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Original Article

Efficacy of SpayVac[®] as a Contraceptive in Feral Horses

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ABSTRACT We tested the efficacy of 2 formulations of the immunocontraceptive SpayVac[®], which packages the immunogen porcine zona pellucida (PZP) and an adjuvant in multilamellar liposomes, as a contraceptive in captive feral horses (*Equus caballus*) for 3 consecutive breeding seasons (Pauls Valley, OK, USA; 2012–2014) following a single inoculation. Annual fertility rates in control adult female horses ($n = 30$ each yr) were 100%, 96.7%, and 100%. In the nonaqueous treatment group, fertility was 16.7% in the first year ($n = 30$) and 75.9% in the second year ($n = 29$), at which point we dropped the group from the study. Fertility rates in the aqueous group were 13.3%, 46.7%, and 43.3% ($n = 30$ each yr). Fifteen of the females in the aqueous group were infertile in all 3 years. Across 11 sampling dates postvaccination, mean PZP antibody titers in serum were 33.7–91.9% greater in nonpregnant females than pregnant females for the aqueous treatment group and 7.8–82.8% greater for the nonaqueous group. However, the 15 consistently infertile females did not necessarily have the greatest antibody titers. Reactions at the injection site occurred in 29.8% of the 84 females that received an injection other than saline solution, but there was no evidence that the reactions were painful or affected mobility. The nonaqueous formulation produced more local reactions than did the aqueous, but presence of PZP did not increase the frequency of reactions above that seen with liposomes + adjuvant. Uterine edema was not found at frequencies greater than would be expected in untreated females. Additional research to explore relationships between vaccine dose, adjuvant, and efficacy is warranted. Published 2017. This article is a U.S. Government work and is in the public domain in the USA.

KEY WORDS *Equus caballus*, feral horse, fertility control, immunocontraception, porcine zona pellucida, SpayVac[®], wildlife contraception.

Feral horse (*Equus caballus*) populations managed by the Bureau of Land Management (BLM) in the western United States often grow rapidly and, in the absence of intervention by BLM, would frequently exceed the carrying capacity of their ranges (Eberhardt et al. 1982, Garrott et al. 1991b, Roelle et al. 2010). The BLM has most often controlled herd size through a gather (round up) and removal process, offering excess horses to the public for adoption. Over time, the supply of horses has exceeded adoption demand, and BLM has moved thousands of animals to long-term holding pastures where they are cared for as long as they live at considerable expense to American taxpayers. In 2007, for example, the cost of holding animals off the range was approximately US\$21 million, or about 67% of the budget for BLM's Wild Horse and Burro Program (U.S. Government Accountability Office 2008:9).

The BLM has long been interested in fertility control as a means of regulating feral horse reproduction and partial

solution to the problem of escalating off-range holding costs. The most promising fertility control agents to date are based on porcine zona pellucida (PZP) glycoproteins extracted from pig (*Sus scrofa*) ovaries. When injected into mammalian species such as the horse, these proteins induce production of antibodies that bind to the surface of the ovum and block sperm penetration and fertilization (Liu et al. 1989). Possible effects on ovarian function have been noted as well (Kirkpatrick et al. 1992, Bechert et al. 2013). The most widely used form of PZP available at the present time is an emulsion registered as ZonaStat-H by the U.S. Environmental Protection Agency for use in horses and burros (*E. asinus*). Unfortunately, ZonaStat-H has 2 important drawbacks relative to use in BLM herds. First, a booster inoculation is required, ideally 3–4 weeks after the primer is administered (Liu et al. 1989). Because darting is rarely feasible on western rangelands, most BLM herds must be gathered in order to provide access to the horses for inoculation. Female horses would need to be held in captivity long enough to administer a booster 3–4 weeks later. Second, ZonaStat-H has a reported duration of efficacy of only 7–10 months, after which annual booster inoculations are required to maintain infertility (Liu et al. 1989, Kirkpatrick et al. 1990, Lyda et al. 2005). Some progress in overcoming

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these drawbacks has been made by substituting time-release pellets, delivered at the same time as the primer injection, for the booster at 3–4 weeks and first annual booster. This formulation, sometimes referred to as PZP-22, has a reported efficacy of 22 months (Turner et al. 2007), but this is still less than ideal from the BLM's perspective.

