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Source: Journal of Wildlife Diseases, 40(3) : 429-433

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-40.3.429

Journal of Wildlife Diseases, 40(3), 2004, pp. 429–433

SAFETY OF *BRUCELLA ABORTUS* STRAIN RB51 IN BLACK BEARS

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ABSTRACT: In two studies conducted from October 1999 to March 2000 and December 2000 to April 2001, adult black bears (*Ursus americanus*) were orally inoculated with 1.4–3.1×10^{10} colony-forming units (CFU) of *Brucella abortus* strain RB51 (SRB51, $n=12$) or 2 ml of 0.15 M NaCl solution (saline, $n=11$). We did not detect a difference ($P>0.05$) in antibody titers to SRB51 in serum obtained before vaccination, at 8 wk after vaccination, or at necropsy at 21 or 23 wk after vaccination between SRB51-vaccinated and nonvaccinated bears. The SRB51 vaccine strain was recovered from tissues obtained at necropsy from one of six SRB51-vaccinated bears in study 1, but none of the six SRB51-vaccinated bears in study 2. Vaccination of black bears with SRB51 did not appear to influence ($P>0.05$) reproductive performance.

Key words: Biosafety, *Brucella,* RB51, serology, *Ursus americanus,* vaccine.

INTRODUCTION

In 1996, a new official calfhood vaccine for cattle, *Brucella abortus* strain RB51 (SRB51), was conditionally approved by the Animal and Plant Health Inspection Service branch of the US Department of Agriculture. The SRB51 strain, a laboratory-derived, lipopolysaccharide O–side chain–deficient mutant (Schurig et al., 1991) of *B. abortus* strain 2308, induces protection in cattle and bison (*Bison bison*) against challenge exposure with virulent *B. abortus* strains (Cheville et al., 1993; Cheville et al., 1996; Olsen et al., 2003). Because SRB51 is being considered as a vaccine for bison in the Greater Yellowstone Area (GYA, USA), studies to evaluate the safety of this vaccine in representative nontarget species needed to be conducted. In the studies reported here, antibody responses, tissue localization and clearance, and reproductive performance were assessed in black bears (*Ursus americanus*) after oral exposure to SRB51.

MATERIALS AND METHODS

Two studies were conducted from October 1999 to March 2000 and December 2000 to April 2001 at a private facility in South Dakota, USA (42°52′N, 93°63′W). Each study used different bears with no animals used in both studies. In October (study 1) or December (study 2), adult black bears were sedated with 500 to 1,000 mg of tiletamine hydrochloride and zolazepam hydrochloride (Telazol®; Fort Dodge Animal Health, Fort Dodge, Iowa, USA) administered intramuscularly by pneumatic dart (Pneudart®, Williamsburg, Pennsylvania, USA). After attainment of lateral recumbency, ear tags were placed for identification, and blood was obtained via jugular venipuncture for serologic analysis. Bears were then orally inoculated by placing the inoculum into the pharyngeal region and holding the head vertically until the bear was observed to swallow. In study 1, bears received either 2 ml of 0.15 M NaCl (saline; $n=6$) or 2 ml of saline containing *B. abortus* strain RB51 (SRB51; $n=6$) orally. In study 2, only bears in the SRB51 treatment group ($n=6$) were anesthetized and inoculated orally. However, control bears ($n=5$) were identified at the time of vaccination for comparison with reproduction in SRB51-inoculated bears. Following oral inoculation, bears were grouped by treatment, with SRB51-vaccinated bears housed separately from nonvaccinated bears.

The SRB51 inoculum in both studies was prepared by dilution of lyophilized SRB51 vaccine (Colorado Serum Company, Denver, Colorado, USA) to approximately 1×10^{10} colony-forming units (CFU) on the basis of viability counts of other vials with the same lot number. Briefly, reconstituted vaccine was serially diluted in saline and plated on tryptose agar plates

containing 5% bovine serum, and standard plate counts were conducted to determine viability. Standard plate counts of the inoculum determined the SRB51 dosage administered.

At 8 wk after oral inoculation in study 1, bears in both SRB51 and control treatments were anesthetized with Telazol, as previously described, and blood was obtained via jugular venipuncture for serologic analysis.

