ebioscience, USA) following manufacturer's instructions. For each cytokine, standards ranging from 0.2 to 1000 pg/ml were performed. Background levels obtained from t=0 were subtracted from the samples and

the differences between the encapsulated and nonencapsulated formulations at each timepoint were determined by ANOVA. Results are expressed as the mean pg/ml of each cytokine.

Statistical procedures. Macrophage infection and survival was expressed as the mean log CFU ± SD for each group. Intensity of infection (bacterial clearance and efficacy of vaccine) at each time point was expressed as mean log CFU±SEM for each group. IgG production was expressed as the mean absorbance ± SEM. Cytokine production *in vitro* was expressed as mean cytokine concentration ± SD for each group of five mice. The significance of differences between groups was determined by analysis of variance (ANOVA). *P* values <0.05 were considered statistically significant.

RESULTS

        B. *abortus* S19Δ*luxR*::kan is more attenuated for survival in macrophages and in mice than its parental strain. To determine the role of the deletion of the *luxR* gene in virulence in S19, J774A.1 macrophages were infected with the marked deletion mutant and compared to the parental strain and to the wild-type *B. abortus* 2308. Using an MOI of 100, the ability of the bacteria to enter and survive within this cell line was evaluated. One hour post- infection (t=0) there was no difference (p>0.05) between the S19 strain or *B. abortus* 2308 and S19Δ*luxR*::kan in the number of bacteria infecting the cell. By 48 hrs post infection, there was a 0.96 log difference (P<0.005) in the number of organisms infecting the cell for S19 vs wild-type 2308 or 1.47 logs difference between S19Δ*luxR*::kan and wild- type 2308. When the marked mutant was compared against the parental strain there was a 0.51 log reduction in the number of bacteria surviving inside the macrophage (P<0.005) (Fig. 25). These results indicate that S19 is not capable of replicating in the same degree when the *luxR* is deleted.

        To determine the effect of S19Δ*luxR*::kan in vivo, mice were inoculated intraperitoneally with 1×10^6 CFU/mice of S19Δ*luxR*::kan or the parental strain S19. Compared to S19, colonization of S19Δ*luxR*::kan in the spleen did not differ (p >0.05) at 1,3,5 and 7 weeks , only at 9 weeks bacterial colonization was reduced to significant (p<0.005) levels compared to S19 (Fig 26). Interestingly, inflammation in the spleen was completely abolished in animals that received the S19Δ*luxR*::kan (Fig 27, 28).
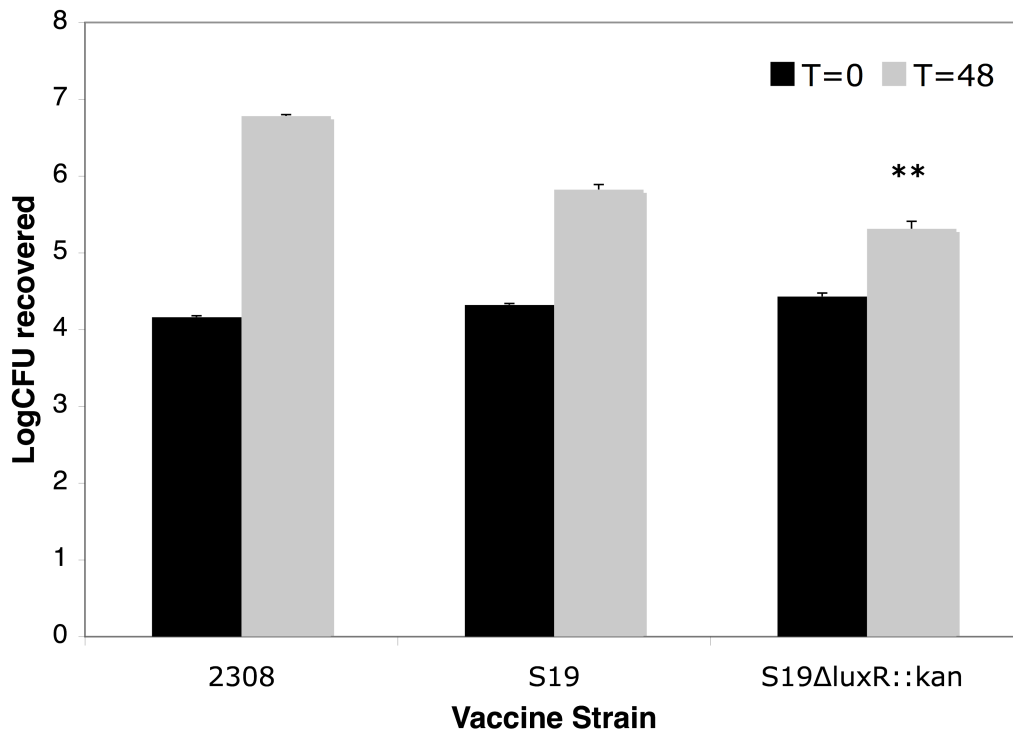
FIG. 25. Survival of the B. *abortus* S19Δ*luxR*::kan mutant in J774A. 1

