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EXPERIMENTAL INFECTION OF NONTARGET SPECIES OF RODENTS AND BIRDS WITH *BRUCELLA ABORTUS* STRAIN RB51 VACCINE

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ABSTRACT: The *Brucella abortus* vaccine strain RB51 (SRB51) is being considered for use in the management of brucellosis in wild bison (*Bison bison*) and elk (*Cervus elaphus*) populations in the Greater Yellowstone Area (USA). Evaluation of the vaccine's safety in non-target species was considered necessary prior to field use. Between June 1998 and December 1999, ground squirrels (*Spermophilus richardsonii*, $n = 21$), deer mice (*Peromyscus maniculatus*, $n = 14$), prairie voles (*Microtus ochrogaster*, $n = 21$), and ravens (*Corvus corax*, $n = 13$) were orally inoculated with SRB51 or physiologic saline. Oral and rectal swabs and blood samples were collected for bacteriologic evaluation. Rodents were necropsied at 8 to 10 wk and 12 to 21 wk post inoculation (PI), and ravens at 7 and 11 wk PI. Spleen, liver and reproductive tissues were collected for bacteriologic and histopathologic evaluation. No differences in clinical signs, appetite, weight loss or gain, or activity were observed between saline- and SRB51-inoculated animals in all four species. Oral and rectal swabs from all species were negative throughout the study. In tissues obtained from SRB51-inoculated animals, the organism was isolated from six of seven (86%) ground squirrels, one of six (17%) deer mice, none of seven voles, and one of five (20%) ravens necropsied at 8, 8, 10, and 7 wk PI, respectively. Tissues from four of seven (57%) SRB51-inoculated ground squirrels were culture positive for the organism 12 wk PI; SRB51 was not recovered from deer mice, voles, or ravens necropsied 12, 21, or 11 wk, respectively, PI. SRB51 was not recovered from saline-inoculated ground squirrels, deer mice, or voles at any time but was recovered from one saline-inoculated raven at necropsy, 7 wk PI, likely attributable to contact with SRB51-inoculated ravens in an adjacent aviary room. Spleen was the primary tissue site of colonization in ground squirrels, followed by the liver and reproductive organs. The results indicate oral exposure to SRB51 does not produce morbidity or mortality in ravens, ground squirrels, deer mice, or prairie voles.

Key words: biosafety, *Brucella abortus*, brucellosis, deer mice, ground squirrels, non-targets, ravens, RB51, voles.

INTRODUCTION

Brucellosis was detected in bison (*Bison bison*) of Yellowstone National Park (YNP; USA) in 1917, when serologic tests revealed *Brucella* spp. antibodies in two bison cows that had recently aborted (Mohler, 1917). In elk (*Cervus elaphus*) populations in YNP, brucellosis was detected as early as 1930 (Murie, 1951). Infected bison and elk that roam outside of YNP have the potential to transmit the disease to brucellosis-free cattle herds in the surrounding Greater Yellowstone Area (GYA). Because of the brucellosis-free status in the states of Idaho, Wyoming, and Montana, there is interest in developing and implementing an effective disease control program for *Brucella abortus* in bison and elk in the GYA.

Management strategies considered for the control and eradication of brucellosis in the GYA include vaccination of wild bison and elk populations. *Brucella abortus* strain RB51 (SRB51), a rough, mutant variant of *B. abortus* strain 2308 (Schurig et al., 1991), is a vaccine candidate for use in wildlife. Strain RB51 has been shown to be efficacious in cattle (Cheville et al.,

1993), and safe in bison (Elzer et al., 1998; Olsen et al., 1999) and elk (Cook et al., 2000; Kreeger et al., 2000). Studies of SRB51 in elk have failed to demonstrate efficacy (Cook, 1999; Kreeger et al., 2000; J.J. Kreeger, unpubl. data); in limited studies, SRB51 has produced protection in bison (Olsen et al., 2000; S.C. Olsens unpubl. data).

