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Authors: Kreeger, Terry J., Miller, Michael W., Wild, Margaret A., Elzer, Philip H., and Olsen, Steven C.

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SAFETY AND EFFICACY OF *BRUCELLA ABORTUS* STRAIN RB51 VACCINE IN CAPTIVE PREGNANT ELK

Terry J. Kreeger,[1,5] Michael W. Miller,[2] Margaret A. Wild,[2] Philip H. Elzer,[3] and Steven C. Olsen[4]

[1] Wyoming Game and Fish Department, 2362 Highway 34, Wheatland, Wyoming 82201, USA
[2] Colorado Division of Wildlife, Wildlife Research Center, 317 West Prospect Road, Fort Collins, Colorado 80526-2097, USA
[3] Department of Veterinary Science, Louisiana State University Agricultural Center, Baton Rouge, Louisiana 70803, USA
[4] Zoonotic Disease Research Unit, National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, 2300 Dayton Ave., Ames, Iowa 50010, USA
[5] Corresponding author (e-mail: tekreege@wyoming.com)

ABSTRACT: *Brucella abortus* strain RB51 is a laboratory-derived rough mutant of virulent *B. abortus* strain 2308 used as a vaccine because it induces antibodies that do not react on standard brucellosis serologic tests. Strain RB51 vaccine was evaluated in pregnant captive elk (*Cervus elaphus*) to determine (1) if it induced abortion and (2) if it protected against abortion following subsequent challenge. The time period of this study (February–June, 1998) was similar to field conditions where elk are vaccinated and possibly exposed to *B. abortus.* Fourteen elk were randomly and equally divided into vaccinated and control groups. The vaccinated group was vaccinated intramuscularly with 1.03×10^{10} colony-forming units (CFU) of strain RB51 and seroconverted postvaccination. Antibodies to strain RB51 were detected by a modification of an existing dot-blot assay. Both groups were challenged 40 days postvaccination with 9.8×10^6 CFU of *B. abortus* strain 2308 administered intraconjunctivally. The first abortion occurred 38 days postchallenge. Abortion occurred in all control elk and in five of seven vaccinated elk 5 to 12 wk postchallenge ($P = 0.23$). Mixed strain RB51 and 2308 infections were present in fetuses and vaginas from the vaccinated group whereas only strain 2308 was cultured from control group fetuses and vaginal swabs. Further evaluation of strain RB51 will be necessary to determine if it will be safe and efficacious in free-ranging pregnant elk.

Key words: Abortion, *Brucella abortus, Cervus elaphus,* dot-blot assay, elk, strain RB51, vaccination.

INTRODUCTION

Brucellosis is a zoonotic disease of domestic and wild ungulates (Hunter and Kreeger, 1998). *Brucella* spp. are faculative intracellular bacteria causing chronic disease that usually persists for life. Mortality in adults is rare, but mortality of offspring may be high in a recently-infected herd. Infection of the female reproductive tract often results in abortion (Enright, 1990).

For economic and health purposes, a cooperative state/federal brucellosis eradication program began in 1934 with the goal of controlling, then eliminating, brucellosis in domestic cattle in the United States. The majority of states are now classified as brucellosis-free (Cheville et al., 1998). However, brucellosis is endemic in elk (*Cervus elaphus*) and bison (*Bison bison*) in the Greater Yellowstone Area (GYA) (Tunnicliff and Marsh, 1935), an ecosystem encompassing Yellowstone National Park, Grand Teton National Park, and surrounding areas in Wyoming, Montana, and Idaho (USA). The presence of brucellosis in wildlife creates a conflict with the national goal of eradication.

Brucellosis vaccines are comprised of living, mutant *Brucella* sp. organisms that infect the host, but are less pathogenic than the parent strain while providing prolonged immunity. Two licensed vaccines are *B. abortus* strain 19 and strain RB51. Strain 19 has been used for decades in cattle and free-ranging elk; however, it induces production of antibodies to the lipopolysaccharide (LPS) O-side chain that are detected in some serologic tests for brucellosis (Stevens et al., 1995). Thus, differentiation between serologic responses following infection and vaccination may be

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difficult. Strain RB51 is a laboratory-derived rough mutant of virulent *B. abortus* strain 2308. It lacks most of the antigenic LPS O-side chain (Schurig et al., 1991) and it does not induce antibodies that react in particle concentration fluorescence immunoassay, card, tube agglutination, or complement fixation tests (Stevens et al., 1994). Because of this, strain RB51 has become the preferred vaccine for cattle and it may become the preferred vaccine for wildlife.