macrophages. Wild-type strain 2308, S19 and *the B. abortus* S19Δ*luxR*::kan mutant
were used to infect J774A.1 macrophages at a multiplicity of infection of 1:100. After 30
min of incubation followed by 1 hr of treatment with gentamicin, infected macrophages
were further incubated for 0 or 48h. Treated cells were lysed, serially diluted and plated
on TSA or TSA/kanamycin plates for CFU determination. Values are the represented as
the mean of three independent experiments ± SEM. Differences in macrophage
colonization by S19 and S19Δ*luxR*::kan are indicated (**P<0.005)
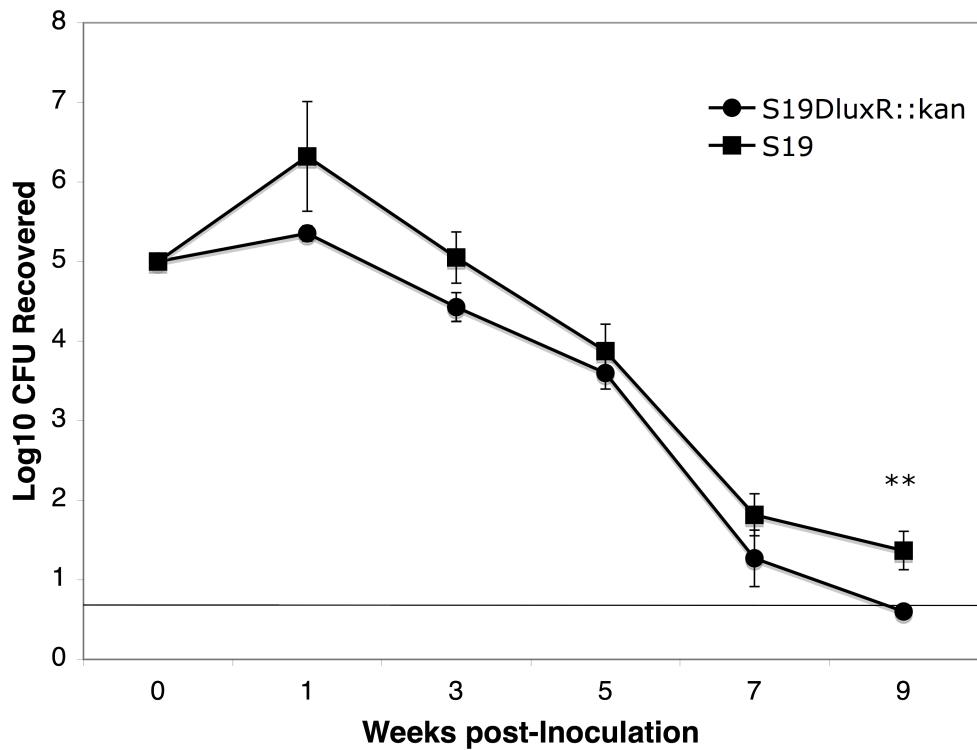
FIG 26. Kinetics of clearance of *B*. *abortus* S19Δ*luxR*::kan after infection. BALB/c mice (n=5/ time point) were infected with 1 x10^6 CFU/mouse of wild-type 2308 or S19Δ*luxR*::kan. At 1,3,5,7 and 9 weeks post-infection, mice were euthanized and the spleens were assessed for bacterial colonization. Values are the means of individual mice ± standard error of the mean. Differences in colonization were determined by ANOVA comparing the wild-type to the mutant (**P<0.005). The solid line represents the limit of detection which is ≥5 CFU.
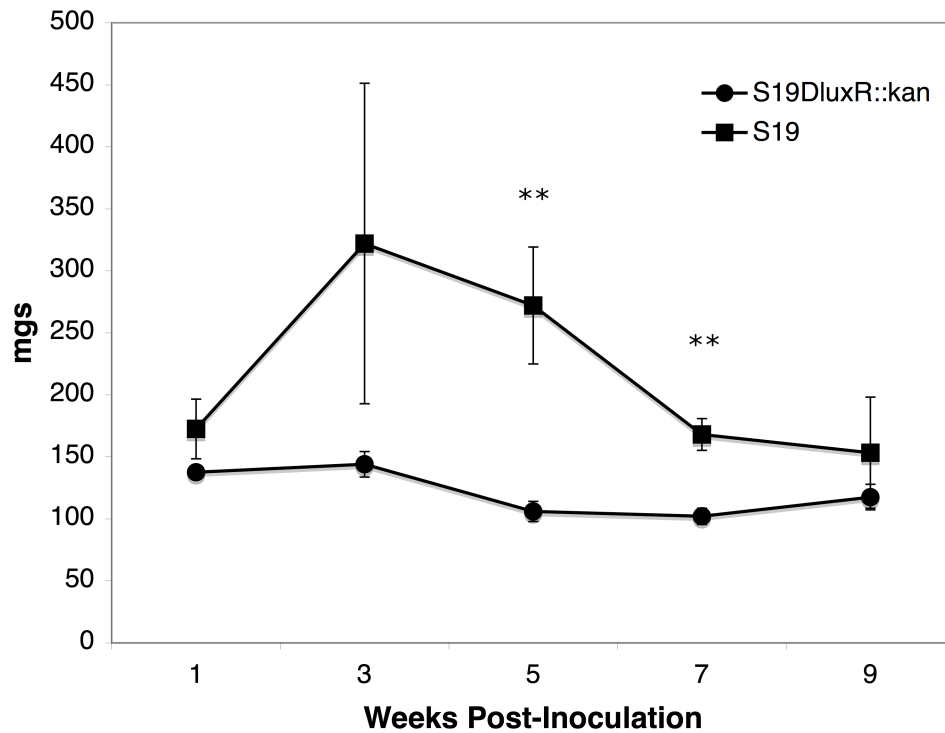
FIG. 27. Spleen weights after *B. abortus* S19Δ*luxR*::kan infection. BALB/c mice

were infected with 2 x10^6 CFU/mouse of wild-type 16M or *luxR::Tn5*. At 1,3,5,7 and 9

weeks post-infection, mice were euthanized and the spleens were weighed to determine

the degree of inflammation conferred by the mutant. Results are expressed as the mean

± standard error of the mean. Differences in weight were determined by ANOVA

comparing the wild-type to the mutant (*P<0.05) (**P<0.005).
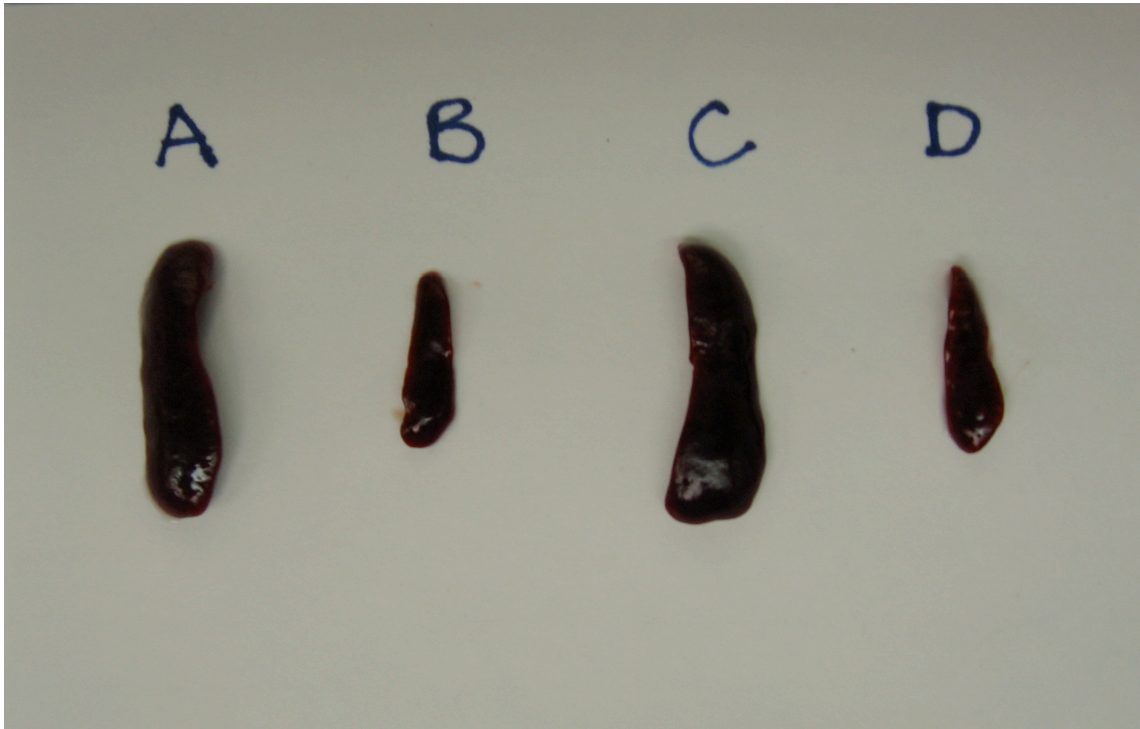
FIG. 28. Spleen morphology in BALB/c mice vaccinated with S19Δ*luxR*::kan. Mice were inoculated with 1×10^5 CFU of either a) B. abortus 2308, b) S19Δ*luxR*::kan, c) S19, control (d) received 100μl of PBS. Animals were euthanized 2 weeks post- inoculation and spleens were weighted, harvested and fixed for histology analysis.