There is concern about the introduction of a live vaccine into the GYA and its potential impact on resident animal populations that include unique genetics and threatened or endangered species. The Greater Yellowstone Interagency Brucellosis Committee (GYIBC; Boise, Idaho, USA) a state and federal interagency committee established to facilitate the development and implementation of brucellosis management plans for elk and bison in the GYA, has identified several non-target species in the GYA in which the safety of any vaccine candidate should be evaluated. These species include representative ungulates, carnivores, rodents, and birds. In nature, they would likely be exposed to the organism via contact with SRB51-contaminated material or scavenging on an infected carcass. Several studies evaluating the safety of SRB51 in non-target ungulates and carnivores have been completed (T. J. Kreeger, pers. comm.). Additionally, a study evaluating the safety of SRB51 in deer mice (*Peromyscus maniculatus*) has been reported (Cook et al., 2001). The purpose of this study was to evaluate the safety of oral exposure of common ravens (*Corvus corax*), deer mice, Richardson's ground squirrels (*Spermophilus richardsonii*), and prairie voles (*Microtus ochrogaster*) to SRB51. These species were selected from the list of representative non-targets developed by the GYIBC.

MATERIALS AND METHODS

Thirteen mature and immature ravens of either sex were captured at the Larimer County Landfill Fort Collins, Colorado (USA; 40°30′N, 105°6′W) and housed in pairs in an outdoor aviary at a Colorado Division of Wildlife facility (Fort Collins, Colorado) from June 1998 until November 1998. Twenty-one mature ground squirrels of either sex captured near Roundup (Montana, USA; 46°27′N, 108°28′W), 21 mature deer mice of either sex selected from a breeding colony located at the National Wildlife Research Center (NWRC; Fort Collins, Colorado, USA), and 21 mature prairie voles of either sex, wild-caught near the NWRC were maintained individually in the Animal Research Building (NWRC, Fort Collins, Colorado) from July 1998 until September 1998, October 1998 until December 1998, and July 1999 until December 1999, respectively. Ravens were exposed to natural lighting and temperature conditions, and the rodent environment was temperature and light controlled (22 C, 12 hr light-12 hr darkness cycle). Ravens, ground squirrels, deer mice, and voles were randomly divided into SRB51-inoculated ($n = 9$, $n = 14$, $n = 14$, $n = 14$) and control ($n = 4$, $n = 7$, $n = 7$, $n = 7$) groups respectively. A commercially available SRB51 vaccine (Colorado Serum Company, Denver, Colorado) was rehydrated according to manufacturer's instructions and diluted in physiologic saline. SRB51-inoculated deer mice and voles orally received SRB51 in a 1 ml volume, whereas SRB51-inoculated ravens and ground squirrels orally received SRB51 in a 0.25 ml volume. Control animals were given the same oral volume of physiologic saline. After incubation at 37 C with 5% CO_2 for 72 hr, vaccine viability was determined by standard plate counts on tryptose agar containing 5% bovine serum.

All animals were observed daily. Oral and rectal swabs were collected from all ravens, ground squirrels, and deer mice prior to inoculation and weekly throughout the study. In voles, swabs were collected 1, 2, 4, 7 and 14 days PI and every 2 wk thereafter. In rodents, fecal swabs were usually accompanied by one or two fecal pellets expelled during the swabbing process. Collected swabs were stored at −69 C prior to bacteriologic evaluation. Based on previous rodent studies (Stevens et al., 1996), control and SRB51-inoculated deer mice and ground squirrels were randomly selected to be euthanized for necropsy at 8 and 12 wk PI; ravens were necropsied at 7 and 11 wk PI and voles were necropsied at 10 and 21 wk PI. At necropsy, tissues were evaluated for gross lesions, and spleen, liver, and reproductive tract (testes, accessory sex glands, uterine horns and uterus) were collected for bacteriologic and histologic evaluation. Additionally, samples of kidney, heart, and lung were collected for histologic evaluation. Histologic specimens were placed in 10% neutral buffered formalin whereas bacteriologic samples were stored at −69 C until processing. Specimens

were sent to the National Animal Disease Center (Ames, Iowa, USA) for bacteriologic evaluation and to the National Veterinary Services Laboratories (Ames, Iowa) for histologic processing.