The Wyoming Game and Fish Department (WGFD; Cheyenne, Wyoming, USA) has vaccinated thousands of elk on feedgrounds in the GYA with a reduced-dose strain 19 vaccine delivered remotely by biobullet (Herriges et al., 1989) and a significant decrease in seropositivity before and after 10 years of vaccination has been documented (S. Smith, pers. comm.). Vaccination with strain RB51 would allow serologic differentiation of vaccinated elk from those exposed to field strain *B. abortus.*

Calfhood vaccination against brucellosis is usually preferred in domestic and wild species because adult vaccination with strain 19 may result in abortion (Davis et al., 1991; Cheville et al., 1996; Palmer et al., 1996). However, preliminary studies have found that RB51 vaccination of pregnant elk resulted in few or no abortions (WGFD, unpubl. data). This finding suggested that vaccination of adult elk on feedgrounds could be an effective means of reducing brucellosis-induced abortions with subsequent reduction of the disease in elk populations. In addition, vaccination of adults would enhance the protection of calfhood vaccination and increase the efficacy of strain RB51. Thus, we examined the safety and efficacy of strain RB51 vaccine to protect elk against abortion under conditions that would mimic potential field applications. Additionally, we developed a modified dot-blot assay to measure antibodies to strain RB51 in elk.

MATERIALS AND METHODS

This study was from February–June 1998 at the Colorado Division of Wildlife's (CDW) Foothills Wildlife Research Facility (Fort Collins, Colorado, USA; 40°35′N, 105°10′W). Fourteen, captive, *Brucella* seronegative, adult female Rocky Mountain elk were used in this study; nine were from the CDW facilities, two were transferred from WGFD research facilities, and three from Idaho Department of Fish and Game facilities. All elk were fed alfalfa hay cubes supplemented with grass hay and a high-energy pelleted supplement (Baker et al., 1998). Water and a trace mineral block were provided *ad libitum.*

All elk were diagnosed pregnant using pregnancy-specific protein analysis (Biotracking, Moscow, Idaho, USA), then randomly and equally divided into vaccinated and control groups. All elk were examined for *Brucella* antibodies using four standard serologic tests (MacMillan, 1990) and determined to be negative prior to initiation of the test. Vaccinated and control groups were housed separately in 4 ha pastures.

On February 26, 1998, the vaccinated group was inoculated intramuscularly with 1.03×10^{10} CFU of strain RB51 vaccine (Colorado Serum Co., Denver, Colorado, USA) administered in the hindquarters. The vaccine dosage was determined by standard plate counts on tryptose agar plus 5% bovine serum. The control group was given sterile saline administered similarly. Elk were observed daily after vaccination for signs of morbidity or abortion.

On 7 April 1998 (40 days postvaccination), all elk were challenged via the conjunctival sac with 9.8×10^6 CFU of *B. abortus* strain 2308, an established virulent strain in cattle (Elzer et al., 1998). The inoculum, prepared as described in Elzer et al. (1994) and diluted in sterile saline, was administered by dropping 50 μl in each eye using a Gilson pipetman P100. The challenge dose was verified by serial dilutions and plating as above with the vaccine dose. Elk were lightly sedated with 40–80 mg xylazine hydrochloride prior to inoculation. The time period of the test was chosen because it represented natural conditions where elk on feedgrounds would normally be vaccinated then possibly exposed to *Brucella*-induced abortions (Herriges et al., 1989).