Evaluation of histological changes in mice inoculated with S19Δ*luxR*::kan.

Due to the significant differences encountered in spleen size between animals

inoculated with S19Δ*luxR*::kan and S19 at 2 weeks post- inoculation, spleens from

mice inoculated with either S19Δ*luxR*::kan, S19, *B. abortus* 2308 or PBS were

evaluated for histological changes associated with the inflammation. The most dramatic

histologic changes of *B. abortus* 2308 and S19 but not in S19Δ*luxR*::kan inoculated

individuals were observed in the liver and the spleen (Fig 29 A, B,C,D, E, F). Degree of

changes ranged from severe to minor inflammatory changes depending on the

treatment group.

In mice inoculated with B. *abortus* 2308, changes observed in the spleen were

severe (occupying approximately 50% of the tissue section examined). The lesions

were characterized by moderate hyperplasia of the marginal zones and lymphoid

follicles having pale centers. The marginal zones were coalescing between adjacent

structure and composed primarily of macrophages and fewer variably degenerate

neutrophils with occasional lymphocytes and plasma cells, which occasionally extended

inside the lymphoid and surrounded vessels. Extensions from the marginal zones into

the red pulp and islands of similar cells were found to occupy up to approximately 60%

of the red pulp. Diffusely, the splenic capsule was taut. Multifocally within lymphoid

follicles was a small amount of intrahistiocytic and extracellular hyperesinophilic and

karyorrhectic debris. Additionally the liver was also compromised. Changes in the liver,

in a random distribution, included inflammatory aggregates composed primarily of

macrophages with varying amounts of lymphocytes and variably degenerate neutrophils

with small amount of hypereosinophilic and karyorrhectic. Bordering the inflammatory

aggregates were degenerating hepatocytes characterized by cytoplasmic hypereosinophilia and karyorrhexis. The inflammatory aggregates ranged from approximately 20 microns to 150 microns. Changes in the lung were inconsistent ranging from normal histologic appearance in one of the subjects to perivascular and peribronchiolar infiltrates of inflammatory cells composed of a small number of macrophages with lesser neutrophils and lymphocytes. The kidneys were generally unremarkable in all three of the members in the wild-type group (data not shown).

Finally, within the S19Δ*luxR*::kan inoculated group, the changes observed in the spleen were mild compared to the other groups. Changes in the spleen were mild enlargement of lymphoid follicles with pale centers containing immature lymphoid cells and rare intracellular and extracellular cellular and karyrrhectic debris. The marginal zone in these animals was only mildly hyperplastic and is composed of primarily histiocytes with lesser plasma cells and fewer lymphocytes and rare neutrophils. Within the red pulp, the plasma cell population was moderately elevated. The liver had multifocal, random, and scatter aggregates of histiocytes, lymphocytes and rare neutrophils that are often only composed of 5 to 10 cells in diameter but in one of the members of the group reach up to approximately 40 cells (50microns). The aggregates were primarily midzonal. There was no apparent and significant changes observed in the lung and the kidney. The epicardial surface of the heart has small aggregates of inflammatory cell composed primarily of lymphocytes with lesser histiocytes and neutrophils and two of the animals had multifocal aggregates of deeply basophilic, granular and amorphous material and a mild increase in fibrous connective tissue.
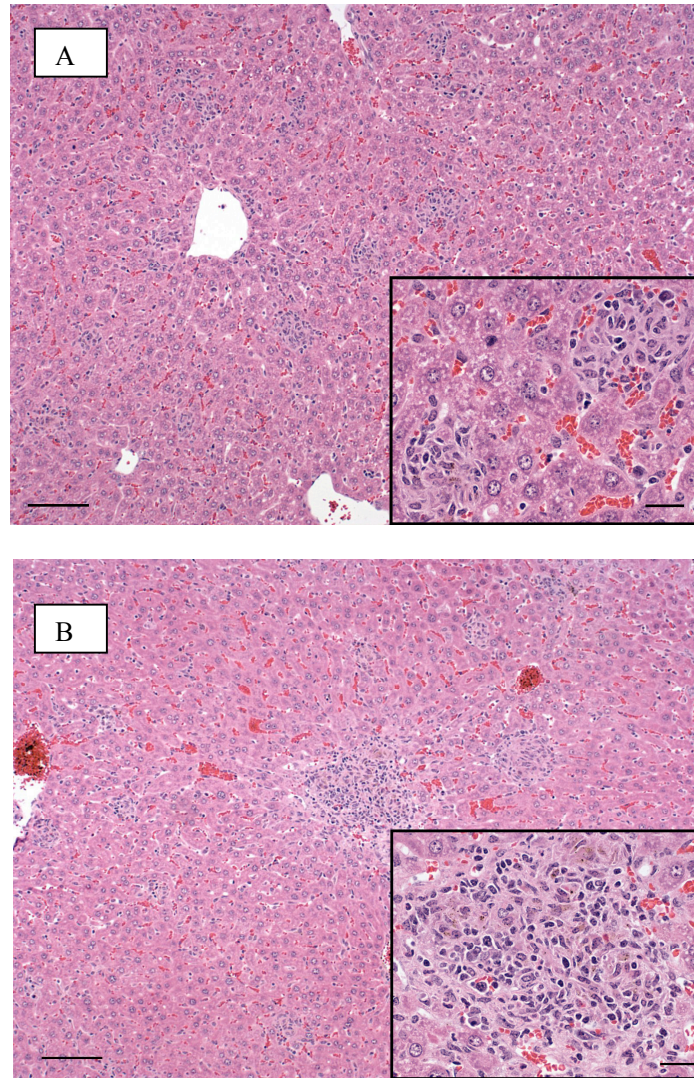
FIG. 29. Histological analysis of spleen (E-H) and liver (A-D) in BALB/c mice inoculated with either S19, S19ΔluxR::kan, _B.abortus_ 2308 or PBS (naïve). Notice the enlargement and coalescing of the white pulp, including marginal zones, and the formation of secondary lymphoid follicles in the spleen of mice vaccinated with either S19 (E) or 2308 (F). There was minimal enlargement of the marginal zone in mice vaccinated with S19ΔluxR (G) or naïve mice (H). In the liver, random distribution of inflammatory foci in mice vaccinated with either S19 (A), or 2308 (B) was observed. Normal liver appearance was observed in mice inoculated with either S19DluxR::Kan or PBS (C, D) Inset. Foci of inflammation are composed primarily of histiocytes. Bar = 100μm, bar = 25μm (inset).Bar = 200μm.