For bacteriologic evaluation, tissues were weighted, minced using a tissue grinder, serially diluted in saline, and placed on a selective medium for SRB51 (Hornsby et al., 2000). Antibiotics in the BR132 selective medium minimize growth of contaminants without inhibiting the growth of SRB51, and thereby enhance the ability to detect small numbers of SRB51 within samples. Oral and rectal swabs were directly plated onto BR132 media. After incubation of plates at 37 C with 5% CO for 72 hr, bacterial cell counts were made from each dilution by standard plate counts. *Brucella* spp. isolates were identified as strain RB51 based on colony morphology, growth characteristics (Alton et al., 1988), rifampin resistance (Schurig et al., 1991), and a *Brucella* spp. specific polymerase chain reaction procedure based on that of Bricker and Halling (1995). Briefly, the reaction mixture consisted of heat-killed cells, 0.2 mM nucleotide mix (Bochringer Mannheim, Mannheim, Germany), 1 × PCR Buffer II (Perkin Elmer, Branchburg, New Jersey, USA), 2.5 U/ml DNA polymerase (Amplitaq Gold, Perkin Elmer, Branchburg, new Jersey), and 1.5 mM MgCl combined with 20 pm per reaction of specific primers for *B. abortus* omp2A as selected from analysis of its sequence (Ficht et al., 1989) (upstream primer GCAACGGTGTTCTTCCACTC and downstream primer GTATCAGGCTACGCAGAAGG). The samples, including positive and negative controls, were cycled (30 sec 95C, 30 sec 44C, 1 min 72C) 30 times in a thermocycler (MJ Research Inc., Watertown, Massachusetts, USA). Products (5 to 8:1 from each reaction mixture) were separated by electrophoresis on a 1.5% agarose gel and analyzed after staining with ethidium bromide.

Formalin-fixed tissues were embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin for light microscopic examination. Spleens and reproductive tracts from deer mice and voles were not collected for histopathology. Assessment of morbidity was based on results of visual and physical examination, including weight gain/loss, evaluation of ocular and nasal secretions, lethargy, fecal characteristics, feed intake, and coat/feather appearance.

RESULTS

Standard plate counts indicated that SRB51-inoculated ravens, ground squirrels, deer mice, and voles received 1.5×10^8, 7.8×10^7, 4.5×10^7, and 1.6×10^9 colony forming units (CFU) of SRB51, respectively.

Two ravens died during the study. The first died during the acclimation period prior to vaccination; the second raven died 2 wk post-vaccination after exhibiting respiratory distress of several days duration. The cause of the first raven's death was not apparent. Lesions in the second raven indicated mycotic pneumonia and serositis. One SRB51-inoculated mouse died immediately after inoculation and one control mouse died at 4 days PI. Deaths were attributed to handling and probable aspiration of the inoculum. One control vole died at 7 wk PI; the cause of death was undetermined.

Bacteriologic cultures of oral and rectal swabs from all species were negative for recovery of SRB51. All tissues collected from voles at necropsy 10 or 21 wk PI were negative for brucellae. SRB51 was isolated from tissues collected at necropsy from one deer mouse, and ten ground squirrels. Two ravens, including one in the control group, were also culture-positive for SRB51 (Table 1, 2). Colony counts varied from 1.0 colony forming units (CFU)/g of tissue cultured to 8.0×10^4 CFU/g. Tissues evaluated from remaining test animals were culture-negative for SRB51.

Gross lesions consisted of splenomegaly and petechiation of the liver and testes in one SRB51-inoculated ground squirrel (CH23). Microscopic examination of tissue sections from ravens, deer mice, and voles revealed no difference between SRB51-inoculated and control animals. Livers of six of the 10 culture-positive ground squirrels contained small to moderate focal granulomas randomly located in the hepatic parenchyma. The granulomas consisted of small and large mononuclear cells and rare multinucleated cells. The granulomas were often accompanied by focal necrosis and/or increased periportal lymphoplasmacytic infiltrates. One SRB51-inoculated vole necropsied at 10 wk had a disseminated

TABLE 1. *Burella abortus* SRB51 culture results (colony forming units (CFU)/g tissue cultured) on tissues from culture-positive ravens, deer mice and ground squirrels.

Species	Animal ID[a]	Necropsy (Wk PI)	Liver	Spleen	Reproductive tract
Common raven	803-T	7	–[b]	–	+
Common raven	805-C	7	–	+++	–
Deer mouse	BZ 13-T	8	++	++	+
Ground squirrel	CH 12-T	8	–	++	–
Ground squirrel	CH 13-T	8	–	+	–
Ground squirrel	CH 15-T	8	+	+++	–
Ground squirrel	CH 17-T	8	++	++++	+
Ground squirrel	CH 18-T	8	–	++++	–
Ground squirrel	CH 22-T	8	–	+	–
Ground squirrel	CH 23-T	12	+++	++++	+
Ground squirrel	CH 25-T	12	+	++++	–
Ground squirrel	CH 28-T	12	–	+++	–
Ground squirrel	CH 29-T	12	++	++++	–

[a] T = SRB51-inoculated; C = Saline inoculated.
[b] – = culture-negative for SRB51; + = <100 colony forming units (CFU)/g; ++ = 100–999 CFU/g; +++ = 1000-10,000 CFU/g; ++++ = > 10,000 CFU/g.

histiocytic pneumonia and lymphoplasmacytic myocarditis of undetermined origin. No lesions were noted in tissues from other animals.

