ORAL VACCINATION WITH MICROENCAPSULED STRAIN 19 VACCINE CONFERS ENHANCED PROTECTION AGAINST BRUCELLA ABORTUS STRAIN 2308 CHALLENGE IN RED DEER (CERVUS ELAPHUS ELAPHUS)

Authors: Arenas-Gamboa, Angela M., Ficht, Thomas A., Davis, Donald S., Elzer, Philip H., Kahl-McDonagh, Melissa, et al.

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Angela M. Arenas-Gamboa,[1,2,4] **Thomas A. Ficht,**[1] **Donald S. Davis,**[1] **Philip H. Elzer,**[3] **Melissa Kahl-McDonagh,**[1] **Alfredo Wong-Gonzalez,**[1] **and Allison C. Rice-Ficht**[1,2]

[1] Department of Veterinary Pathobiology, Texas A&M University and Texas Agricultural Experiment Station, College Station, Texas 77845-1114, USA

[2] Department of Molecular and Cellular Medicine, Texas A&M Health Science Center, College Station, Texas 77845-1114, USA

[3] Department of Veterinary Science, Louisiana State University, Ag Center Baton Rouge, Louisiana 70803, USA

[4] Corresponding author (email: aarenas@cvm.tamu.edu)

ABSTRACT: Bison (*Bison bison*) and elk (*Cervus elaphus nelsoni*) in the Greater Yellowstone Area (GYA), USA, are infected with *Brucella abortus*, the causative agent of bovine brucellosis, and they serve as a wildlife reservoir for the disease. Bovine brucellosis recently has been transmitted from infected elk to cattle in Montana, Wyoming, and Idaho and has resulted in their loss of brucellosis-free status. An efficacious *Brucella* vaccine with a delivery system suitable for wildlife would be a valuable tool in a disease prevention and control program. We evaluated Strain 19 (S19) in a sustained release vehicle consisting of alginate microspheres containing live vaccine. In a challenge study using red deer (*Cervus elaphus elaphus*) as a model for elk, alginate, a naturally occurring polymer combined with a protein of *Fasciola hepatica* vitelline protein B was used to microencapsulate S19. Red deer were orally or subcutaneously immunized with 1.5×10^{10} colony-forming units (CFUs) using microencapsulated S19. Humoral and cellular profiles were analyzed bimonthly throughout the study. The vaccinated red deer and nonvaccinated controls were challenged 1 yr postimmunization conjunctivally with 1×10^9 CFUs of *B. abortus* strain 2308. Red deer vaccinated with oral microencapsulated S19 had a statistically significant lower bacterial tissue load compared with controls. These data indicate for the first time that protection against *Brucella*-challenge can be achieved by combining a commonly used vaccine with a novel oral delivery system such as alginate-vitelline protein B microencapsulation. This system is a potential improvement for efficacious *Brucella*-vaccine delivery to wildlife in the GYA.

Key words: *Brucella abortus*, brucellosis, *Cervus elaphus*, encapsulation, red deer, vaccine.

INTRODUCTION

Brucella abortus, a facultative intracellular gram-negative bacterium, is the etiologic agent of bovine brucellosis, which is also a zoonotic disease of nearly worldwide distribution (Boschiroli et al., 2001).The bacterium is mainly transmitted from infected animals to other animals and humans through contact with contaminated animal tissues, excretions, and secretions (Corbel, 1997). Human brucellosis (undulant fever) can be a severe and debilitating disease requiring prolonged treatment and often leaving permanent sequelae (Pappas et al., 2005). Moreover, brucellosis is a major cause of direct and indirect economic losses for livestock industries.

In the United States, *B. abortus* has been largely eradicated in cattle, but it continues to be a problem in bison (*Bison bison*) and elk (*Cervus elaphus nelsoni*) in the Greater Yellowstone Area (GYA), USA (Davis and Elzer, 2002). The prevalence of brucellosis within these wildlife populations constitutes a health problem mainly because of the threat of free-ranging wildlife transmitting bovine brucellosis to cattle and possibly to humans. Within the past few years, several *B. abortus*-infected cattle herds have been identified in Wyoming, Idaho (Holland, unpublished), and Montana (Cross et al., 2007) in which the source of the infection was presumed to be bison or elk (Holland, 2004). The continued prevalence of *B. abortus* in the GYA and surrounding states can, in large

part, be attributed to the wildlife management practices of supplemental feeding implemented during the winter. The winter feed grounds in the GYA have resulted in significant over congregations of elk and increased the risk of intraspecific and interspecific transmission of *B. abortus* (Davis and Elzer, 2002).