C.

D.
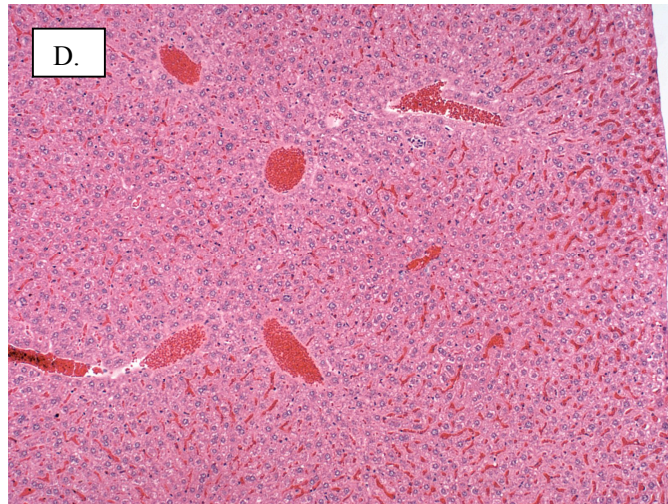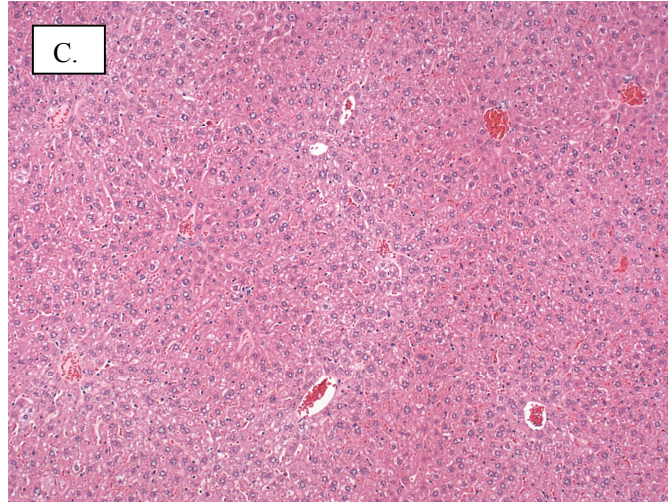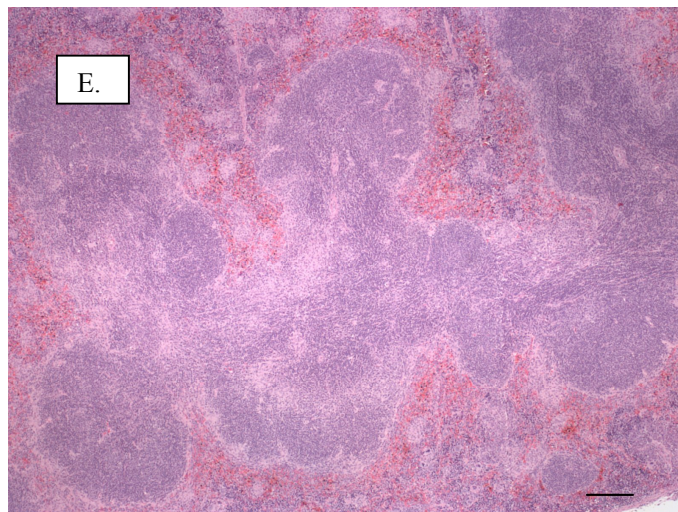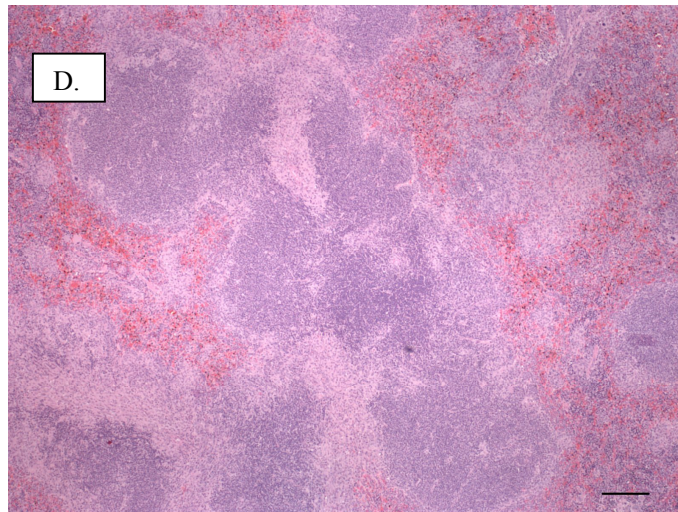
FIG. 29 (continued)

FIG. 29 (continued)

G.

H.
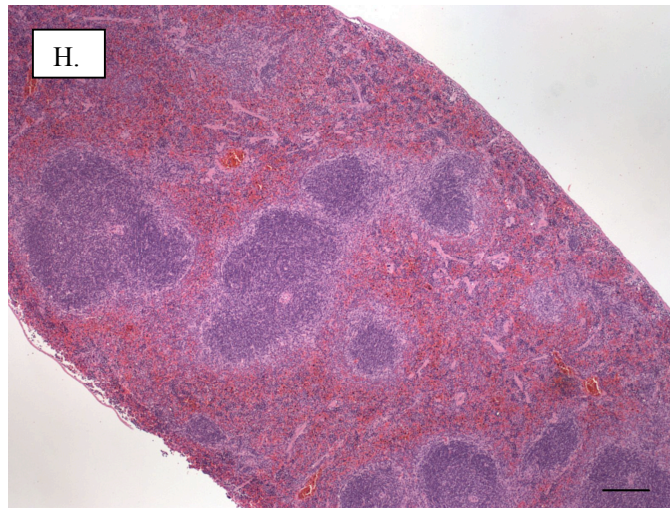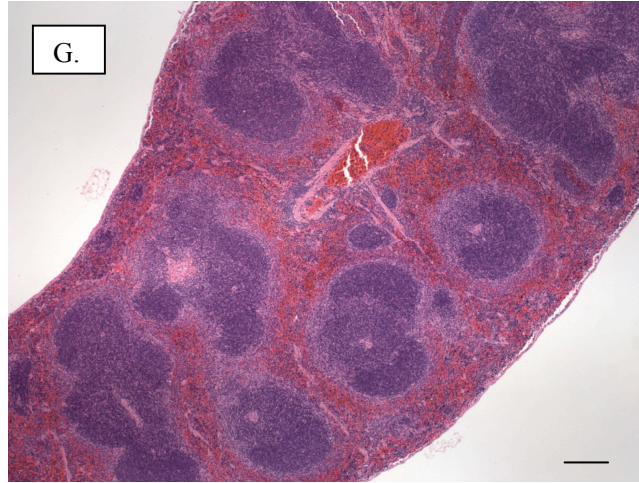
FIG. 29 (continued)

Evaluation of immune protection provided by S19Δ*luxR*::kan. In order to

determine the efficacy of the S19Δ*luxR*::kan mutant as a vaccine, the level of protection

provided by equal numbers of either S19Δ*luxR*::kan or S19 was evaluated against B.

