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Experimental Infection of Richardson's Ground Squirrels (*Spermophilus richardsonii*) with Attenuated and Virulent Strains of *Brucella abortus*

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ABSTRACT: A previous investigation of the safety of *Brucella abortus* strain RB51 (sRB51) in various nontarget species suggested that Richardson's ground squirrels (*Spermophilus richardsonii*) may develop persistent infections when orally inoculated with the vaccine. In the present study, sRB51, *B. abortus* strain 19 (s19), and virulent *B. abortus* strain 9941 (s9941) were administered orally to Richardson's ground squirrels to further characterize *B. abortus* infection in this species. Six groups of nongravid ground squirrels were orally inoculated with 6×10^8 colony forming units (cfu) sRB51 ($n=10$), 2.5×10^4 cfu s19 ($n=10$), 2.5×10^7 cfu s19 ($n=6$), 1.3×10^6 cfu s9941 ($n=5$), 2.1×10^8 cfu s9941 ($n=5$), or vaccine diluent (control; $n=4$). One of five animals in the lower-dose s19 group and two of three animals in the higher-dose s19 group showed persistence of bacteria in various tissues at 14 wk postinoculation (PI). At 18 wk PI, one of five animals in the sRB51 group and one of five animals in the high-dose s9941 group were culture positive. Although we did detect some persistence of *B. abortus* strains at 18 wk, we found no evidence of pathology caused by *B. abortus* strains in nonpregnant Richardson's ground squirrels based on clinical signs, gross lesions, and microscopic lesions.

Key words: *Brucella abortus*, brucellosis, nontarget, Richardson's ground squirrel, *Spermophilus richardsonii*, strain 19, strain RB51, wildlife vaccination.

Brucellosis is a highly important disease in humans, wildlife, and livestock worldwide. In the US, bison (*Bison bison*) and elk (*Cervus elaphus*) are considered sources of *Brucella abortus* for domestic cattle in the Greater Yellowstone area (GYA), which includes portions of the states of Wyoming, Montana, and Idaho. Based on epidemiologic evidence, wild elk have been identified as the source for *B. abortus* in an Idaho cattle herd (Hillman, 2002). In addition, both elk and bison are capable of transmitting *B. abortus* to cattle in experimental settings (Davis et al., 1990; Thorne et al., 2002). Considerable efforts by numerous researchers have been devoted to investigating the efficacy and safety of *B. abortus* strain RB51 (sRB51), an attenuated rough strain (Schurig et al., 1991) in wild ungulates. Strain RB51 is safe for use in bison, and, although this vaccine has produced mixed results against abortion in this species (Elzer et al., 1998; Olsen et al., 1998, 1999; Elzer et al., 2002; Olsen and Holland, 2003; Olsen et al., 2003), it is currently being considered for use in vaccination of bison in Yellowstone National Park. In elk, this vaccine does not appear to provide any protection from abortion (Cook et al., 2000; Kreeger et al., 2000). However, recombinant strains of sRB51 are currently being investigated for efficacy in elk (P. Nol, unpubl. data). Research programs involving live vaccines intended for use in wildlife require that safety studies be carried out in potential nontarget species. In such a study, Januszewski and others (2001) noted that Richardson's ground squirrels (*Spermophilus richardsonii*), an abundant species of ground squirrel in the GYA, maintained persistent infections for 12 wk after oral inoculation with 7.8×10^7 colony forming units (cfu) of sRB51 (6/7 and 4/7 were culture positive in various tissues at 8 and 12 wk, respectively). In addition, 6/10 culture positive squirrels had gross liver lesions, and one animal had lesions in the testes, although no clinical signs were observed, and it was not determined if these lesions were specifically induced by

sRB51 infection. The presence of persistent infections and lesions in these animals was unexpected, as this was not found in other rodent species tested in this study, including deer mice (*Peromyscus maniculatus*) and prairie voles (*Microtus ochrogaster*). We were therefore interested in determining how long various *B. abortus* strains (including field strain) could persist in Richardson's ground squirrels and whether any of these strains would induce pathology. Additional knowledge regarding how *Brucella* strains may affect this species would be very useful, not only in the event that a live attenuated strain of *B. abortus* is utilized in a field vaccination program in the GYA, but also in determining if this species could be adversely affected by exposure to field strain *B. abortus* or serve as a reservoir for *B. abortus* strains in general. In addition, we also hoped to gather information regarding the potential of Richardson's ground squirrels to serve as a model for brucellosis using an attenuated strain as a surrogate for virulent strains. Such a model would be helpful as another screening tool for vaccines and treatments for brucellosis while avoiding the high costs of a biosafety level three facility. In the present study, we orally infected one group of nonpregnant Richardson's ground squirrels with sRB51, two groups of ground squirrels with *B. abortus* vaccine strain 19 (s19) at two dose levels, and two groups with field strain 9941 (s9941) at two dose levels. Strain 9941 was originally isolated from an infected cattle herd in the GYA, the source of which was attributed to wildlife. We followed these squirrels over the course of 14 and 18 wk after infection. We aimed to determine whether oral inoculation of *B. abortus* could induce disease in Richardson's ground squirrels, if shedding of particular strains occurred after ingestion, and to investigate the duration of persistence of the three strains after infection.