Current vaccine strains used to control brucellosis in cattle have proven to be less effective in wildlife. Data from controlled experiments suggest that Strain 19 (S19) prevents abortion in only 30% of immunized elk (Davis and Elzer, 2002), whereas RB51, when given in conventional format, is ineffective in elk (Thorne et al., 1980; Kreeger et al., 2002a; Cook et al., 2002), and of significantly reduced efficacy in bison (Davis and Elzer, 2002). Reduced efficacy of the vaccine strains arises from difficulties with vaccination of wildlife species but also from the delivery method used to immunize the animals. Although the efficacy of S19 in elk is not comparable with its performance in cattle, S19 has been used since 1985 in Wyoming elk, and it is presently the only available alternative. The method used to immunize elk in Wyoming is by remote ballistic injections of biodegradable hollow pellets containing lyophilized S19 from distances of up to 30 m (Olsen et al., 2006). Issues associated with this technique include excessive time, labor, logistics, and elevated cost as well as inconsistencies in injection site, route of injection, and amount of vaccine delivered.

It is generally acknowledged that the removal of winter feeding is not a viable strategy for the short-term prevention and control of brucellosis in wildlife in the GYA. Alternative prevention and control measures such as improvement of vaccine efficacy and more suitable techniques to deliver vaccines to free-ranging wildlife need further investigation not only to reduce the prevalence of brucellosis in wildlife but also to reduce the risk of transmission from infected wildlife to cattle and possibly humans.

We have investigated the possibility of delivering the currently licensed vaccine S19 in a controlled microencapsulated format. We have demonstrated previously an increased efficacy when live *Brucella* vaccine candidates are delivered in microspheres containing alginate, a naturally occurring biopolymer combined with vitelline protein B (VpB) of the sheep liver fluke, *Fasciola hepatica*. This protein possess an unusual enzymatic resistance to breakdown, modifying the erosion time of the capsule content (Arenas-Gamboa et al., 2008). In the present study, we evaluated the vaccine efficacy of S19 when delivered by oral or subcutaneous routes.

MATERIALS AND METHODS

Animals

Fifty-four 1- to 2-yr-old female red deer (*Cervus elaphus elaphus*) were bought from a privately owned tuberculosis- and brucellosis-free herd. This species was chosen as an animal model for Rocky Mountain elk (*Cervus elaphus nelsoni*) because of its close genetic relationship, ease of handling, and animal husbandry. Upon arrival, animals were retested for anti-*Brucella* immunoglobulin G (IgG) levels (total IgG) by enzyme-linked immunosorbent assay (ELISA), dewormed (using Moxidectin and Cydectin, Wyeth, Madison, Wisconsin, USA), and allowed to acclimate for 3 mo. For experimental purposes, the red deer were randomly assigned into six treatment groups ($n=9$). All animal care and experimental procedures were performed in compliance with institutional animal care and guidelines as required at Texas A&M University.

B. abortus S19 and strain 2308 cultures

Bacteria used in these experiments include B. *abortus* S19 used as the vaccine 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 hr. Bacteria were harvested by scraping off of the plate surface into phosphate-buffered saline (PBS). The harvested bacteria were pelleted by centrifugation at 3,000 × G, and the pellet was resuspended in fresh PBS. This step was repeated two more times and the concentration of bacteria was determined by optical density using a standardized curve of optical