abortus wild-type challenge at 20 weeks post- vaccination. To try to enhance the

efficacy of the S19Δ*luxR*::kan mutant, the strain was also encapsulated into alginate

VpB microbeads at the same dose. At 21 weeks post- vaccination (one week after

challenge), mice vaccinated with the S19Δ*luxR*::kan had statistically significant

decrease in bacterial load in the spleen relative to naïve mice with a 3.06 log (p <0.005)

reduction for the mutant and a 5.01 log (p <0.005) reduction for S19. Interestingly when

the mutant was given in a microencapsulated format, the efficacy of the vaccine was

increased by 0.75 logs (p < 0.005) compared to the nonencapsulated S19Δ*luxR*::kan

vaccinated mice (Fig 30). By encapsulating the organism, the S19Δ*luxR*::kan vaccine

efficacy was enhanced to a level that S19 and S19Δ*luxR*::kan were not statistically

significant between each other (P>0.15). Importantly, safety in immunization was

increased in mice vaccinated with the mutant strain, as these mice did not exhibit the

splenomegaly that the S19 vaccine caused in these animals (P<0.005) (Fig 31).
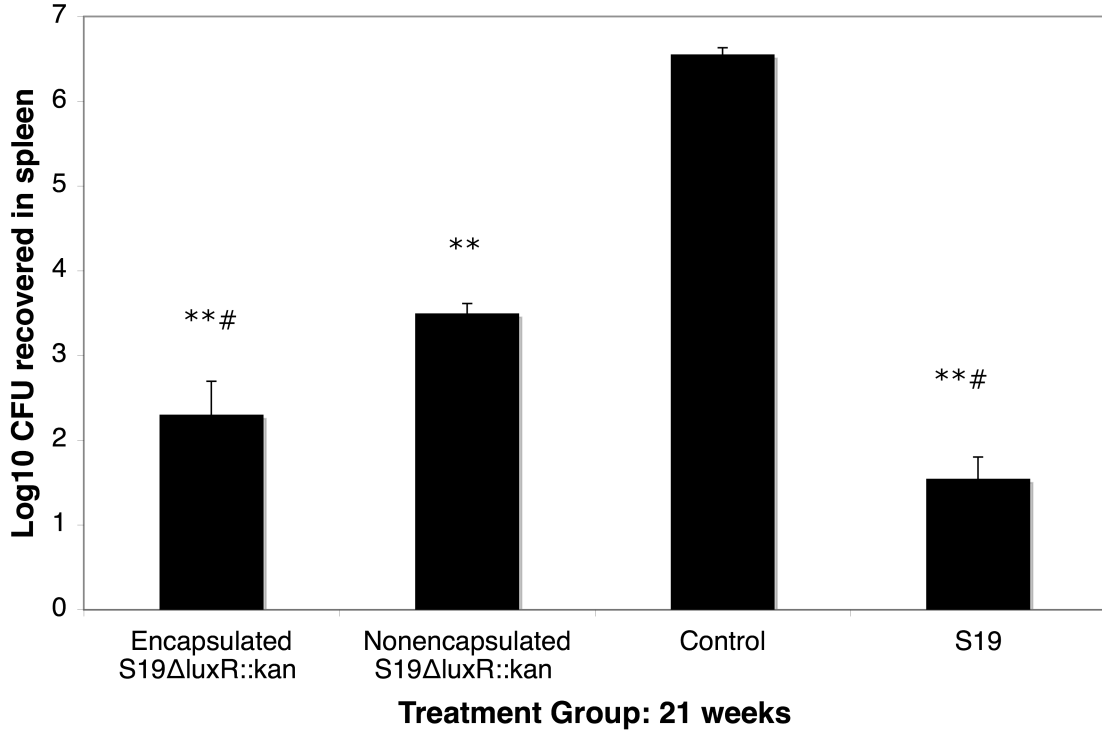
FIG. 30. Immunization efficacy of *B. abortus* S19Δ*luxR*::kan vaccine formulations.

BALB/c mice were immunized i.p. with 1x 10^5 of either nonencapsulated *B abortus*

S19Δ*luxR*::kan or encapsulated *B. abortus* S19Δ*luxR*::kan. Control groups received

empty capsules or S19 . After 20 weeks, mice were challenged i.p. with 1 x 10^5 CFU

wild-type 2308. One week post challenge, mice were euthanized, spleens harvested

and the bacterial load was determined. Values are reported as the mean $\log_{10}$ recovery

of 2308 from spleens. Differences in colonization between the vaccinated

groups and naïve mice was determined by ANOVA (**$P<0.005$). Differences between

encapsulated and nonencapsulated vaccine was determined by ANOVA (# $P<0.005$).
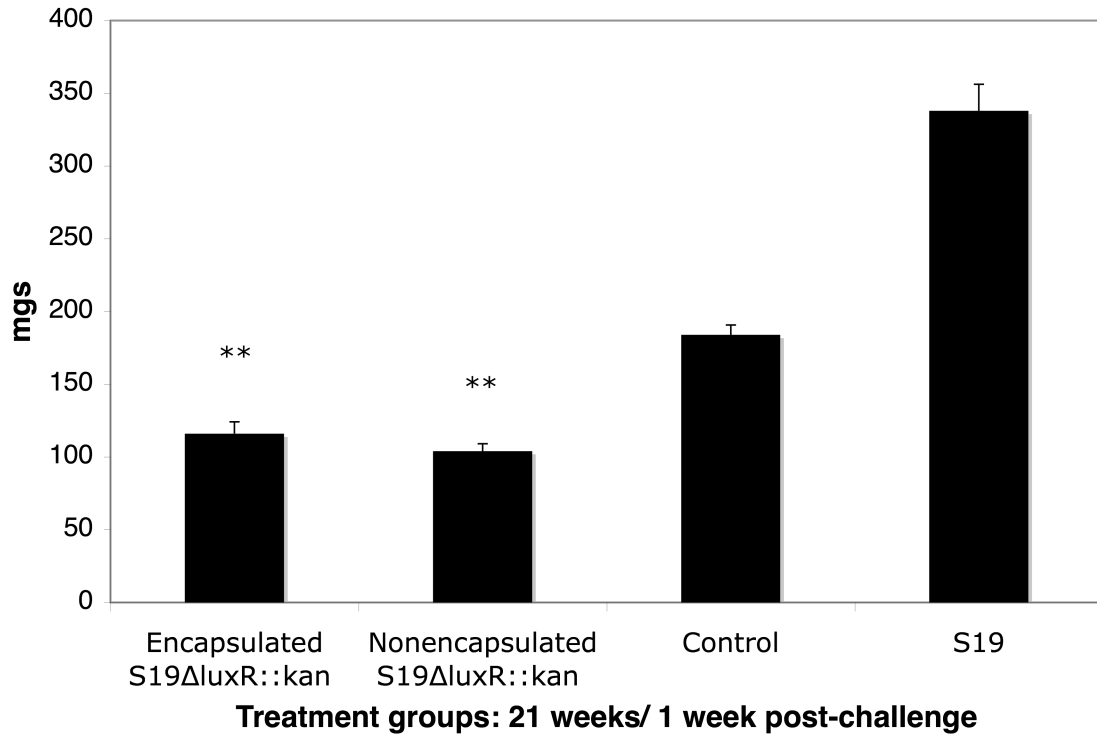
FIG. 31. Spleen weights after *B. abortus* wild-type challenge in animals

previously vaccinated with S19Δ*luxR*::kan. BALB/c mice were vaccinated with 1x10^5

CFU/mouse of S19, encapsulated S19Δ*luxR*::kan or nonencapsulated S19Δ*luxR*::kan.