Forty-one wild Richardson's ground squirrels were obtained from Roundup, Montana, USA (46.445°N, 108.541°W), in June 2004. Squirrels were captured in Tomahawk 48.3×17.1×17.1 cm single-door traps and transported to an indoor laboratory animal facility at the US Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), National Wildlife Research Center (NWRC), Fort Collins, Colorado, USA. On arrival, all animals were weighed, treated with ivermectin (0.1 mg by mouth; Ivomec, Meriel, Deluth, Georgia, USA), and given a topical insecticide dust (Drione Insecticide, Bayer Environmental Laboratories, Montvale, New Jersey, USA). At NWRC, squirrels were held under a 2-wk quarantine period upon arrival. Two weeks after arrival, 10 squirrels were transported from NWRC to a biosafety level three (BL-3) animal facility at the USDA, Agricultural Research Service, National Animal Disease Center (NADC), Ames, Iowa, USA. Squirrels were housed in standard 26.7×48.3×20.3 cm plastic rat cages. All squirrel housing, care, and experimental procedures were approved by the NWRC and the NADC Institutional Animal Care and Use Committee guidelines. Total acclimation time for the NWRC animals prior to inoculation was 26 days. The 10 animals taken to NADC acclimated there for 18 days prior to inoculation.

Brucella abortus strain RB51 and strain 19 used in this experiment were commercial vaccine strains obtained from Colorado Serum Company, Denver, Colorado, USA. Strain RB51 and s19 were reconstituted and diluted to desired concentrations with the provided manufacturer's diluent. *Brucella abortus* strain 9941 was grown on tryptose agar and harvested after 48 hr by aspiration using saline. The suspension was adjusted by use of a spectrophotometer (Bausch and Lomb, Rochester, New York, USA) to the desired concentrations. Actual concentrations of viable bacteria within inoculums were determined by serial dilution and standard plate counts.

Animals were randomly assigned to treatment groups stratified by sex. At NWRC, 10 squirrels were orally inoculated with 6×10^8 cfu sRB51, six squirrels were inoculated with 2.5×10^7 cfu s19, 10 squirrels received 2.5×10^4 cfu s19, and four squirrels were given vaccine diluent provided by the manufacturer. One squirrel that had been assigned to the higher-dose s19 group had to be euthanized prior to inoculation due to illness. At the NADC BL-3 animal facility, five squirrels were orally inoculated with 1.3×10^6 cfu s9941, and five squirrels were inoculated with 2.09×10^8 cfu s9441. Each squirrel received 250 µl of inoculum directly into the mouth via tuberculin syringe. All squirrels were observed twice daily for clinical signs for 21 days and once daily thereafter.

Prior to inoculation, and at 4, 8, and 13 wk postinoculation, whole blood was collected from animals inoculated with sRB51 and s19 only. Up to 0.5 ml of blood was collected via the femoral or saphenous vein using a 0.5-ml insulin syringe, and blood was allowed to clot in a 0.5-ml polypropylene microcentrifuge tube (USA Scientific Inc., Ocala, Florida, USA). Blood was centrifuged at 8,900 × G for 20 min (Eppendorf 5415D, Brinkmann, Westbury, New York, USA). Clots were stored in polypropylene tubes at −80 C until bacterial analysis. Due to logistical reasons, blood collection was not performed in squirrels housed at NADC.

To fulfill biosafety requirements at NWRC and to collect shedding data, cage litter swabs were collected from cages on days 3, 6, 9, 13, 17, and 22 PI. Rectal swabs were collected from two randomly selected squirrels from each of the three inoculated groups on the above days as well. All swabs were directly plated on Kudzas Morse (KM) agar within 2 hr of collection and incubated at 37 C with 5% CO_2 for 72 hr. Plates were placed at 4 C until analysis. Plates were observed for growth of *Brucella*, and any suspicious colonies were analyzed by polymerase chain reaction (PCR). Shedding data were not collected from squirrels housed at NADC.