A third formulation of PZP known as SpayVac[®], produced by ImmunoVaccine Technologies Inc. (IVT; Halifax, NS, Canada) using a proprietary liposome technology, has been reported as having long-term, single-dose efficacy in several species, including grey seals (*Halichoerus grypus*; Brown et al. 1997a), fallow deer (*Dama dama*; Fraker et al. 2002), and most recently feral horses (Killian et al. 2008, Fraker and Brown 2011). Killian et al. (2008), for example, reported contraceptive efficacy of 100%, 83%, 83%, and 83% in 4 years following inoculation of 12 feral horses in Nevada, USA, with SpayVac. This result sparked renewed interest within the BLM to conduct further investigations on the efficacy of SpayVac in feral horses.

The 3 main objectives of our study were to 1) quantify the efficacy of SpayVac in preventing pregnancy and foaling over a maximum of 5 breeding seasons postinjection when females are exposed to breeding males in a pasture setting; 2) determine the relationship between PZP serum antibody titers and fertility; and 3) assess the frequency and severity of injection-related and general side effects, if any, following intramuscular injection by hand. In addition, for the 4 years of their study, Killian et al. (2008) reported that 70–100% of females treated with SpayVac showed some degree of uterine edema. Although we do not view uterine edema as a serious contraindication, others have indicated concern, so a fourth objective was to estimate the frequency and degree of uterine edema in females treated with SpayVac. Finally, Bechert et al. (2013) reported that 93% of females that they treated with SpayVac ceased estrous cycling within 3–4 months after vaccination. Our fifth objective was therefore to determine what proportion of our SpayVac-treated females stopped cycling.

STUDY AREA

We conducted our captive breeding trial at BLM's wild horse and burro adoption facility near Pauls Valley, Oklahoma, USA. Adult female horses resided year-round in 3 pastures of approximately 12 ha each, and were accompanied by breeding males from approximately the first week in June (2011) or the first week in May (2012 and 2013) to the first week in October (all years). During the remainder of the year, space limitations forced us to house the males in a separate facility in Nebraska, USA. We transported males to and from their winter quarters by trailer, moving them back to Oklahoma in the spring 2–3 weeks before beginning the breeding trial for that year. We immunized the females and drew blood samples in a processing facility consisting of a series of alleys and pens and a padded hydraulic squeeze chute located 100–300 m from the pastures.

Horses fed on pasture bermudagrass (*Cynodon* sp.) *ad libitum* and alfalfa hay provided at a rate of approximately 6.4 kg/animal/day. FlaxLic[®] protein/fat supplement (New

Generation Feeds, Belle Fourche, SD, USA) and Ranch House trace mineralized salt blocks (United Salt Corporation, Houston, TX, USA) were also provided *ad libitum*. Bureau of Land Management personnel annually immunized horses against eastern and western equine encephalomyelitis, equine influenza type A₂, the EHV-1 and EHV-4 strains of equine herpes virus, and tetanus (Fluvac Innovator[®] 5; Zoetis, Florham Park, NJ, USA); West Nile virus (Vetera[®] WNV; Boehringer Ingelheim Vetmedica, St. Joseph, MO, USA); *Streptococcus equi* (Strepvax[®] II; Boehringer Ingelheim Vetmedica); and rabies (Imrab[®] 3; Merial Limited, Duluth, GA, USA). Additionally, BLM personnel dewormed horses at least annually (more frequently for specific susceptible individuals) using Ivermax[™] Equine Oral Liquid (RXV Products, Westlake, TX, USA).

METHODS

Vaccine Preparation

We conducted all work on this study under the auspices of a protocol (#2011-06) approved by the Animal Care and Use Committee at the Fort Collins Science Center, U.S. Geological Survey, Fort Collins, Colorado, USA. We chose 2 formulations of SpayVac (an aqueous emulsion and a nonaqueous form) for testing based on a recommendation from a companion study on safety and mechanism of action conducted at Oregon State University, Corvallis, Oregon, USA (Bechert et al. 2013). Personnel at IVT prepared the vaccines as described by Brown et al. (1997b). Briefly, they obtained frozen pig ovaries from SiouxPreme Packing Co. (Sioux Center, IA, USA) and isolated PZP by grinding thawed ovaries and passing the resulting mixture through a series of nylon mesh screens with successively decreasing pore sizes to recover oocytes. They then homogenized the oocytes to release PZP, mixed the PZP at a rate of 200 µg/dose with phosphate buffered saline (pH = 7.4), and added 0.2 g lecithin and 0.02 g cholesterol (Lipoid, Newark, NJ, USA) per dose to form multilamellar liposomes. One half of the PZP-liposome mixture was added to Modified Freund's Adjuvant (MFA; Calbiochem, San Diego, CA, USA; 1:1 v/v) to form a water-in-oil emulsion (the aqueous vaccine; 1 mL/dose). The second half of the PZP-liposome mixture was lyophilized and then reconstituted with MFA to form the nonaqueous vaccine (0.5 mL/dose). Vehicle controls were produced in the same manner but without the PZP antigen. Saline controls consisted of 1 mL of phosphate-buffered saline. Technicians at IVT loaded all vaccines and control doses into individual syringes and delivered them to us frozen on dry ice.