At 20 weeks post-vaccination, mice were challenged with 1x10^5 CFU/mouse of wild-type

2308. One week post- challenge, mice were euthanized and the spleens were weighed

to determine the degree of inflammation conferred by the infection. Results are

expressed as the mean ± standard error of the mean. Differences in weight were

determined by ANOVA comparing the S19 vaccine strain with the mutant (**P<0.005).

Encapsulated S19Δ*luxR*::kan elicited stronger humoral responses than the nonencapsulated vaccine. Serum collected at 0,3, 7 and 21 weeks post-vaccination (1 week post-challenge) was assayed for the presence of *Brucella*-specific IgG1 vs IgG2a antibodies by ELISA. Immunization with encapsulated S19Δ*luxR*::kan elicited stronger IgG1 and IgG2a (1:1 ratio) responses that the nonencapsulated mutant ($p < 0.005$) (Fig. 32,33). A higher IgG1 induction was seen throughout the course ($p > 0.05$). In this case, an induction of higher and sustained antibody levels coincides with increased protection of encapsulated S19Δ*luxR*::kan. One week post-challenge, animals vaccinated with the mutant elicited a stronger IgG1 responses (2.36: 1 ratio). Further studies to correlate IgG isotype and cytokine profile needs to be conducted.

S19Δ*luxR*::kan mutant failed to induce proinflammatory cytokines. To correlate the mild inflammatory response observed in mice vaccinated with the S19Δ*luxR*::kan mutant with cytokine expression in vivo, animals were bled at 0,3,7 and 21 weeks post vaccination (1 week post-challenge). Animals receiving the mutant by either formulation failed to induce TNFα at any timepoint (Fig. 34), consistently with the lack of inflammation seen in this animals by histopathology.
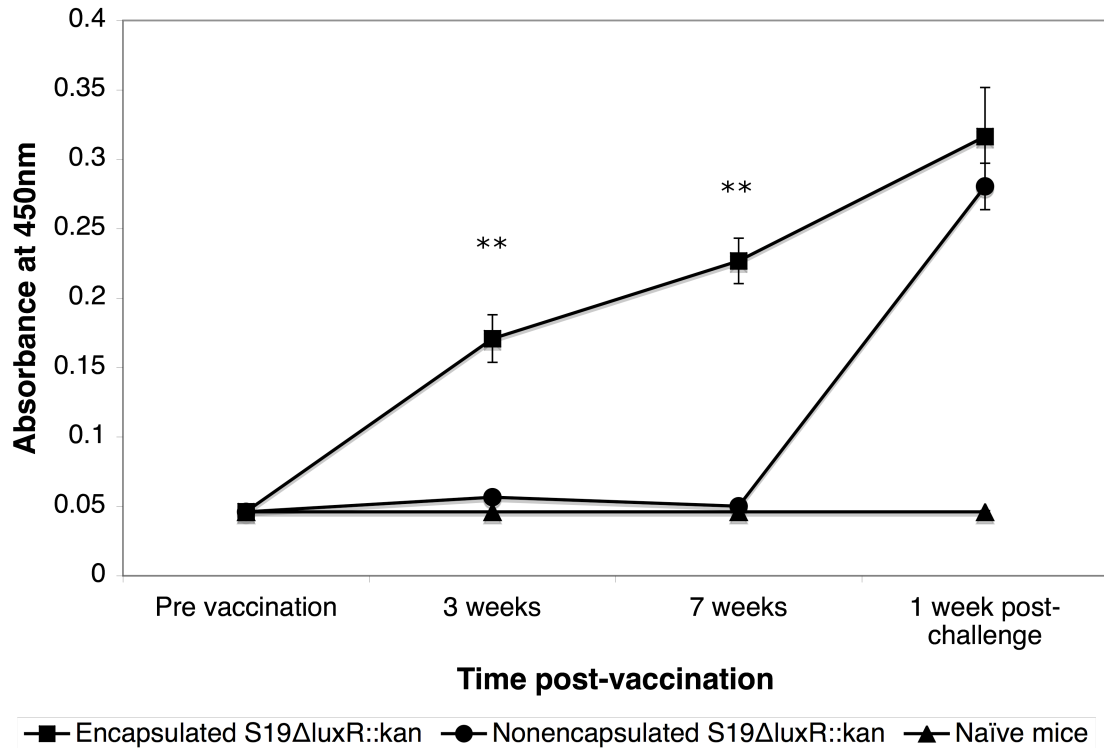
FIG 32. IgG1 anti-*Brucella* antibodies in serum from mice immunized with S19Δ*luxR*::kan. BALB/c mice were inoculated IP with 1x 10^5 CFU of either nonencapsulated S19Δ*luxR*::kan or encapsulated S19Δ*luxR*::kan. Control group received empty capsules. At 0,3,7 and 21 weeks post vaccination (1 week post-challenge), serum samples were collected and used for IgG1 determination by ELISA. Results are shown as the mean ± standard deviations of absorbance at A_{450}.
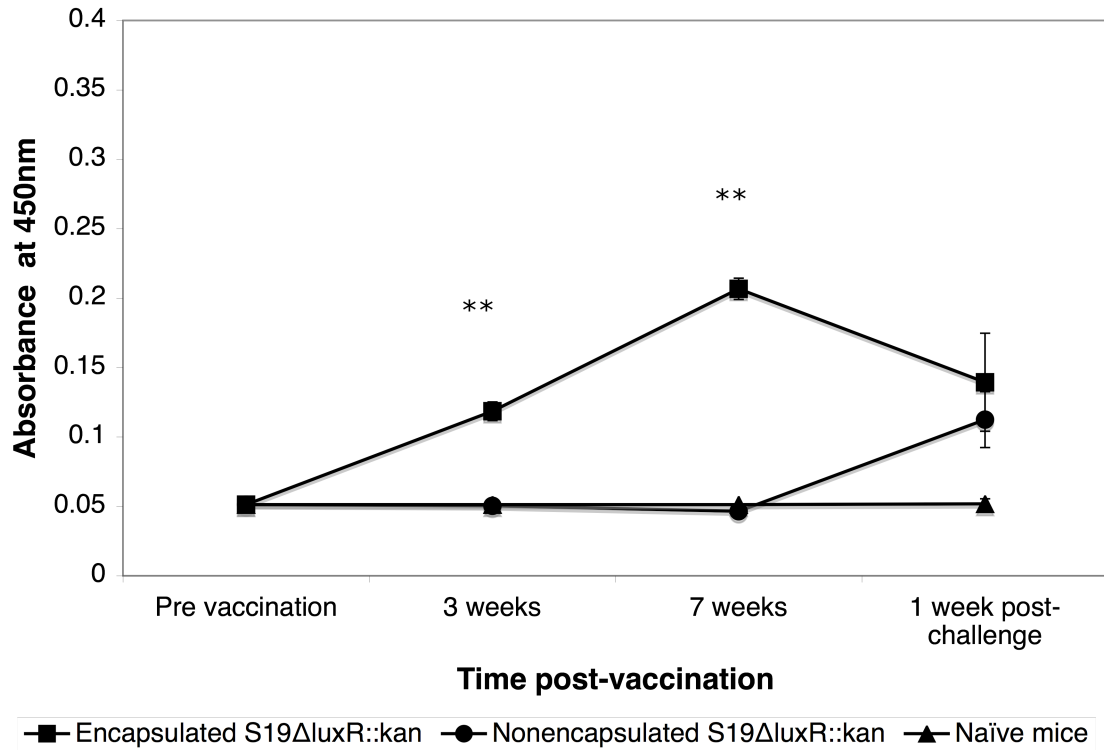
FIG 33. IgG2a anti-*Brucella* antibodies in serum from mice immunized with