At 14 wk PI, 15 animals representing all four groups housed at NWRC were placed under isoflurane anesthesia, euthanized with CO_2, and bled postmortem via cardiac puncture. These blood samples were centrifuged at 2,800 × G for 20 min in a Beckman CS-6R Centrifuge (Beckman Coulter Inc., Fullerton, California, USA). Animals were then necropsied, during which pharyngeal lymphoid tissue, lung, heart, liver, spleen, kidney, mesenteric lymph nodes, and reproductive tract (testes, accessory sex organs, uterus, and uterine horns) were taken for culture and histopathology. At 18 wk PI, the remaining 15 animals at NWRC and all the animals inoculated with s9941 at NADC were euthanized and processed as described already. Samples collected for culture were placed in sterile plastic Whirl-Pak sampling bags (Nasco, Ft. Atkinson, Wisconsin, USA) and stored at −80 C. Frozen specimens collected at NWRC were sent on dry ice to the NADC for bacteriologic evaluation. Samples collected for histopathologic examination were placed in 10% neutral buffered formalin and sent to the National Veterinary Services Laboratories (Ames, IA, USA) for histologic processing. Special staining (Giemsa, Gram, and Grocott's Silver) of formalin-fixed tissues was performed when lesions were identified to determine presence of *B. abortus* or other organisms.

Tissues and blood/blood clots collected for culture were allowed to thaw, and tissues were weighed and ground in glass tissue grinders with 2 ml of phosphate buffered saline (PBS). Two-hundred microliters of the resulting fluid were placed on each of three plates containing selective media for *B. abortus* (KM, tryptose agar with 15% heat-inactivated bovine serum albumin, and brilliant green B132; Hornsby et al., 2000). The plates were incubated at 37 C with 5% CO_2 and

TABLE 1. Number of animals positive for *Brucella abortus* at 14 and 18 wk postchallenge over total number of animals tested.

Time point (wk)	*Brucella abortus* strain (dose)					
	sRB51 (6×10^8 cfu[a])	s19 low dose (2.5×10^4 cfu)	s19 high dose (2.5×10^7 cfu)	s9941 low dose (1.3×10^6 cfu)	s9941 high dose (2.09×10^8 cfu)	Control (diluent)
14	0/5	1/5	2/3	na	na	0/2
18	1/5	0/5	0/3	0/5	1/5	0/2

[a] cfu = colony forming units.

monitored for growth. The presence of *Brucella* spp. was confirmed using PCR, as described next.

A *Brucella* spp. PCR based on that of Bricker and Halling (1995) and described in Januszewski et al. (2001) was performed to verify isolation of *B. abortus*. Briefly, selected colonies were picked into 196 μl of sterile double-distilled water. Five microliters prepared sample were used in the PCR reaction (50 μl total). Polymerase chain reaction was performed using a DNA thermalcycler (iCycler, Biorad, Hercules, California, USA; or MJ Research Inc., Watertown, Massachusetts, USA) and AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Foster City, California, USA). Positive controls consisted of sRB51 and s19 bacteria. Ten microliters of reaction products were size fractionated through a 2% agarose gel (Sigma, St. Louis, Missouri, USA) in 1X TAE buffer (40 mM Tris acetate, 1 mM EDTA) and analyzed after staining with acridine orange (Molecular Probes, Inc., Eugene, Oregon, USA) or ethidium bromide.

None of the swabs taken from cage litter was positive for *Brucella* spp. for any of the six time periods during which cage samples were taken. Two of six rectal swabs taken on day 2 PI from animals inoculated with 2.5×10^7 cfu s19 grew *Brucella* spp., confirmed by PCR. No other rectal swabs were positive throughout the remainder of the 3-wk sampling period.

One animal inoculated with 2.5×10^4 cfu s19 was culture positive on both spleen and pharyngeal lymphoid tissue at 14 wk PI, and two animals inoculated with 2.5×10^7 cfu s19 were culture positive on mesenteric lymph node and lung samples, respectively, at 14 wk PI. One squirrel inoculated with 2.09×10^8 cfu s9941 was positive on blood culture at 18 wk PI. One sRB51-inoculated animal was positive on pharyngeal lymphoid tissue at 18 wk PI. A summary of these data can be found in Table 1.

On examination, all squirrels displayed copious subcutaneous and abdominal fat and had markedly enlarged, pale livers. All livers displayed hepatocellular vacuolization with a pronounced centrilobular pattern consistent with lipidosis. Livers of two squirrels had small, multifocal areas of pleocellular infiltrates consisting of small and large mononuclear cells, suggesting mild hepatitis (2.5×10^4 cfu s19, n=1; 2.5×10^7 cfu s19, $n=1$). One animal in the sRB51 group had focal necrotizing hepatitis.