Sera collected at vaccination and at challenge were tested for antibodies to strain RB51 using a dot blot assay (Olsen et al., 1997) modified for use in elk. Samples were evaluated without knowledge of vaccination status. The secondary antibody used in this assay was determined by absorption of elk sera on 0.45 μm nitrocellulose (Schleicher and Schuell Inc, Keene, New Hampshire, USA) and screening with the following peroxidase-conjugated antibodies: rabbit anti-bovine IgG-heavy and light chain spe-

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cific; rabbit anti-sheep IgG-heavy and light chain specific; rabbit anti-goat IgG-heavy and light chain specific; rabbit anti-bovine IgG-Fc fragment specific; goat anti-human IgG-heavy and light chain specific (Jackson Immunoresearch, West Grove, Pennsylvania, USA); goat anti-bovine IgG-heavy chain specific; peroxidase-labeled protein A from *Staphlococcus aureus* (Kirkegaard and Perry Laboratories Inc., Gaithersburg, Maryland, USA); goat anti-canine IgG (Crawley Down, Sussex, UK); swine anti-goat IgG (Boehringer Manheim Biochemicals, Indianapolis, Indiana, USA); sheep anti-bovine IgG1; and sheep anti-bovine IgG2 (The Binding Site, Birmingham, UK). Based on this preliminary work, rabbit anti-goat IgG-heavy and light chain specific was selected as the secondary antibody because it demonstrated the strongest immunoreactivity to elk sera.

The procedure for conducting the dot-blot assay was as follows. *Brucella abortus* strain RB51 was grown on tryptose agar (Difco Laboratories, Detroit, Michigan, USA) containing 5% bovine serum for 48 hr at 37 C. Bacteria were removed from the agar by aspiration using saline, killed by irradiation (1.4×10^6 rads), washed in saline, and stored in 1 ml aliquots at −70 C. Antigen stock solutions for the assay were made by spectrophotometric adjustment of killed strain RB51 to 20% transmission at 600 nm. Thirty μl of the appropriate suspension was added to each well of a 96-well microfiltration unit (Bio-Rad Laboratories, Richmond, California, USA) containing a nitrocellulose membrane (Schleicher and Schuell, Inc.) with a pore size of 0.45 μm. The membrane was dried by applying a vacuum after 30 min incubation at 20–22 C (room temperature). Each well then received 100 μl of 0.02M Tris, 0.5M NaCl buffer (Tris-NaCl, pH 7.5) containing 0.25% fish gelatin (Tris-FG, Norland Laboratory, New Brunswick, New Jersey, USA). After 30 min incubation, the nitrocellulose was dried and washed twice by vacuum filtration using 100 μl of Tris-NaCl containing 0.3% Tween 20 (Tris-NaCl-Tween, Sigma Chemical Co, St. Louis, Missouri, USA). Ten-fold dilutions of serum samples were prepared in Tris-FG beginning at a 1:20 dilution. Serum from goats vaccinated with *Brucella melitensis* strain 16M served as positive controls for each assay. Goat serum was used because there was no source for a secondary antibody against elk IgG. Several commercial secondary antibodies were screened to identify which had the strongest cross-reaction with elk antibodies. Because the secondary antibody selected for use was a rabbit anti-goat IgG peroxidase conjugate, goat serum was the ideal positive control. Goat serum against *B. melintensis*, obtained from previous studies (S. Olsen, unpubl. data), was found to react against *B. abortus* strain RB51 in the dot blot assay. Although goat anti-serum against strain RB51 would have been ideal, this was not available at the time of this study.

Wells to which no serum was added served as negative controls for each assay. Elk sera was not used for negative controls because sera were evaluated blindly. Because the vaccination status of a sample was unknown, using elk serum as a negative control was not possible. Thirty μl of each serum dilution and positive controls were added to separate wells of the microfiltration unit. Following a 30-min incubation, the nitrocellulose was dried by vacuum and washed five times with 100 μl per well of Tris-NaCl-Tween. The peroxidase-conjugated secondary antibody was diluted 1/2,000 in Tris-FG and a 100 μl aliquot added to all wells. After 30 min incubation, the wells were washed five times with Tris-NaCl-Tween and dried for 30 sec under vacuum. The nitrocellulose was removed from the microfiltration unit and incubated for 10 min in Tris-NaCl containing 0.5 mg of 4-chloro-1-napthol/ml and 0.15% H_2O_2 (Sigma Chemical Co, St Louis, Missouri, USA). Membranes were dried in the dark for 12 hr at 22 C before interpretation. Results were expressed as the highest serum titer that produced a visible color reaction that was interpreted to be no less than 50% of the intensity of the color reaction produced by the positive control sera.

