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EFFECTS OF DELIVERY METHOD ON SEROLOGICAL RESPONSES OF BIGHORN SHEEP TO A MULTIVALENT *PASTEURELLA HAEMOLYTICA* SUPERNATANT VACCINE

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ABSTRACT: The safety and efficacy of a remotely delivered multivalent *Pasteurella haemolytica* supernatant vaccine (serotypes A2 and T10) were examined in captive Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*). Twenty bighorn sheep were grouped according to baseline leukotoxin neutralizing antibody titers (≤ 2 or >2 $\log_2^{-1}$) and vaccination history (previously vaccinated or unvaccinated). Within these groups, animals were randomly assigned to one of two delivery treatments: hand injection (control) or biobullet implantation. All bighorns received a single dose from the same lot of vaccine ($n = 10$/treatment); four additional animals were injected intramuscularly with 0.9% saline as unvaccinated sentinels. Mild, transient lameness one day after hand injection or biobullet implantation was the only adverse effect. Serum neutralizing antibody titers to *P. haemolytica* leukotoxin differed between delivery treatments ($P = 0.009$) and among baseline titer/vaccination history groups ($P = 0.013$). Neutralizing titers were higher among hand-injected bighorns. Although neutralizing titers were lower among implanted bighorns than hand-injected controls at 1 wk ($P = 0.002$) and 2 wk ($P = 0.021$) after vaccination, seroconversion rates in response to implantation (6/10) and hand injection (9/10) did not differ ($P = 0.303$). Agglutinating antibody titers to T10 were high and did not vary over time or between delivery treatments. Agglutinating antibody titers to A2 in the hand-injected controls were not different ($P \geq 0.07$) than those in bighorns vaccinated with biobullet implantation. These data demonstrate that although hand injection elicits higher absolute titers, biobullet implantation may also stimulate effective antibody responses to *P. haemolytica* supernatant vaccine. Further evaluation of biobullet vaccination against pneumonic pasteurellosis in free-ranging populations of wild bighorn sheep is warranted.

Key words: Bighorn sheep, *Ovis canadensis*, *Pasteurella haemolytica*, pasteurellosis, vaccine delivery, vaccination.

INTRODUCTION

Pneumonic pasteurellosis is a serious disease in bighorn sheep. Pneumonia epidemics are thought to have contributed to limiting the abundance of bighorn sheep (*Ovis canadensis canadensis*) in North America over the last century (Buechner, 1960; Post, 1962; Hobbs and Miller, 1992). *Pasteurella* spp. are arguably the most common pathogens isolated from bighorns during these epidemics (Potts, 1937; Marsh, 1938; Post, 1962; Onderka and Wishart, 1984). Successful long-term bighorn sheep management is impeded by the current inability to prevent or control mortality caused by pneumonic pasteurellosis (Onderka and Wishart, 1984; Festa-Bianchet, 1988; Hobbs and Miller, 1992).

Despite intensive study of the bighorn pneumonia complex, few tools for its management have emerged. Vaccination has been suggested as a potential management tool for many years. However, the success of previous attempts to vaccinate bighorn sheep against pasteurellosis has varied. Early vaccines either failed in application or were never fully evaluated or incorporated into bighorn management programs (Howe, 1964; Foreyt, 1992; Foreyt and Silflow, 1996); one modified-live *P. haemolytica* A1 vaccine apparently caused pasteurellosis in healthy bighorn sheep (Onderka et al., 1988). More recently, Miller et al. (1997) described a multivalent *P. haemolytica* vaccine (A1, A2, T10) that stimulated marked elevations in antibody

TABLE 1. Treatment allocation, including animal identification (the last two digits indicate the year of birth) and sex of study animals. Saline sentinels were A85, C92, M88, and E88. Animals were housed according to sex and reproductive status. All ewes with lambs (including A85, C92, and M88) were housed together with the 1997 lambs in one pasture. Ewes without lambs (including E88) were housed in another pasture which shared fences with the ram pasture.