Necropsy

At 23 wk after oral inoculation in study 1, SRB51-inoculated bears and their cubs and control bears were anesthetized in their dens with Telazol via pneumatic dart or intramuscular injection. In a similar manner, SRB51-inoculated bears and their cubs in study 2 were anesthetized in their dens at 21 wk after inoculation. After sedation, blood was obtained for serology via jugular venipuncture. All SRB51-inoculated bears and their cubs in both studies were then euthanized with intravenous injections of sodium pentobarbitol (Sleepaway, Ft. Dodge Labs, Ft. Dodge, Iowa) and necropsied. Control bears were only sedated in study 1, and all were allowed to recover from anesthesia.

Maternal tissues collected at necropsy from SRB51 vaccinates for both bacteriologic and histologic evaluation included: mammary gland, liver, lung, spleen, kidney, and uterus. Maternal samples obtained for bacteriologic examination only included vaginal, conjunctival, nasal, and rectal swabs; blood; tonsil; and hepatic, parotid, popliteal, prescapular, medial retropharyngeal, mediastinal, internal iliac, mandibular, mesenteric, and supramammary lymph nodes. Bacteriologic samples from cubs included blood, spleen, lung, liver, kidney, mesenteric lymph node, and cerebrospinal fluid. Histologic techniques also were used to examine cardiac samples from both sows and cubs and fetal liver, spleen, and kidney samples. All tissues collected for histologic evaluation were fixed in neutral buffered 10% formalin embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Special stains (Congo red, silver, acid-fast, or Prussian blue; Sheehan and Hrapchak, 1980) were performed on sections in which examination of hematoxylin and eosin sections suggested that additional histologic techniques were warranted.

Bacteriologic evaluation

Tissue samples were triturated in saline and placed on tryptose agar plates containing 5% bovine serum and a selective medium for SRB51 (RBM) that contains antibiotics, including rifampicin (Hornsby et al., 2000). The antibiotics in RBM media minimized growth of contaminants without inhibiting growth of SRB51, thereby enhancing the ability to detect small numbers of SRB51 within samples.

Following incubation of plates at 37 C and 5% CO_2 for 7 days, SRB51 was identified by colony morphology, growth characteristics (Alton et al., 1988), and resistance to rifampicin (Schurig et al., 1991). Isolates were confirmed as SRB51 in a polymerase chain reaction (PCR) procedure with the use of primers specific for SRB51 (Vemulapalli et al., 1999). Briefly, the reaction mixture consisted of heat-killed cells, 0.2 mM nucleotide mix (Boehringer Mannheim, Mannheim, Germany), 1× PCR Buffer II (Perkin Elmer, Branchburg, New Jersey, USA), 2.5 U/ml DNA polymerase (Amplitaq Gold, Perkin Elmer), and 1.5 mM MgCl combined with 0.2 μM per reaction of specific primers (upstream primer GCAACGGTGTTCTTCCACTC and downstream primer GTATCAGGCTACGCAGAAGG) for *B. abortus* omp2A as selected from analysis of its sequence (Ficht et al., 1989). The samples, including positive and negative controls, were cycled (30 sec at 95 C, 30 sec at 44 C, 1 min at 72 C) 30 times in a thermocycler (MJ Research Inc., Watertown, Massachusetts, USA). Products were analyzed by electrophoresis on a 1.5% agarose gel after staining with ethidium bromide.

Serologic evaluation

Blood samples were allowed to clot, and serum was separated by centrifugation. Serum was divided into 1-ml aliquots, frozen, and stored at −70 C. Serologic titers of animals to SRB51 were determined by dot-blot assay (Olsen et al., 1997) in which γ-irradiated SRB51 is used as antigen, and peroxidase-labeled protein A (1:100 dilution; Kirkegaard and Perry Laboratories Inc., Gaithersburg, Maryland, USA), rabbit anti-canine IgG heavy- and light-chain antibodies (1:5,000; Jackson Immunoresearch Laboratories, West Grove, Pennsylvania), or rabbit anti-goat IgG heavy- and light-chain antibodies (1:10,000 dilution; Jackson Immunoresearch Laboratories) were used as secondary antibodies.