density (Klett units) vs. colony-forming units (CFUs). 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} CFUs/ml of the vaccine strain S19 were prepared as described previously for live bacteria (Abraham et al., 1996), with several modifications. In brief, enumerated live S19 vaccine strain (total 1.5×10^{11} for 10 doses) was resuspended in a total of 100 µl of 3-[*N*-morpholino] propanesulfonic acid (MOPS) buffer (10 mM MOPS and 0.85% NaCl, pH 7.4) and mixed with 10 ml of alginate solution (1.5% sodium alginate, 10 mM MOPS, and 0.85% NaCl, pH 7.3). Three-hundred-micrometer spheres were obtained by extruding the suspension through a 200-µm nozzle into a 100 mM calcium chloride solution and stirred for 15 min using the Inotech encapsulator I-50 (Inotech Biosystems International, Rockville, Maryland, USA). To permanently cross-link the capsule, microspheres were resuspended in MOPS buffer supplemented with 0.05% (w/v) poly-L-lysine (molecular weight 22,000) for 15 min. After 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 adding 1 mg of VpB to the bacteria-alginate suspension. All capsules were stored at 4 C in MOPS buffer until use.

Bacterial enumeration and viability

To determine the bacterial content per milliliter of alginate particles, 1 ml of capsules was removed from the encapsulator before permanent cross-linking with poly-L-lysine or VpB with poly-L-lysine. The capsules were allowed to settle and washed twice with MOPS buffer, and then particles were dissolved using 1 ml of depolymerization solution (50 mM Na_3-citrate, 0.45% NaCl, and 10 mM MOPS, pH 7.2) with stirring for 10 min. Bacterial number (colony-forming units per milliliter) per milliliter of capsules was determined by plating onto TSA plates.

Immunization of red deer

Red deer were randomly distributed into six treatment groups ($n=9$). Animals were given a single dose of vaccine containing either 1) encapsulated S19 without VpB (SC), 2) encapsulated S19 with alginate and VpB (SC), 3) encapsulated S19 without VpB (oral), or 4) encapsulated S19 with alginate and VpB (oral). Control groups received 1.5×10^{10} CFUs of either 1) nonencapsulated S19 (SC) or 2) 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 before vaccination, and 6, 12, and 17 days postvaccination. Serum samples were evaluated for total IgG 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, Nalge Nunc International, Rochester, New York, USA) at a protein concentration of 25 µg/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) bovine serum albumin to prevent nonspecific binding. The blocking buffer was removed, and deer serum samples diluted 1:100 in blocking buffer were incubated 2 hr at room temperature with rocking. After three more washes, horseradish peroxidase-conjugated goat anti-deer IgG (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA) was added to the wells at a dilution 1:1,000 and incubated at room temperature for 1 hr. After incubation, plates were washed as described above, and the peroxidase substrate *o*-phenylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, Missouri, USA) was added following the manufacturer's instructions and incubated for no more than 20 min. The reaction was stopped by the addition of 50 µl of 0.5 M NaOH, and the absorbance measured at 450 nm (A_{450}). All assays were performed in triplicate and repeated at least two times.

Lymphocyte proliferation assay from peripheral blood mononuclear cells

At 17 wk postvaccination, mononuclear cells were isolated from the buffy coats of peripheral blood as described previously, with some modifications (Waters et al., 2002). In brief, 2×10^5 cells/well were seeded in 96-well plates (Falcon, BD Biosciences, San Jose, California, USA) in RPMI medium containing 10% (v/v) fetal bovine serum, 1 mM L-glutamine, and 1 mM nonessential amino acids. Cells were stimulated with 1) *B. abortus* S2308 (wild-type) lysate at a concentration of 12.5 µg/ml, 2) concanavalin A (5 µg/ml), or 3) medium alone during incubation for 6 days at 37 C in atmosphere containing 5% (v/v) CO_2. After

incubation, 1 µCi of *methyl*-[³H]thymine was added to each well, and incubation was continued for an additional 18 hr. The cells were harvested onto glass fiber filters using a 96-well plate cell harvester, and the incorporated [³H]thymidine was determined using a liquid scintillation counter (Beckman Coulter, Fullerton, California, USA). Data are represented as mean counts per minute (cpm) ± SD.