S19Δ*luxR*::kan. BALB/c mice were inoculated IP with 1x 10^5 CFU of either

nonencapsulated S19Δ*luxR*::kan or encapsulated S19Δ*luxR*::kan. Control group

received empty capsules. At 0,3,7 and 21 weeks post vaccination (1 week post-

challenge), serum samples were collected and used for IgG1 determination by ELISA.

Results are shown as the mean ± standard deviations of absorbance at A_{450}
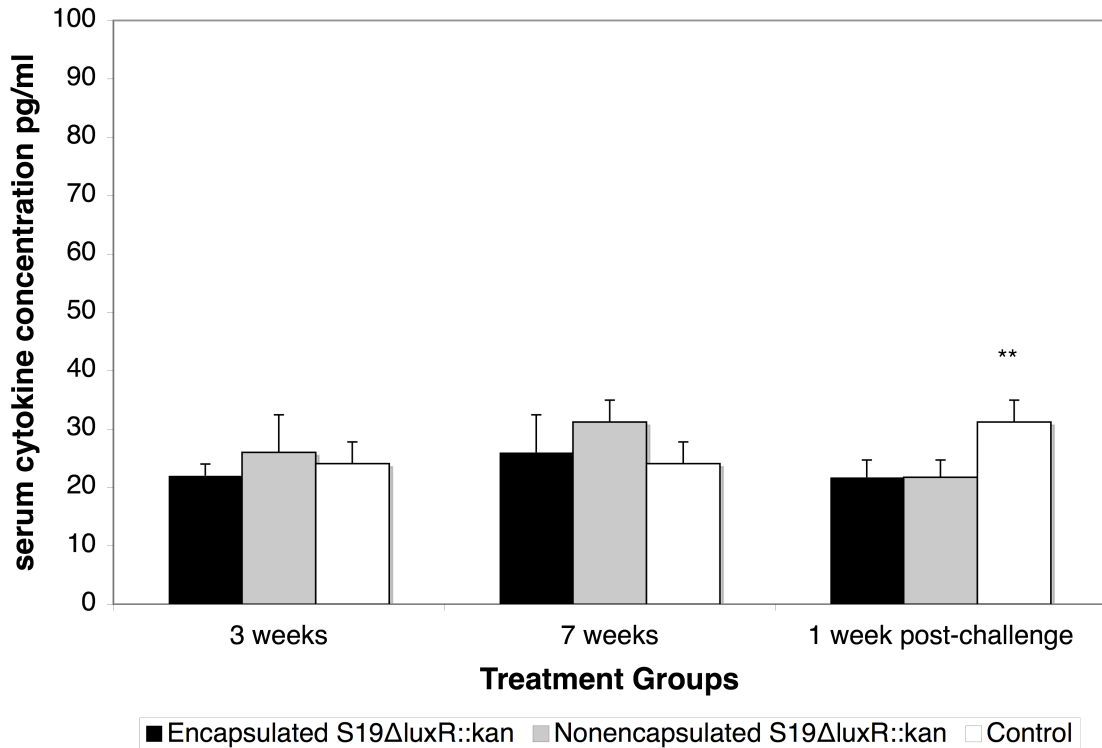
FIG 34. TNFα production in BALB/c mice immunized with S19Δ*luxR*::kan. Mice

were vaccinated with 1 x 10^5 CFU of either nonencapsulated B. *abortus* S19Δ*luxR*::kan,

encapsulated B. *abortus* S19Δ*luxR*::kan. Control group received empty capsules. Mice

were bled at 0,3,7 weeks post-vaccination and 1 week post-challenge and TNFα

determination from the serum was performed by ELISA. Results are represented as the

mean cytokine concentration ± SDEV for each group of five mice. The significance of

differences between nonencapsulated and encapsulated formulations at each timepoint

was determined by ANOVA (**P<0.005).

DISCUSSION

The development of vaccines to control brucellosis has proven to be a challenge for years. Extensive use of S19 vaccine has played an enormous role in reducing the disease in cattle but, it became clear that this vaccine in its existing form is of limited use in controlling the disease in humans and in wildlife populations (15). Years of investigations has lead to a better understanding of *Brucella* virulence and the correlates of protective immunity, so that vaccines superior to S19 can be developed. The observation that the highest levels of protection are obtained when the host is immunized with live vaccines, indicates that persistence and vaccine viability are key aspects required for an efficacious anti-brucellosis vaccine (37, 82).

Previous studies in our lab, have identified genes related to survival and virulence using transposon-based mutagenesis strategies. Among the genes identified, B. *melitensis luxR* mutants have been evaluated for survival in macrophages and the mouse model to confirm attenuation and immune potential. We have previously demonstrated that B. *melitensis luxR* mutants are suitable vaccine candidates due to their ability to generate protection in BALB/c mice. Also, by using this mutant we were able to increase safety by preventing splenomegaly in inoculated mice. Currently available vaccine strains S19 and Rev-1 induce splenomegaly in mice (67). In this study, we generated a marked deletion mutant in the S19 vaccine strain with the aim of increasing safety and possible use of this vaccine in other populations.