Block	Hand injection	Biobullet	History
1	Q92 F M91 F T88 F E994 F	Q94 F E89 F E392 F C994 M	Previously vaccinated; baseline leukotoxin titer > 2
2	S96 F	C89 F	Previously vaccinated; baseline leukotoxin titer ≤ 2
3	U96 M E997 F C297 M	A95 F M397 M M897 F	Never vaccinated; baseline leukotoxin titer ≤ 2
4	E497 F R96 M	A97 F O96 M	Never vaccinated; baseline leukotoxin titer > 2

titers to leukotoxin and surface antigens. Kraabel et al. (1998) subsequently demonstrated that the immune response generated by this vaccine was protective when bighorn sheep were challenged intratracheally with a pathogenic strain of *P. haemolytica* T10. Strains of *P. haemolytica* biotype T are also referred to as *P. trehalosi* in the literature (Sneath and Stevens, 1990).

In addition to simply having effective vaccines, practical alternatives for delivery are needed before vaccination can become a useful wildlife management tool. In order to implement an effective vaccination program against pasteurellosis in free-ranging bighorn sheep, alternative methods to traditional intramuscular hand-injection are required. Individual capture and restraint of wild animals is labor-intensive and expensive. Also, capture and restraint procedures are stressful (Miller et al., 1991), and could compromise an individual animal's ability to respond to vaccination. Remote implantation using a biobullet would alleviate this need for individual capture. This experiment was designed to compare the serological responses of captive bighorn sheep to an experimental multivalent *P. haemolytica* supernatant vaccine (A2 and T10) when delivered by traditional intramuscular hand-injection or biobullet implantation.

MATERIALS AND METHODS

Twenty captive Rocky Mountain bighorn sheep were used in this experiment. All sheep were housed at the Colorado Division of Wildlife's Foothills Wildlife Research Facility (Fort Collins, Colorado, USA; 40°35′N, 105°10′W) throughout the study. Grass/alfalfa hay mix and a pelleted high-energy supplement were provided as prescribed under established feeding protocols for bighorn sheep in respective age/sex classes (Miller, 1990); fresh water and mineralized salt blocks were provided *ad libitum*. The resident bighorn herd was lungworm free (M. W. Miller, unpubl. data).

The sheep were divided into four blocks (3 to 12 sheep/block) according to their vaccination history (previously vaccinated or not) and their baseline *P. haemolytica* leukotoxin neutralizing antibody titers (>2 or ≤2 $\log_2^{-1}$). From these blocks, sheep were randomly assigned to one of the two treatment groups: hand-injection or biobullet implantation. (n = 10/group) (Table 1).

The experimental supernatant vaccine was prepared using four strains of *P. haemolytica*; formulation was similar to that of another vaccine previously tested in bighorn sheep (Miller et al., 1997; Kraabel et al., 1998). Two strains of *P. haemolytica* serotype A2 were used. Both were of domestic sheep origin; one strain was recovered from a domestic sheep in New Zealand (Alexander et al., 1995) and the other was chosen for its demonstrated pathogenicity in

bighorn sheep (Foreyt et al., 1994; Foreyt and Silflow, 1996). Similarly, two strains of *P. haemolytica* serotype T10 were used; one strain was from a domestic sheep (USDA *P. haemolytica* JF2) and one was from a bighorn (Kraabel et al., 1998). The culture supernatants from each strain were prepared in the same way. An overnight (O/N) culture of bacteria on a blood agar plate was used to inoculate a small volume of RPMI 1640 (ICN Biomedicals, Inc., Costa Mesa, California, USA) containing 0.5% Brain Heart Infusion Broth (BHIB) (Difco Laboratories, Detroit, Michigan, USA). This culture was incubated O/N at 37 C, shaking at 100 rpm. This primary culture was used to inoculate 1800 ml of RPMI 1640 in a 2 L fermenter (The Virtis Company, Inc., Gardiner, New York, USA). Fermenter conditions were set at 37 C and 200 rpm. The start optical density (OD) was between 0.1 and 0.2 at 525 nm. The culture was fermented to a stop OD of 0.58–0.70 at 525 nm (approximately 2–4 hr). The culture was then centrifuged (6000 × g) and the supernatant was passed through a 0.2 μm filter (Gelman Scientific, Rexdale, Ontario, Canada). The recovered supernatant was concentrated about 20 times using a Millipore Minitan System® with a 5000 MWC membrane (Millipore Corporation, Bedford, Massachusetts, USA).