Challenge and efficacy of vaccination

One year postvaccination, two to three animals from each group were housed in BL3-facility buildings (five to six animals per building) and acclimated for 2 wk before challenge. Animals were exposed conjunctivally using a challenge dose of 1×10^9 CFU/deer of *B. abortus* wild-type 2308 as confirmed by plating serial dilutions onto TSA plates. Two weeks postchallenge, 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 min using a stomacher. In addition, submandibular, mesenteric, and mammary lymph nodes were extracted, and each lymph node was homogenized in 1 ml 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 [Oxoid, Wesel, Germany]). Three to 5 days postinoculation, bacterial counts were enumerated. Results are represented as the mean±SEM CFUs/g tissue. For lymph nodes, results are represented as the mean±SEM CFUs/lymph node.

Statistical procedures

Immunoglobulin G levels elicited by vaccination are expressed as the mean±SD A_{450} for each group. For determination of cellular responses, cpm from each group is expressed as the mean±SD cpm. Efficacy of vaccination was expressed as mean±SEM log CFUs for each group. The significance of differences between groups was determined by analysis of variance using Prism software (GraphPad Software Inc., San Diego, California, USA). A *P* value <0.05 is considered statistically significant.

RESULTS

Encapsulated S19 elicited *Brucella*-specific IgG responses

Serum collected at different times postvaccination was assayed for *Brucella*-specific antibodies by ELISA. Immunization with S19 elicited an IgG response in subcutaneously vaccinated animals that was significantly different from naïve animals by 6 wk postvaccination (*P*<0.005). Animals that received the oral vaccine elicited lower detectable IgG levels at all time points, but 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 wk postvaccination, indicating a delayed release compared with all of the other groups, for which IgG peaked at 6 wk postvaccination (Fig. 1).

Encapsulated S19 enhances cellular immune responses

To determine the possible effect of encapsulation on the cellular response elicited in red deer vaccinated with S19, a cellular proliferation assay was performed. Animals were bled 17 wk postvaccination, and blastogenesis was performed using cells isolated from the buffy coat. Only animals receiving the encapsulated vaccine in which VpB was added to the core formulation via a subcutaneous route exhibited statistically significant proliferative responses compared with naïve animals (*P*<0.05). Animals receiving nonencapsulated vaccine failed to mount significant cellular responses (Fig. 2).

Evaluation of immune protection provided by encapsulated *B. abortus* S19

To determine the effect of encapsulation on immunization, the level of protection provided by vaccination with either encapsulated or nonencapsulated S19 was evaluated against wild-type challenge at 1 yr postvaccination. Recovery of challenge organism in spleen, lung, liver, and submandibular, mesenteric, and mammary lymph nodes was evaluated. Two weeks postchallenge, only red deer vaccinated orally with the encapsulated vaccine containing VpB in the formulation had a statistically significant lower bacterial load
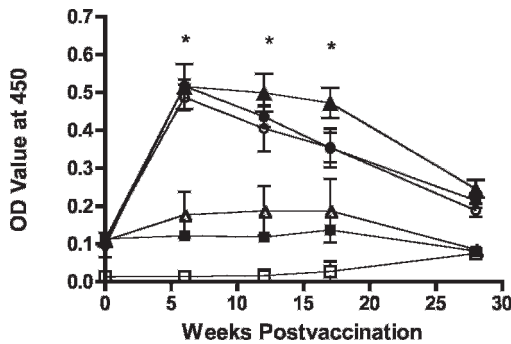
FIGURE 1. Immunoglobulin G (IgG) anti-*Brucella* antibodies in serum from deer vaccinated with vaccine strain 19 (S19). Red deer ($n=9$) were vaccinated subcutaneously (SC) or orally with 1.5×10^{10} colony-forming units of 1) unencapsulated *Brucella abortus* S19, 2) encapsulated *B. abortus* S19 without vitelline protein B (VpB), 3) encapsulated *B. abortus* with VpB, 4) encapsulated oral S19 without VpB, 5) encapsulated oral S19 with VpB, or 6) empty capsules (control). Serum samples collected 0, 6, 12, and 17 wk postvaccination were analyzed for IgG by enzyme-linked immunosorbent assay. Results are shown as means±SD of absorbance (OD=optical density) at 450 nm. *Values are statistically different ($P<0.005$) from the control by analysis of variance at each time point (S19/alginate SC S19/alginate vpB SC, S19 SC). Closed squares = S19/alginate oral; open squares = control nonvaccinated; open triangles = S19/alginate/VpB oral; closed triangles = S19/alginate SC; open circles = S19/alginate/VpB SC; closed circles = S19 SC.
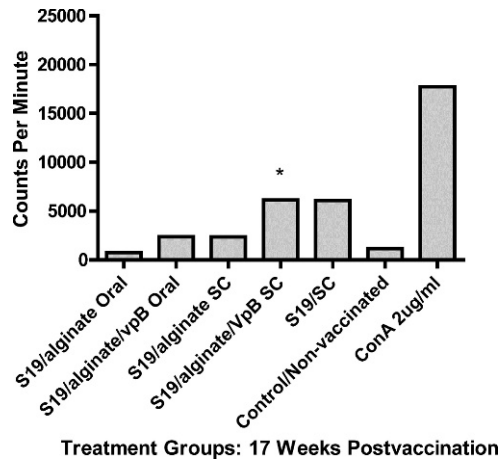