S19Δ*luxR*::kan mutant was evaluated for survival and attenuation in the macrophage and mouse model. As shown in this study, S19Δ*luxR*::kan was defective for survival in macrophages and cleared faster in BALB/c mice. In mice, during the initial

weeks post inoculation, the mutant and the parental strain did not elicit significant differences but S19Δ*luxR*::kan was completely cleared from the spleen of mice by 9 weeks. In contrast S19 inoculated mice, had   1.36 logs of recoverable bacterial by 9 weeks. S19Δ*luxR*::kan safety was further revealed by the lack of splenomegaly in inoculated mice. Even at the initial weeks post inoculation (1,3 and 5) when the bacterial load in the spleen was similar, the mean spleen weights in S19Δ*luxR*::kan was 137mgs vs. 172mg at 1 week, 144mgs vs. 322 mgs at 2 weeks and 106mgs vs. 272 mgs at 3 weeks for S19Δ*luxR*::kan or S19 respectively. Histological analysis supported this finding by indicating that animals that receive the S19Δ*luxR*::kan mutant did not elicit the degree of inflammatory response observed in S19 vaccinates. Furthermore, inflammatory changes observed in other organs including the liver was totally abolished with the mutant. In accordance with this results, the proinflammatory cytokine TNFα could not be detected in animals vaccinated with the mutant. This difference in inflammatory response exhibited by S19Δ*luxR*::kan provide an opportunity to evaluate its use as an improved vaccine candidate.

In vitro studies using antigen presenting cells have demonstrated that microencapsulated antigens are taken up and processed differently as compared to nonencapsualted materials (26). Similarly, in vivo data has shown that microencapsulation serves to modify the uptake, trafficking and processing of antigens (69). Additionally, recent reports demonstrate that persistence of the vaccine strain in the host is needed for the development of a suitable and long term immunity (37). Consistent with this, S19 vaccine exhibit only modest attenuation, meaning they survive longer in the host and produce the unwanted side effects such as the severe

inflammation reported here (fig 28). To try to enhance immunization efficacy, we investigated the vaccine potential of S19Δ*luxR*::kan when delivered in a controlled release vehicle. For this purpose, alginate a polysaccharide extracted from algae was used in combination with the protein vitelline protein B (VpB) derived from the parasite *Fasciola hepatica* as the capsular material used to entrap the S19Δ*luxR*::kan mutant (61, 73, 75). By encapsulating the organism we wanted to create the means of increased persistence (similar to the S19) but without causing the inflammation observed with this vaccine.

Protection studies against wild-type challenge with either S19 or S19Δ*luxR*::kan strains protected mice significantly, but the efficacy of the vaccine was reduced in the S19Δ*luxR*::kan mutant. This indicated that the *luxR* gene in S19 is necessary to induce a complete immunity towards *Brucella* infections. The reduced efficacy was successfully compensated by delivering the mutant vaccine in a sustained, microencapsulated format. Corroborating the observation of persistence as a function of vaccine efficacy. Encapsulation of live attenuated organisms is an interesting approach to improve immunization efficacy in potential vaccine candidates. It is important to mention that the viability of the bacteria was not compromised due to the mild conditions exposed during this procedure.

The degree of protection was conferred by either the encapsulated or nonencapsulated S19Δ*luxR*::kan mutants was compared to humoral profiles. Immunization with encapsulated S19Δ*luxR*::kan induced higher IgG1 and IgG2a levels compared to the nonencapsulated S19Δ*luxR*::kan mutant. Contrary to what was expected, 1 week post-challenge, animals that received the encapsulated vaccine,

mounted a stronger IgG1 response. Further investigations to determine the cytokine profiles elicited by these mice needs to be conducted to explain this finding.

A *luxR* knockout was created in the S19 vaccine and investigated for its potential use as an improved vaccine candidate. Vaccination with a sustained release vehicle to enhance vaccination efficacy was evaluated utilizing the live S19Δ*luxR* in encapsulated alginate microspheres containing a non-immunogenic eggshell precursor protein of the parasite *Fasciola hepatica* (Vitelline protein B, VpB). BALB/c mice were immunized intraperitoneally with either encapsulated or unencapsulated S19Δ*luxR* at a dose of 1×10^5 CFU per animal to evaluate immunogenicity, safety, and protective efficacy. Humoral responses post-vaccination indicate that the vaccine candidate was able to elicit an anti-*Brucella* specific IgG response even when the vaccine was administered in an encapsulated format. Safety was revealed by the absence of splenomegaly in mice that were inoculated with the mutant. Finally, a single dose with the encapsulated mutant conferred higher levels of protection compared to the unencapsulated vaccine. These results suggest that S19Δ*luxR* is safer than S19, induces protection in mice, and should be considered as a vaccine candidate when administered in a sustained release manner.

SUMMARY AND CONCLUSION

Brucellosis is an important zoonotic disease of nearly worldwide distribution. The occurrence of the infection in humans is largely dependent on the prevalence of brucellosis in animal reservoirs, including wildlife. The current vaccine strains used for cattle, S19 and RB51 have proven ineffective in protecting humans and wildlife populations including bison (*Bison bison*) and elk (*Cervus nelsoni*) from infection and abortion. To test possible improvements in vaccine efficacy, a novel approach of immunization was examined using alginate microspheres containing a non-immunogenic eggshell precursor protein of the parasite *Fasciola hepatica* (vitelline protein B, VpB) to deliver new live vaccine candidates.

Brucella species defective in the *luxR* gene, were identified as attenuated in macrophages and mice, and were chosen as targets to create vaccine strains. This gene is involved in known virulence mechanisms of the type IV secretion system. Additionally current vaccine strains were re-evaluated in Red Deer.

In the mouse model, the *luxR::Tn5 B. melitensis* and S19Δ*luxR*::Km mutants proved to be suitable vaccine candidates based on its reduced virulence in macrophages and in BALB/c mice and ability to generate protection in the mouse model. We were able to demonstrate an improvement in the efficacy of the vaccine when the immunogen was given using a controlled release vehicle. In the case of *luxR*::Tn5 vaccinates, encapsulated immunized mice produced higher concentrations of circulating interleukin 12 (IL12) and gamma interferon (IFNγ), suggesting an induction of a Th1 response.

The deer model was necessary to assess the use of encapsulation in target species that are naturally infected with *Brucella*. Due to the fact that current vaccine strains do not confer a good protection against infection in Red Deer, a different methodology of vaccination using microencapsulation was investigated. The efficacy of both S19 and RB51 was enhanced when the vaccine was given in a sustained release format. Together, these results suggest that microencapsulation of live *Brucella* produces an enhanced delivery vaccine system against brucellosis increasing the efficacy of poorly-performing nonencapsulated vaccine candidates.

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VITA

ANGELA MARIA ARENAS GAMBOA

ADDRESS

440 Reynolds Medical Building. Room 420

College Station, Texas 77843-1114

EDUCATION

Ph.D. Veterinary Microbiology, Texas A&M University- May 2007

DVM. La Salle University- February 2002