Vaccine was comprised of equal amounts of each of the four concentrated supernatants and was adjuvanted with $Al(OH)_3$ (Merial Inc., Athens, Georgia, USA) and Quil A (Cedarlane, Hornby, Ontario, Canada) (23 and 0.005% of vaccine total, respectively). A 2 ml dose was used for intramuscular hand-injection. A quantity of adjuvanted vaccine was lyophilized and the equivalent dry weight of a 2 ml dose (0.03 g) was packed into biobullets (Ballistic Technologies Inc., Newcastle, Oklahoma, USA) according to the manufacturer's suggestions.

Hand-injected sheep were vaccinated intramuscularly in the left hip. Biobullets were fired at a shaved target on the hindquarters of sheep restrained in a chute at a distance of approximately 4 m. In order to monitor the herd for changes in antibody titers not related to vaccination, four additional animals were injected intramuscularly with 2 ml of physiological saline.

All animals were bled with little or no restraint immediately prior to vaccination and again at 1, 2, 4, 8, and 12 wk post-vaccination. Sera were removed and stored at −20 C until the end of the study period.

Levels of leukotoxin neutralizing antibodies in bighorn sera were measured using a previously described in vitro assay (Miller et al., 1997). Neutralizing titers were expressed as the reciprocal $\log_2$ dilution that yielded ≥50% neutralization of toxicity. Seroconversion was defined as ≥2 increase in titer. Levels of serum antibodies against serotype-specific surface antigens were measured in a direct microagglutination assay (Shewen and Wilkie, 1982) using washed formanilized *P. haemolytica* serotypes A2 or T10 as the antigen. Agglutinating titers were expressed as the reciprocal $\log_2$ of endpoint dilutions.

Data were analyzed using least squares analysis of variance for general linear models (SAS Institute, Inc., 1995) with a repeated measures structure and randomized complete block design. All comparisons used a pre-established $\alpha = 0.05$ to assess statistical significance. Sample sizes (n = 10/treatment group) were sufficient ($\beta = 0.2$) to detect ≥3 $\log_2^{-1}$ differences in antibody titers among groups.

RESULTS

No adverse effects were observed, save for mild transient lameness 1 day after vaccination in most sheep. No serum antibody changes were observed during the study period in the four animals injected with saline. Serum neutralizing antibody titers to *P. haemolytica* leukotoxin differed by baseline titer/vaccination history ($P = 0.013$) and between delivery treatments ($P = 0.009$) (Fig. 1A). Neutralizing titers were higher among hand-injected bighorns. Although neutralizing titers were lower among implanted bighorns than hand-injected controls for 1 wk ($P = 0.002$) and 2 wk ($P = 0.021$) after vaccination, seroconversion rates (≥2 increase in titer at 2 wk post vaccination) in response to implantation (6/10) and hand injection (9/10) did not differ ($P = 0.303$). Agglutinating antibody titers to the surface antigens found on *P. haemolytica* serotype T10 were high and did not vary over time or between delivery treatments (Fig. 1B). Agglutinating antibody titers to the surface antigens on *P. haemolytica* A2 did not differ between the hand-injected controls and the bighorns vaccinated with biobullet implantation ($P = 0.07$). Baseline titer/vaccination history had a significant effect on agglutinating antibody response to the surface antigens of *P. haemolytica* serotype A2 ($P = 0.0001$). Animals that had not