FIGURE 2. Lymphocyte proliferative responses of peripheral blood mononuclear cells from deer immunized with S19. Red deer ($n=9$) were vaccinated subcutaneously or orally with 1.5×10^{10} colony-forming units of 1) unencapsulated *Brucella abortus* S19, 2) encapsulated *B. abortus* S19 without vitelline protein B (VpB), 3) encapsulated *B. abortus* with VpB, 4) encapsulated oral S19 without VpB, 5) encapsulated oral S19 with VpB, 6) empty capsules (control), and 7) positive control (concanavalin A stimulation). Twelve weeks postvaccination, peripheral blood mononuclear cells were isolated, cultured at 37 C for 6 days, and pulsed for 18 hr with 1 μCi of *methyl*-[³H]thymine. Results are expressed as mean counts per minute±SD. *Value is statistically different from the control by analysis of variance ($P<0.05$).

in the spleen relative to naïve deer. Animals vaccinated using this formulation had a 1.85-log lower ($P<0.0005$) bacterial burden (Fig. 3A) compared with naïve animals. Colonization of wild-type 2308 in lung and liver was not significantly different between the control and the immunized groups, but animals that received the encapsulated oral vaccine with VpB had a lower bacterial burden ($P>0.05$; Fig 3B, C). Submandibular lymph nodes that were closer to the site of inoculation exhibited the highest CFU counts compared with the other sites (Fig. 4A–C). 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 significantly lower bacterial burden in the submandibular lymph nodes ($P<0.005$; Fig. 4A).