Other lesions observed in these animals included pyogranuloma in the kidney (2.5×10^4 cfu s19, $n=1$), focal necrotizing pancreatitis (sRB51, n=1), multifocal interstitial nephritis (sRB51, n=1; 2.5×10^4 cfu s19, $n=2$), focal nephrosis (2.5×10^4 cfu s19, $n=1$), and two animals had sparse mononuclear cellular infiltrates in the lumen of the oviduct and uterus (sRB51, $n=2$). None of the lesions found in any of the squirrels could be attributed to *B. abortus* infection based on culture and/or special staining.

Our study indicates that Richardson's ground squirrels experience minimal clinical signs and pathology associated with

oral ingestion of field or vaccine strains of *B. abortus* at challenge levels ranging from 2.5×10^4 cfu to 6×10^8 cfu. We intentionally used a one-log-higher dose for sRB51 than that used by Januszewski and others (2001) in order to increase our chances of inducing persistent infection. The target s19 and s9941 dose rates for the present study were based upon traditional challenge doses (1×10^7 cfu) of virulent *B. abortus*. While the s9941 doses achieved target levels, we failed to do so with the s19 doses, which we intended to be at 1×10^6 cfu and 1×10^8 cfu for low and high dose, respectively. Despite the fact that this study did not evaluate s19 infection in ground squirrels at intended challenge levels, this information still alleviates concerns that this species could be severely impacted by *B. abortus* infection with either attenuated or virulent strains of *B. abortus*.

Although a typical oral vaccine dose could reach as high as 1×10^{11} cfu, we elected to use the lower doses to avoid the possibility of high mortality rates during our experiment in order to collect long-term data. Based on our observations however, dose rates of 1×10^{10} or 10^{11} cfu probably would not have produced the severity of disease that we initially expected. If a live *B. abortus* strain were to be proposed for use in the field as an oral vaccine, however, safety trials at the higher dose levels would need to be considered with the vaccine strain.

There was a general lack of persistence of the three strains of *B. abortus* in Richardson's ground squirrels at 18 wk PI. It is unknown if and when the three animals that were culture positive at 14 wk and the two positive animals at 18 wk may have cleared the infection if given more time. As we could not detect any evidence of shedding of sRB51 or s19 beyond 2 days PI, and only two of the 25 animals cultured at 18 wk showed evidence of persistent infection, our data suggest that even if infected, ground squirrels are unlikely to be severely affected by *B. abortus* or serve as sources of *Brucella* in the field.

Our results indicate that the Richardson's ground squirrel would not serve as a satisfactory model for brucellosis based on our oral inoculation trial. Not only did these animals not experience disease due to infection by sRB51, or the more virulent s19, Richardson's ground squirrels also do not appear to be susceptible to field strain *B. abortus*. Wild rodents throughout the world have been used as models for evaluation of infection and persistence of various *Brucella* species (Gorban and Grekova, 1978; Miller and Neiland, 1980; Cook et al., 2001); however, susceptibility to *Brucella* spp. infection varies greatly among rodent species, and there is currently no known rodent model utilizing attenuated strains of *Brucella*. Miller and Neiland (1980) found that Arctic ground squirrels (*Citellus parryii*) were readily infected by intraperitoneal (IP) *B. suis* biovar 4, but they did not experience significant pathologic changes; lemmings (*Dicrostonyx* spp.) did develop significant disease. Deer mice (*Peromyscus maniculatus*) inoculated both orally and IP with approximately 1×10^8 cfu sRB51 were able to clear infection by 9 wk and exhibited no clinical signs (Cook et al., 2001; Januszewski et al., 2001). Comparable results have also been seen in inbred BALB/c mice, wherein oral and IP administration of 10^6 to 10^8 cfu sRB51 could only induce tissue persistence ranging from 4 to 8 wk. This is in contrast to virulent strain 2308, which persisted for at least 20 wk subsequent to IP inoculation in BALB/c mice, a commonly used inbred strain of laboratory mice (Stevens et al., 1994, 1995). Although Richardson's ground squirrels do appear to have greater persistence of *B. abortus* strains after oral infection, in comparison to some rodent species, this persistence is not of a consistent nature or of adequate duration for use as a laboratory infection model.

In summary, *B. abortus* strains RB51, 19, and 9941 do not produce disease in

nonpregnant Richardson's ground squirrels when given orally at challenge doses ranging from 2.5×10^4 cfu to 6×10^8 cfu. Although Richardson's ground squirrels seem to harbor *B. abortus* for a slightly longer period of time in comparison to some other rodent species, our data indicate that this species is unlikely to act as a reservoir or source of infection for either vaccine or wild type strains of *B. abortus*. Based on these results, the Richardson's ground squirrel would be a poor model for oral *Brucella* infection as well. These data provide useful information for researchers and those charged with the management of brucellosis in wildlife in the GYA.

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