Treatments and Blood Sampling

We randomly selected 90 females—ages 4 ($n = 68$), 5 ($n = 18$), 6 ($n = 2$), or 7 ($n = 2$)—from 120 females that were present at the Pauls Valley facility when we initiated our study. All females were thus at or near prime breeding age (Garrott et al. 1991a, Roelle et al. 2010), and all were open (nonpregnant) at the time of enrollment by virtue of separation from males during the previous breeding season. Age was either known precisely (i.e., removed from the range as a foal or born in captivity) or estimated by tooth eruption

and wear (American Association of Equine Practitioners 2002). Bureau of Land Management personnel uniquely marked each female on the left hip with a 4-digit freeze mark in numerals 7.6 cm high. We assigned each female to 1 of 3 experimental groups (aqueous vaccine, nonaqueous vaccine, and control) in a stratified-random fashion such that each group was equally represented in each of the 3 pastures. We further subdivided the 30 females in the control group into aqueous (4 F/pasture), nonaqueous (4 F/pasture), and saline (2 F/pasture) groups. After allowing all materials to thaw for approximately 30 min at room temperature, we delivered vaccines and sham doses on 30 March 2011 by hand injection in the left rump while each female was restrained in a padded hydraulic squeeze chute.

At the time of vaccination and on 7 additional occasions in 2011 (3 May, 7 Jun, 6 Jul, 2 Aug, 7 Sep, 5 Oct, and 14 Dec), we drew 10 mL of blood from each female in serum separator vacutainers by jugular venipuncture for PZP titer assays. We also drew blood samples on 7 March 2012, 9–10 January 2013, 6 March 2013, and 13–14 November 2013. Following collection, we allowed the blood samples to clot for approximately 2 hr at room temperature. We then centrifuged the samples at 1,300 G for approximately 12 min and removed 2 aliquots of approximately 2 mL of serum by pipette. We placed each aliquot in a 10-mL polypropylene transport vial labeled with the date and horse identification number, froze the samples overnight in a household freezer, and shipped them on dry ice to Fort Collins, Colorado, where they were stored at -80°C until being shipped on dry ice by overnight courier for titer assays.

To corroborate our foaling observations (see below), we drew an additional 10 mL of blood from each female in a plain red-top vacutainer on 12 December 2011, 9–10 January 2013, and 13–14 November 2013. We separated serum from these samples as described above and shipped the frozen samples to the Endocrinology Lab, Animal Health Diagnostic Center, Cornell University, Ithaca, New York, USA, for pregnancy testing. Personnel at the lab assayed the samples for pregnant mare serum gonadotropin and estrone sulfate and characterized each female as open, pregnant, or inconclusive.

Female Horse Health and Condition

During each blood draw through 7 March 2012, we observed females carefully for general health and palpated each injection site to ascertain whether a reaction had occurred. We characterized each site as follows: none = no reaction apparent; swelling = a diffuse raised area, perhaps irregular in shape, may be firm, but does not appear or feel hard; lump = a distinct, roughly circular nodule that appears or feels hard; abscess = a draining sore or pocket of fluid or pus. We characterized the severity of each reaction on a scale from 0 to 3, using the same scoring system both in palpation of the injection sites and pasture observations (see below): 0 = no reaction observable or palpable; 1 = reaction (swelling, lump, or abscess) present, but no pain or lameness noticeable; 2 = reaction present, mild to moderate lameness or response to palpation; 3 = reaction present, severe lameness or strong response to palpation. On 30

March 2011 and 7 March 2012, we also estimated body condition of our females using the system of Henneke et al. (1983). We assigned each female a score from 1 to 9 following observation in the chute and palpation of fat and soft tissue of the neck, back, ribs, and rump.