DISCUSSION

Wildlife vaccination represents a practical tool for the control of transmissible diseases that represent a health risk to livestock or humans. Common issues with vaccines for wildlife include the need for good protective immunity, minimization of side effects, safety, ease of handling and administration, and a low cost of production and delivery. Current vaccination strategies used to control brucellosis in the GYA do not fulfill these requirements: *B. abortus* S19 vaccine only confers protection in 30% of elk; it can produce abortion in a low percentage of vaccinated animals; and it is ballistically delivered, increasing the cost and risk of administra-
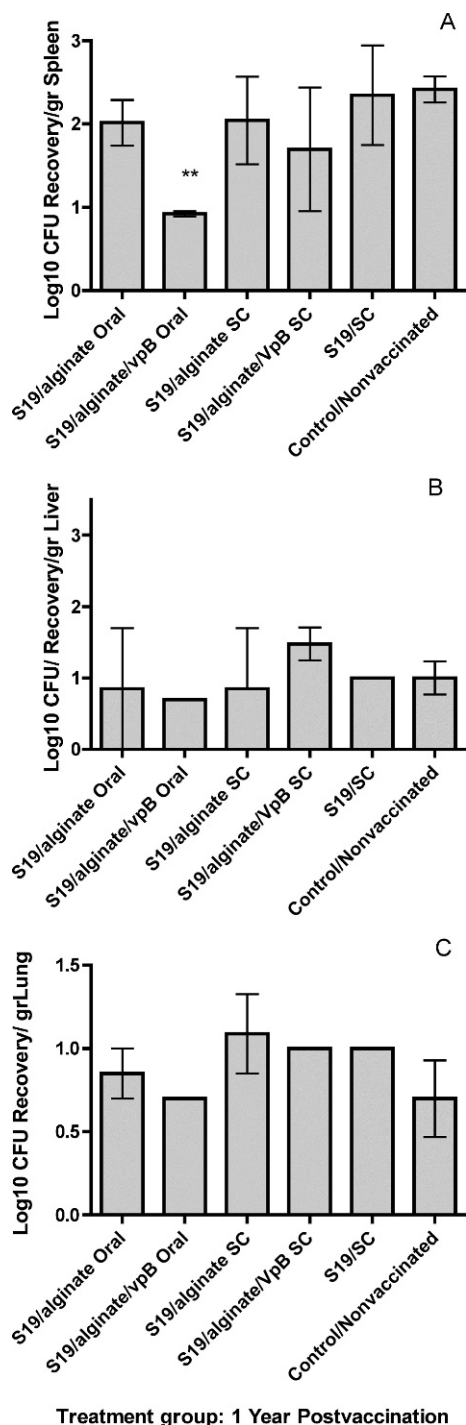
FIGURE 3. Vaccination efficacy against 2308 wild-type challenge. Red deer were vaccinated subcutaneously or orally with 1.5×10^{10} colony-forming units (CFUs) of 1) unencapsulated *Brucella abortus* S19, 2) encapsulated *B. abortus* S19 without vitelline protein B (VpB), 3) encapsulated *B. abortus* with VpB, 4) encapsulated oral S19 without VpB, 5) encapsulated oral S19 with VpB, or 6) empty capsules (control). One year postvaccination animals were conjunctivally infected with 1×10^9 CFUs of wild-type 2308. Two weeks postinfection, animals were euthanized; spleens were harvested; and the bacterial count per gram of A) spleen, B) liver, and C) lung was determined. Values are reported as the mean $\log_{10}$ recovery of 2308 from the organs. Difference in colonization between the vaccinated and control was determined by analysis of variance. **Value significantly different from control ($P<0.01$).

tion. By using microencapsulation techniques, we studied the ability to enhance S19 efficacy and provide an easier method to administer the vaccine to wildlife such as elk.

Experimental evidence suggests that live vaccines are more efficacious against brucellosis than killed vaccines (Schurig et al., 2002; Ko, 2003). Vaccination results using *B. abortus* S19 and RB51 and challenge with 2308 suggest that the persistence of the *Brucella* vaccine strain is also critical to confer protection (Ko, 2003; Kahl-McDonagh et al., 2006). 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 by the encapsulation process (Arenas-Gamboa et al., 2008). Also, we tested the ability of the vaccine to protect against virulent 2308 when the immunogen is given by an oral route. Oral administration was analyzed because the most cost-effective method of vaccinating wildlife would be to incorporate the vaccine into an oral 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. 1). Animals that received VpB in the formulation orally mounted higher IgG levels than red deer not receiving VpB in the formulation.

This greater IgG response may reflect the resistance of capsules with VpB to proteolytic breakdown in the stomach. (Rice Ficht et al., 1992).

Previous investigations suggest that induction of specific cellular-mediated immune responses after immunization is important for the establishment of a protective immune response in elk (Cook et al., 2002; Olsen et al., 2006). Studies performed with the encapsulated S19 indicated that by 17 wk, only alginate/VpB capsules administered subcutaneously were able to stimulate a statistically significantly higher cellular response compared with naïve animals. Red deer that received the same formulation orally did show enhanced cellular responses, but these were not statistically significant ($P<0.1$). These data suggest that by incorporating the S19 vaccine into microcapsules, conditions necessary to trigger a cellular response were provided. Further studies adjusting the VpB concentration for the oral formulation are needed to obtain the response observed with subcutaneous vaccination.