Following challenge, elk were observed twice daily for abortion or other indications of reaction to the challenge dose. Calving was assisted if signs of labor exceeded 12 hr or if an abnormal presentation was observed. Aborted fetuses were collected immediately and frozen, then necropsied and tissues cultured at later date. Following normal parturition, calves were observed for ≥10 days to assess health and fitness after which they were blood sampled for serology, euthanized, necropsied, and tissues cultured. Vaginal swabs (Culturette,™ Becton Dickinson, Cockeysville, Maryland, USA) were collected and placed in modified Amies with charcoal or modified Stuart's media, frozen, and cultured. Upon abortion or delivery, adult elk were blood sampled for antibodies against strains 2308 and RB51. Seroconversion to strain 2308 was determined using standard plate agglutination, standard tube, rivanol, and complement fixation tests. Titers were considered positive based on criteria established by Morton et al. (1981). When possible, milk samples were also taken and cultured.

The primary culture medium consisted of *Brucella* agar (Difco Laboratories, Detroit, Michigan, USA) with the addition of 7,500 I.U.

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TABLE 1. Results of pregnant elk vaccinated with *Brucella abortus* strain RB51 on February 26, 1998 then challenged on 7 April 1998 with *B. abortus* strain 2308. Antibodies against strain RB51 were measured in cows at time of challenge; antibodies against strain 2308 were measured in cows at time of birth/abortion.

Group	Strain RB51 antibody titer[a]	Strain 2308 antibody titer[a]	Abortions	Live births	Culture status[b]	
					Fetus[c]	Vagina[c]
Vaccinated	7/7	5/7	5/7	2/7	2/4	4/6
Control	0/7	7/7	7/7	0/7	5/5	6/6

[a] Strain RB51 antibody titer positive if ≥1:640 as measured by dot blot assay; 2308 antibody titer positive based on the criteria of Morton et al. (1981).
[b] Not all fetal or vaginal cultures analyzed due to sample contamination.
[c] Cultures from vaccinated elk were both strains RB51 and 2308; cultures from control elk were strain 2308.

bacitracin, 1,800 I.U. polymixin B, 30-mg cyclohexamide, and 0.000125% crystal violet per liter. Culture swabs were streaked across the entire plate in one direction, then streaked at right angles again covering the entire plate. Plates were inverted and incubated at 37 C under 10% CO_2, for a minimum of 6 days. Suspect bacterial colonies were removed and streaked for isolation on Columbia blood agar with 5% sheep blood (Hardy Diagnostics, Santa Maria, California, USA) and MacConkey agar plates (Hardy Diagnostics, Santa Maria, California, USA). Isolates which were unable to grow on MacConkey agar were then identified using standard bacteriological methods (Quinn et al., 1994) and any *Brucella* sp. were differentiated by carbon utilization panels (Biolog, Inc., Hayward, California, USA).

The null hypothesis tested was that vaccination with *B. abortus* strain RB51 does not protect against abortion in elk challenged with virulent strain 2308. Differences in abortion rates between vaccinated and control groups were compared by one-sided Fisher's exact test for two proportions at a significance level of alpha ≤0.1. Antibody titers to strain RB51 vaccine were compared by one-way ANOVA at a significance level of $P \leq 0.05$. Means are reported with standard errors.

RESULTS

No morbidity was observed from the time of vaccination to challenge. Antibody titers to strain RB51 were significantly higher ($P = 0.00004$) in vaccinated elk (8,533 ± 1,080) compared to controls (125 ± 34). None of the control elk had strain RB51 antibody titers >1:160, whereas all the vaccinated elk had titers ≥1:640. Several of the vaccinated elk and all of the control elk seroconverted to strain 2308 after challenge (Table 1). The first abortion occurred on 15 May 1998 (77 days postvaccination; 38 days postchallenge) from a vaccinated elk. Ten aborted fetuses were collected between 15 May and 26 June. One fetus from each group was never found and was presumed to have been aborted based on vaginal examination and consumed by the cow.

Sample sizes were sufficient to detect relatively large (>50%) reductions in abortion rates among vaccinated elk ($1 - \beta \geq 0.43$, where abortion rates for controls were ≥0.86). Two of seven elk from the vaccinated group and none of the elk from the control group gave birth to viable calves. Vaccination did not lower abortion rates ($P = 0.23$). No *Brucella* spp. were cultured from either viable calf, but one calf had antibodies against strain 2308. Neither calf had antibodies against strain RB51.