Statistics

Because of logarithmic conversions, titers with a value of 0 were analyzed with a value of 1. Serologic responses of bears in both studies were compared over all times by an analysis of variance procedure with titers to each secondary antibody evaluated separately (SAS Institute Inc., Cary, North Carolina, USA). Means for individual treatments were separated by a least significant difference procedure ($P<0.05$).

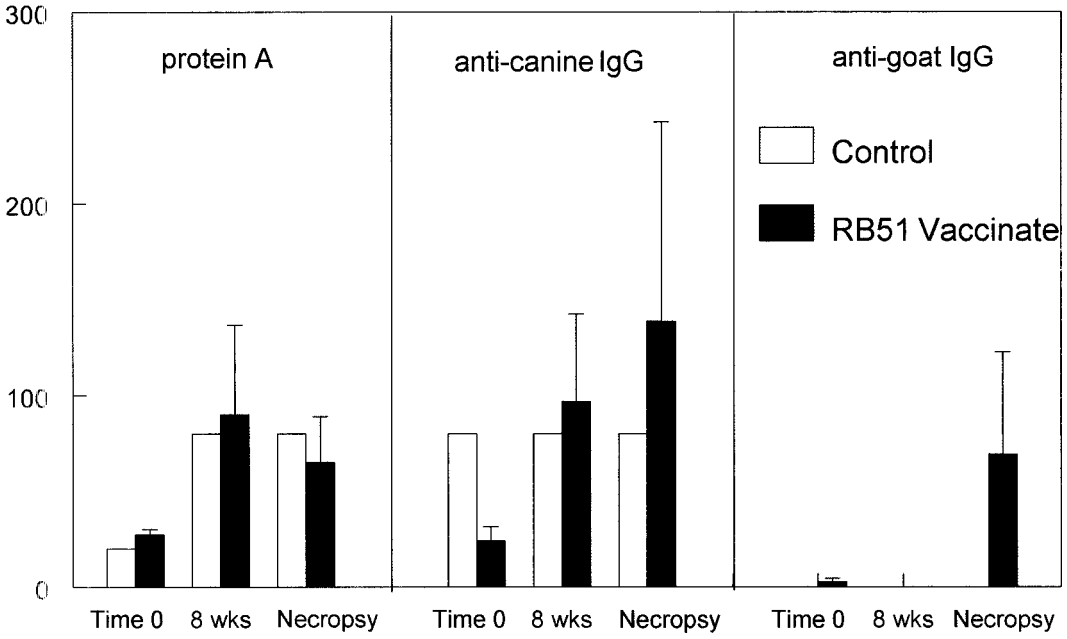

FIGURE 1. Serologic responses of nonvaccinated (control) bears or bears orally inoculated with $1.4–3.1\times10^{10}$ colony forming units of SRB51 to γ-irradiated SRB51 in a dot-blot assay in which peroxidase-conjugated protein A, rabbit anti-canine IgG, or rabbit anti-goat IgG were used as secondary antibodies. Antibody responses were determined before vaccination (time 0; $n=12$ for SRB51 and $n=6$ for control treatments), 8 wk after vaccination ($n=6$ for SRB51 and control treatments), and at necropsy 21 or 23 wk after vaccination ($n=12$ for SRB51 and $n=6$ for control treatments). Responses are presented as mean titer±SEM.

A 95% confidence interval was predicted from the bacteriologic data by a Clopper-Pearson procedure (SAS Institute Inc.).

RESULTS

The concentration of viable SRB51 bacteria within the inocula for studies 1 and 2 were determined by standard plate counts to be 1.4×10^{10} and 3.1×10^{10} CFU, respectively.

At necropsy in the first study, twin cubs were found with one SRB51-inoculated bear and one nonvaccinated bear. No other bears in SRB51 or control treatments were pregnant or had cubs present at the time of evaluation. In study 2, three of the five paired controls had cubs (two triplets and one set of twins) at the time of necropsy, whereas three of six SRB51-inoculated bears had cubs (three sets of twins).