To determine the degree of protection against virulent 2308 challenge, two to three animals from each group were housed in large animal BL-3 facility buildings. Because of federal regulations and difficulty in housing these animals, group sizes were very limited. Among animals exposed to a conjunctival dose of

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abortus with VpB, 4) encapsulated oral S19 without VpB, 5) encapsulated oral S19 with VpB, or 6) empty capsules (control). One year postvaccination, animals were conjunctivally infected with 1×10^9 CFUs of wild-type 2308. Two weeks postinfection, animals were euthanized; lymph nodes were harvested; and the bacterial count per lymph node A) submandibular, B) mammary, and C) mesenteric was determined. Values are reported as the mean $\log_{10}$ recovery of 2308 from the different lymph nodes. Significance of difference in colonization between the vaccinated and control was determined by analysis of variance. *Value significantly different from control ($P<0.05$).
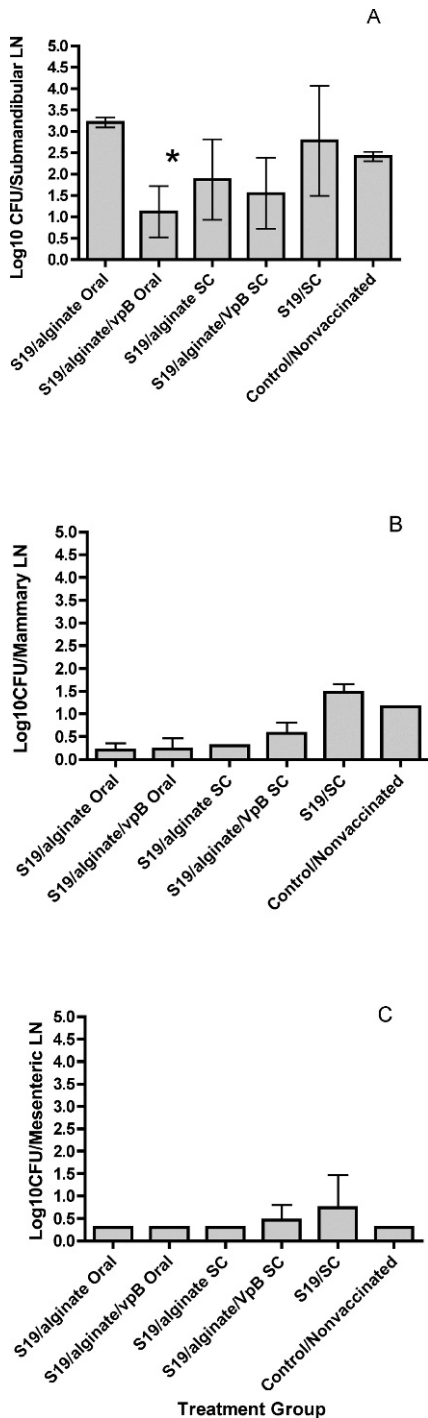
FIGURE 4.   Bacterial dissemination after conjunctival 2308 wild-type challenge. Red deer were vaccinated subcutaneously or orally with 1.5×10^{10} colony-forming units (CFUs) of 1) unencapsulated *Brucella abortus* S19, 2) encapsulated *B. abortus* S19 without vitelline protein B (VpB), 3) encapsulated *B.*

1×10^9 CFUs 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 with the control. In accordance with this observation, this group also exhibited the lowest numbers of recoverable bacteria in lungs and livers. Bacterial numbers in the submandibular lymph nodes also were diminished ($P<0.005$). These results suggest the induction of mucosal immunity by the oral vaccine. Further experiments to determine the induction of mucosal immunity as well as cytokine production by alginate microspheres in red deer are underway.

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. Because 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 observations made by other groups, in which alginate was proven to resist low pH degradation (Wee and Gombotz, 1998). The exact mechanism of protection and delivery remains to be determined. These results are promising for the development of an oral bait vaccine.

This study was conducted over a year, and animals were vaccinated only once. If the vaccine is given as oral bait, animals may be naturally boosted by consuming multiple doses, thus enhancing vaccine efficacy. Also, the conjunctival dose used for challenge may be higher than natural exposure of elk to B. *abortus* in the field. If the field is less severe, protection against field challenge may be greater than 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 underway.

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 virulent challenge was sustained for at least 1 yr postvaccination. This methodology of vaccination might be a safe and effective means of preventing and controlling brucellosis in the GYA and may represent an alternative to enhance the efficacy of other vaccines that are currently in use.

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