Serum Assays

Technicians at IVT used an enzyme-linked immunosorbent assay to determine PZP antibody titers in horse serum. They coated 96-well polystyrene microtiter plates (Bio-Rad, Hercules, CA, USA) with PZP antigen (prepared in-house, $1\ \mu\text{g}/\text{mL}$) and incubated them overnight at 4°C . They then washed the plates with a Tris wash buffer (Tris-buffered saline/Tween-20) and blocked them with 3% gelatin (Bio-Rad) for 30 min at 37°C . Following another wash, they added a standard horse serum sample and unknown horse sera samples to the wells and incubated the plates at 4°C overnight. They diluted unknown serum samples and a positive reference serum sample to 1/25,000 and added each sample to the plates in triplicate. They then washed the plates, added a secondary antibody, Protein G (Calbiochem) conjugated to alkaline phosphatase enzyme, to each well at a dilution of 1/1,000, and incubated the plates for 1 hr at 37°C . Following another wash, they incubated the plates for 1 hr at 37°C with a substrate solution containing 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) at a concentration of 1 mg/mL. They then used a microtiter plate reader (ASYS Hitech GmbH, Eugendorf, Austria) to measure the optical density or absorbance of each well at a wavelength of 405 nanometers. They calculated titers of the unknown serum samples as the average absorbance of the triplicate unknown samples expressed as a percentage of the average absorbance of the triplicate positive reference sample. The positive reference sample was from a female previously vaccinated (in a separate study) with SpayVac and determined to have a strong, PZP-specific antibody response. The positive reference sample was considered to have a titer of 100%.

Male Horses

We randomly selected 12 adult males, 6–9 years of age at the start of the study, from a group gathered at the Adobe Town/Salt Wells Creek herd management complex in Wyoming, USA, in October 2010. We gelded and dropped 1 of these males from the study because of his aggression. We allowed 2 months following inoculation for anti-PZP antibody titers to build and introduced 3 randomly selected males into each pasture with the females on 7 June 2011. We anticipated that the pastures would be large enough for each male to acquire a stable harem from the 30 females present and coexist with minimal aggression, but this turned out not to be the case. Fighting was not uncommon, and in 2 of the pastures breeding activity was dominated by a single male. On 6 July 2011, we randomly assigned a single male to each pasture and developed a random rotation such that pastures received a new male approximately every 2 weeks, and all males had a rest period of ≥ 2 weeks following each session in a pasture. Following the first rotation, we eliminated another male because he was never observed breeding a female. In 2012

and 2013, we used a new randomly assigned order each year and each pasture session lasted approximately 3 weeks. Two males died of causes unrelated to the study, one due to impaction and rupture of the large colon and the other due to a spinal injury, during the winter of 2012–2013, leaving only 8 in the rotation in 2013.

Pasture Observations

A technician who was blind to the treatment status of individual females observed each pasture ≥ 2 hr/day and ≥ 3 days/week to record information on injection-related (2011 only) or other health issues, estrous and breeding behavior, and foaling. We tried several vantage points or modes of transportation (e.g., from the fence surrounding the pasture, from a truck in the pasture, mounted on a domestic horse) for making these observations, ultimately settling on a golf car as the best platform; using the car, we were able to move within a few meters of the females while evoking little reaction other than curiosity. We recorded the nature and severity of reactions to the injections using the system described above. We characterized estrous and breeding behavior on a scale from 0 to 5 as follows: 0 = no evidence of estrous or breeding behavior observed; 1 = warm = squatting, winking, or urination observed, but female is not completely receptive to the male; 2 = hot = squatting, winking, or urination observed and female is receptive to the male; 3 = mounting observed; 4 = penetration observed; and 5 = ejaculation (flagging of the male's tail) observed. The main purpose of these observations was simply to ensure that females were being bred. We associated foals with individual dams based on nursing by the foal that was readily accepted by the female and protective behavior by the female, especially during the first few days and weeks of the foal's life. Female–foal pairings were observed ≥ 3 times/week from birth through the end of the breeding season.

Uterine Edema

On 9–10 January 2013 and 13–14 November 2013, we examined each female in the study per rectum using a Universal Medical Systems 900 (Bedford Hills, NY, USA) portable ultrasound and a 5-MHz linear transducer. Uterine edema was characterized as none, slight, moderate, or extensive. During these examinations, we also recorded information on follicular development, stage of estrous cycle, and pregnancy. All examinations were performed while females were restrained in a squeeze chute; none required sedation.

Cyclicity

On 3 June, 10 June, 17 June, 23 June, and 1 July 2014, we drew 10-mL samples of blood in a plain red-top vacutainer from each female treated with the aqueous formulation of SpayVac. We extracted serum as previously described and shipped it to the Endocrinology Lab, Animal Health Diagnostic Center, Cornell University for progesterone analyses. Females with < 0.5 ng/mL progesterone in all 5 samples were considered to be not cycling.