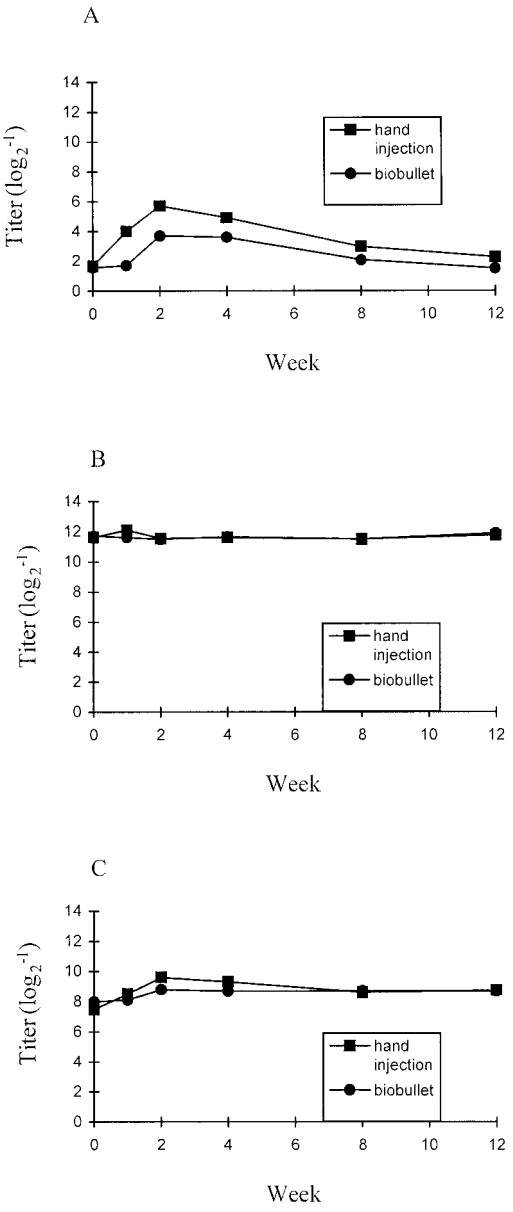

FIGURE 1. Mean antibody titers ($\log_2^{-1}$) over time in bighorn sheep vaccinated with multivalent *Pasteurella haemolytica* supernatant vaccine. A. Bighorn sheep vaccinated by intramuscular hand-injection had significantly higher leukotoxin neutralizing antibody titers than those animals vaccinated by biobullet implantation. B. Agglutinating antibody titers to the surface antigens on *Pasteurella haemolytica* serotype T10 did not vary between treatments or over time. C. Agglutinating antibody titers to the surface antigens on *P. haemolytica* serotype A2 in animals vaccinated by hand-injection were not significantly different from those in animals vaccinated remotely.

been previously vaccinated and had low baseline titers showed the most dramatic response to vaccination.

DISCUSSION

Recent experiments using *P. haemolytica* supernatant vaccine in bighorn sheep (Miller et al., 1997; Kraabel et al., 1998) have provided data supporting the use of this type of vaccine in a field application. Traditional hand injection would be appropriate if animals were being individually handled for other purposes such as tagging or radio-collaring. However, for general bighorn sheep herd health management programs, hand injection is not a practical method of vaccine delivery. Remote implantation using biobullets is used extensively in elk (*Cervus elaphus*) and less so in bighorns and other species (Angus, 1989; Herriges et al., 1991; Jessup et al., 1992; DeNicola et al., 1996). The advantages of biobullet use include cost-effectiveness and a seemingly minimal adverse effect on the treated animals. Another possibility for vaccine presentation is through the oral route. Bighorn sheep have been treated effectively with oral anthelmintics and antibiotics for many years (Schmidt et al., 1979; Coggins, 1988; Bailey, 1990). New technology using poly(methacrylic) hydrogels or alginate microspheres as vehicles for oral administration of vaccines to ruminants appears potentially effective (Bowersock et al., 1994a, b, 1998). Although the authors have completed initial studies investigating similar delivery in bighorn sheep, further testing is required before valid comment can be made regarding efficacy.