At necropsy in study 1, 23 wk after vaccination, SRB51 was recovered from the internal iliac, retropharyngeal, and mesenteric lymph nodes from one of six SRB51-inoculated bears. This bear had no cubs and was not pregnant at the time of evaluation. The SRB51 vaccine strain was not recovered from fetal tissues obtained at necropsy from the two cubs of the SRB51 vaccinate. In study 2, SRB51 was not recovered from any tissue obtained at necropsy from any of the six SRB51-inoculated bears or their cubs.

Regardless of secondary antibody, titers of bears orally inoculated with SRB51 did not differ ($P>0.05$) from dot-blot titers of nonvaccinates at any sampling time (Fig. 1). In both studies, one of six SRB51-inoculated bears had titers at necropsy that were four dilutions greater than the titer of paired serum obtained before vaccination. In both of these bears, the highest titers were noted when the anti-canine antibody was used as the secondary antibody. When compared with the serum obtained before inoculation, the bear that was culture positive for SRB51 at necropsy in

study 1 did not demonstrate a significant increase in titer to SRB51 at 8 wk after inoculation or at necropsy with any of the secondary antibodies used in this study.

With the exception of small, subacute, multifocal tonsillar abscesses in one SRB51-inoculated sow in study 1, no gross lesions were noted at necropsy.

Histologic evaluation of lung sections from eight SRB51-vaccinated adult bears (five in study 1 and three in study 2) revealed multifocal, perivascular, and, to a lesser extent, peribronchiolar accumulations of moderate numbers of histiocytes. The cytoplasm of these histiocytes were expanded by an amorphous basophilic material that also contained numerous birefringent crystals. In some adult bears, multifocal accumulations of low numbers of lymphocytes were found in interstitial areas of the kidney or were randomly distributed in hepatic parenchyma.

Special stains of histologic sections of lung indicated that lesions were compatible with pneumoconiosis. The etiology of the particulate material in pulmonary sections was not conclusively determined but most likely was inhaled environmental debris. With these exceptions, no significant histologic lesions were noted in examination of remaining maternal tissues and all fetal tissues.

DISCUSSION

The results of these studies suggest that oral exposure to 10^{10} CFU of SRB51 is not associated with adverse clinical, histologic, or reproductive effects in bears. Although bears in both treatments had poor conception rates in the first study, reproduction performance of bears in both treatments in each study were similar.

Our data also suggest that in a small percentage of bears (one of 12, 8%; 95% confidence interval, 0.2–38.5%), SRB51 can persist for up to 23 wk after oral inoculation. Failure to recover SRB51 from mucosal swabs, mammary gland, supramammary lymph nodes, or uterine and fetal tissues in any SRB51-vaccinated bear in our studies suggests that if bears are orally exposed to SRB51, it is unlikely that they will transmit the strain laterally or vertically. When evaluated with the bacteriologic data, the poor serologic responses noted in our studies might indicate SRB51 is not well adapted to colonize or replicate in tissues of black bears and therefore does not effectively induce immunologic responses or cause clinical effects. Alternatively, SRB51 might not efficiently attach and invade mucosal surfaces of black bears after oral exposure.

Data from others have suggested that bears in areas with endemic brucellosis might undergo at least transient infection with field strains of *B. abortus.* Grizzly (*Ursus arctos horribilis*) and black bears sampled in Montana (USA) near Yellowstone National Park demonstrated positive responses on a panel of brucellosis serologic tests, whereas samples from bears in northern Montana, in an area without brucellosis, were negative on the same panel of tests (K. Aune, pers. comm.). Although there are no data at this time to determine whether exposure to *B. abortus* has any effects on survival or reproduction of bears in the GYA, our data and studies in other species (Januszewski et al., 2001; Kreeger et al., 2002) suggest that SRB51 is safe in black bears and is unlikely to be more pathogenic in bears than *B. abortus* field strains that are currently present in the GYA environment.

ACKNOWLEDGMENTS

We thank A. Duit, D. Pringle, A. Jensen, K. Casey, and D. Buffington for their technical assistance.

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Received for publication 3 September 2003.