No differences in clinical signs, appetite, weight grain or loss, or activity were observed between control and SRB51-inoculated animals in all four species.

DISCUSSION

The negative culture results of the oral and rectal swabs indicate a lack of persistent shedding of the SRB51 organism by any of the species tested. The SRB51 organism may have been shed by ravens, ground squirrels, and deer mice during the first few days PI and was therefore not detected as the first PI swabs were collected at one wk PI. Surprisingly, the modified collection protocol that was designed to detect early shedding in the voles did not result in positive cultures. The limited sensitivity of direct plating of fecal swabs may not have allowed the detection of fecal shedding of low numbers of SRB51 organisms.

TABLE 2. *Brucella abortus* SRB51 culture-positive animals per total number animals in group necropsied.

Species	First group[a] controls	First group vaccinates	Second group[b] controls	Second group vaccinates
Common raven	1/2	1/5	0/2	0/5
Deer mouse	0/3	1/6	0/3	0/7
Ground squirrel	0/4	6/7	0/3	4/7
Prairie vole	0/3	0/7	0/3	0/7

[a] First group was necropsied at 7 wk PI (common ravens), 8 week PI (deer mice and ground squirrels) and 10 wk PI (prairie voles).
[b] Second group was necropsied at 11 wk PI (common ravens), 12 wk PI (deer mice and ground squirrels), and 21 week PI (prairie voles).

The cause of SRB51 infection in the control raven is undetermined. The organism may have been transferred to the bird via direct contact as the infected control raven was in an aviary room adjacent to the SRB51-inoculated ravens; a small area of the separating wall consisted of woven wire. Alternatively, the organism could have been transferred via contaminated clothing, feed bowls, or capture nets and drapes, or conceivably by wild deer mice which frequented the outdoor aviary.

Tissue colonization of SRB51 appears to persist in ground squirrels for a greater period of time than in the ravens, deer mice, and voles. However, the SRB51 organism

did not produce any clinical signs of illness in any of the species studied. Clearance of SRB51 by 12 wk in the deer mice and by 10 wk in the voles is consistent with results from previous studies in mice. In one study, BALB/c mice infected with 5×10^7 CFU of SRB51 intraperitoneally (IP) cleared the infection between 30 and 60 days PI, whereas those infected with 1×10^4 CFU of virulent *B. abortus* strain 2308 harbored the bacteria through 60 days PI (Palmer et al., 1996). In another study BALB/c mice showed clearance of 9.2×10^6 CFU of SRB51 from spleens in <6 wk and persistance of 1.2×10^5 CFU of strain 2308 for >20 wk (Stevens et al., 1994). In a third mouse study, SRB51 antigen persisted for 4 wk in parotid lymph nodes after oral inoculation with 5×10^8 CFU, but the organism was not recovered from the spleen between 2 and 12 wk PI. (Stevens et al., 1996) In a recent deer mouse study, orally inoculated mice receiving 1×10^8 CFU SRB51 cleared the infection by 6 wk and mice receiving a similar inoculum IP cleared the infection by 9 wk (Cook et al., 2001) Granulomas similar to those noted in the ground squirrel livers have been observed in *B. abortus* strain 19- inoculated BALB/c mice (Enright et al., 1990) and *Brucella suis* biovar 4-inoculated ground squirrels (*Citellus parryii*) (Miller et al., 1980).

In summary, oral exposure to SRB51 did not produce observed morbidity or mortality in ravens, ground squirrels, deer mice, or prairie voles. Neither fecal nor oral shedding was detected. Ravens, deer mice, and voles cleared the infection by 8 to 10 wk. However, SRB51 infection persisted in some ground squirrels for at least 12 wk. Further investigation to determine the duration of SRB51 infection in ground squirrels is merited.

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