Strains RB51 and 2308 were cultured from two aborted fetuses from vaccinated elk; no cultures were obtained from the other two fetuses due to contamination. Strain 2308 was cultured from five fetuses from the control elk; one fetus was contaminated. Strains RB51 and 2308 were also cultured in vaginal swabs from the vaccinated group whereas cultures from the control group were only strain 2308 (Table 1). Milk samples were obtained from only three of the vaccinated elk and none of the control elk; strain 2308 was cultured from one milk sample.

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DISCUSSION

Elk vaccinated as calves with strain RB51 and then challenged with the same dose of strain 2308 used in this study mostly aborted 6 to 8 wk after challenge (W. Cook, unpubl. data). The first abortion in our study did not occur until 11 wk postvaccination. This longer period to abortion could have been a function of the lower pathogenicity of strain RB51. Culture of the fetus was inconclusive because of contamination. However, the dam had not seroconverted to strain 2308. Subsequent abortions in the vaccinated group had mixed RB51 and 2308 cultures. Thus, abortion strictly due to strain RB51 vaccination cannot be ruled out.

Strain RB51 vaccine did not provide significant protection from abortion following challenge. Abortion occurred in all control elk and in five of seven vaccinated elk 5 to 12 wk postchallenge. A previous study of elk vaccinated as calves with 1×10^9 CFU strain RB51 and then challenged when pregnant also failed to demonstrate significant protection (W. Cook, unpubl. data). Reasons for lack of protection in our study could be due to (1) insufficient vaccine; (2) excessive challenge dose; (3) insufficient time between vaccination and challenge for cell-mediated immunity (CMI) to develop; or (4) lack of strain RB51 efficacy in elk.

The vaccine dose used in this study was the same efficacious dose used in cattle calves (Cheville et al., 1996). It was believed that any higher dose would increase the risk of vaccine-induced abortions. Because vaccinated elk had antibody titers to strain RB51, it was unlikely that the lack of protection from abortion was due to an insufficient vaccine dose.

The challenge dose was also chosen to provide comparative data with previous cattle and elk trials (McEwen et al., 1939; Manthei and Carter, 1950; W. Cook, unpubl. data). In cattle trials, challenge with $\geq 1.0 \times 10^7$ CFU strain 2308 generally resulted in 90–100% abortion rates (Manthei and Carter, 1950). Although the challenge dose used in this study was reasonable, it resulted in 100% of control elk aborting as it did in previous studies (W. Cook, unpubl. data). It could be argued that a challenge dose causing consistent 100% abortion rates in unprotected animals is higher than seen in natural infections. If true, the challenge dose could have been unrealistic and strain RB51 protection from abortion could have been greater if a lower challenge dose had been used.

Protection from infection and elimination of *B. abortus* most likely requires CMI responses (Nicoletti and Winter, 1990). The temporal development of CMI in elk has not been examined. In cattle, it took 10 to 12 wk after vaccination with strain RB51 for lymph node cells to proliferate when incubated with strain 2308 (Stevens et al., 1995). This delay in proliferative response could have been due to vaccine-induced lymphocyte depletion (Araya et al., 1989). Thus, it is possible that in the 40 days that elapsed between vaccination and challenge in this study, insufficient CMI responses had developed in elk to prevent infection. If true, vaccination in the field would have to be conducted earlier than current practices in order to develop adequate CMI.

We could be left to conclude that strain RB51 is simply not efficacious in elk. In our study, RB51 vaccine was 28% (2/7) effective in preventing abortions. If this level of protection was truly characteristic of strain RB51 vaccine in pregnant elk, this relatively low level may be beneficial from a disease management viewpoint. A recently-developed, individual-based, stochastic model indicated that this level of vaccine efficacy would still result in a decline in population seroprevalence over time (Gross et al., 1998). A decline in seroprevalence would indicate fewer infected animals which would have fewer *Brucella*-induced abortions and, thus, a decreased likelihood of disease transmission.

This type of study needs to be repeated and expanded. More time needs to be al-

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lowed between vaccination and challenge to allow for development of CMI. If more time proves beneficial, then this time period would have to be incorporated into any field application. Also, increased vaccine dosages or multiple vaccinations need to be assessed.

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