Leukotoxin neutralizing serum antibodies are required for protection against *P. haemolytica* pneumonia (Shewen and Wilkie, 1983; Kraabel et al., 1998). The highest leukotoxin titers were observed in bighorn sheep receiving vaccine by traditional hand injection. Bighorn sheep vaccinated remotely via biobullets had lower leukotoxin neutralizing antibody titers than the hand-injected controls (Fig. 1A). How-

ever, there was no significant difference in rates of seroconversion ($\geq 2 \log_2^{-1}$ increase in titer) between implanted bighorns and hand-injected animals. Serological responses to hand-injection were consistent with previous data (Miller et al., 1997). The cause of diminished antigenic stimulation in the implanted sheep was not determined. It is possible that lyophilizing or other aspects of preparing and handling the adjuvanted product somehow reduced potency; although pilot studies in domestic sheep suggest that this is unlikely (H. J. McNeil, unpubl. data). Perhaps the way in which the biobullets degrade and release the enclosed material requires that a greater antigenic load be delivered. This possibility deserves further investigation.

Agglutinating serum antibodies to the surface antigens on *P. haemolytica* are also required for protection (Shewen and Conlon, 1993). The high agglutinating antibody titers to the surface antigens of *P. haemolytica* T10 (Fig. 1B) found in this study are consistent with previous studies (Miller et al., 1997; Kraabel et al., 1998), as well as with the results of other bighorn sheep sera tested in this laboratory. *Pasteurella haemolytica* T10 is the most common serotype of *P. haemolytica* found as a commensal in the nasopharynx of bighorns (Wild and Miller, 1991; Queen et al., 1994). Many of these are believed to be non-pathogenic (Wild and Miller, 1991; Sweeney et al., 1994; Kraabel et al., 1998). This would explain the high base-line agglutinating antibody titers in all groups, regardless of vaccine history. Avirulence could be due to lack of leukotoxin production in these strains (Green et al., 1999).

Hand-injected sheep had only slightly higher agglutinating antibody titers to the surface antigens of *P. haemolytica* serotype A2 than the remotely vaccinated animals (Fig. 1C). The relatively high agglutinating antibody titers to A2 probably reflect previous exposure to *P. haemolytica* A2, either by vaccination or natural colonization. The vaccination history/baseline titer affected the magnitude of an individual's potential agglutinating antibody response to the surface antigens on *P. haemolytica* A2. Animals that had never been previously vaccinated and had a baseline leukotoxin neutralizing titer of <2 showed the greatest antibody response to vaccination. However, animals maintaining a high agglutinating titer are less likely to show a detectable increase in circulating agglutinating antibodies after vaccination. This is a characteristic of all assays that use doubling dilutions to titrate serum. High agglutinating titers alone are insufficient to confer protection; however in combination with neutralizing antibodies against leukotoxin, titers are correlated with protection (Shewen and Wilkie, 1983).

Although protection from challenge was not evaluated in this study, evidence suggests that animals vaccinated remotely may respond adequately, albeit animals vaccinated by traditional intramuscular hand-injection attained higher absolute titers. Kraabel et al. (1998) observed that bighorn sheep which survived intratracheal challenge with a pathogenic strain of *P. haemolytica* serotype T10 had leukotoxin neutralizing antibody titers of ≥ 3.5. Animals vaccinated by intramuscular hand-injection or biobullet implantation had comparable leukotoxin neutralizing titers. Even though implants stimulated lower absolute titers, animals with a primed immune system can mount a protective anamnestic response in the face of subsequent challenge against *P. haemolytica* (Kraabel et al., 1998).

These data demonstrate that this experimental multivalent *P. haemolytica* supernatant vaccine is safe for use in bighorn sheep when delivered either by traditional intramuscular hand-injection or remote implantation using a biobullet. The vaccine may stimulate antibody responses sufficient to confer protective immunity when delivered by intramuscular hand-injection or biobullet implantation.

This vaccine has since been used to remotely vaccinate wild free-ranging bighorn sheep (M. W. Miller, unpubl. data). Al-

though no efficacy data are available at this time, no adverse effects have been reported. The remote application of this vaccine as an important component of bighorn sheep herd health management programs warrants further investigation.

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