Analyses

We had the following information available for assessing drug efficacy: foal observations (2012 and 2013), palpation

and ultrasound (2013 and 2014), and hormonal pregnancy tests (all 3 yr). In the hormonal pregnancy tests, there were 20 cases where the results were inconclusive; in 18 of those 20 cases, no foal was observed. It thus seemed important to include those cases in our analyses, by using the other 2 criteria, to obtain the best estimate of drug efficacy. We therefore performed all of our analyses on a composite outcome measure in which a female was judged to be fertile if any of the 3 criteria (ultrasound, hormone test, foal observation) was positive. Use of this composite measure allowed us to include females whose hormone assays were inconclusive, but likely had only a small effect on our overall results, because results from the various measures of fertility were in complete agreement in 220 (92.1%) of 239 cases. We also performed duplicate analyses (data not shown) using only the results of hormonal pregnancy testing (because data were available for all 3 yr); in no case did the results differ substantively from those presented here for the composite measure of fertility.

We compared the binomial composite measure of fertility across the 2 treatment groups (aqueous and nonaqueous) with logistic regression when we were interested in estimating treatment group differences after adjusting for effects of years since treatment, body condition, and pasture assignment. We did not include female age in our models, but rather controlled for the effects of age through randomization and restricting enrollment to females 4–7 years of age. We used Akaike's Information Criterion (AIC; Burnham and Anderson 2002) to select those models better supported by the data. Because control females were fertile in 89 of 90 possible instances, the logistic model estimates were limited to comparisons of aqueous and nonaqueous groups. We also compared fertility in the control group with each of the treated groups without any additional covariates using Fisher's exact tests for the homogeneity of proportions fertile in each year. Comparisons of 2 treatment groups with Fisher's exact test provided a corresponding 95% confidence interval on the odds ratio. In addition, we compared injection-site reactions across the control and treatment groups, and by formulation (aqueous vs. nonaqueous, treated and control females combined) and ingredient (liposomes plus adjuvant with PZP or without PZP, treated and control females combined) with logistic regression and Fisher's exact test for homogeneity of proportions.

RESULTS

There was the potential for females in the control group to be fertile in 90 cases (30 F \times 3 yr) during the course of our study. Using the composite measure, fertility occurred in 89 cases, indicating that males were able to cover females adequately with the rotation schedules that we used. Thus, differences in pregnancy rate or composite fertility rate between control and treatment groups can likely be attributed to the treatment, rather than to a failure of the males to breed.

The aqueous formulation of SpayVac was effective in 2012 as evidenced by a fertility rate of only 0.133 foals/female as compared with 1.00 foals/female in the control group (odds ratio 95% CI = [29.8, ∞], $P < 0.001$; Table 1). Fertility in

Table 1. Fertility rates in treated and control adult female feral horses enrolled in a captive breeding trial of the immunocontraceptive SpayVac at Pauls Valley, Oklahoma, USA, 2012–2014.

Year	Aqueous ^a			Nonaqueous ^a			Control	
	Fertility rate ^b	<i>n</i>	<i>P</i> ^c	Fertility rate ^b	<i>n</i>	<i>P</i> ^c	Fertility rate ^b	<i>n</i>
2012	0.133	30	<0.001	0.167	30	<0.001	1.00	30
2013	0.467	30	<0.001	0.759	29	0.026	0.967	30
2014	0.433	30	<0.001	Withdrawn			1.00	30

^a Aqueous and nonaqueous refer to 2 formulations of SpayVac.

^b Fertility rate is expressed as proportion of females. Fertility was determined through hormone assay (pregnant mare serum gonadotropin and estrone sulfate), rectal palpation and ultrasound, and direct observation of foals. A female was considered fertile if any of the 3 measures was positive.

^c *P*-value is the probability that the fertility rate is the same in the treatment and control groups as determined by Fisher's exact test.

the aqueous group increased in 2013, but remained different from rates in the control group (odds ratio 95% CI = [4.1, 1,424.5], *P* < 0.001), which also occurred in 2014 (odds ratio 95% CI = [7.7, ∞], *P* < 0.001). Fertility rate in the nonaqueous group differed statistically from that of the control group in 2012 (odds ratio 95% CI = [24.5, ∞], *P* < 0.001) and 2013 (odds ratio 95% CI = [1.0, 428.9], *P* = 0.03). In 2013, however, the difference was quite small biologically (nonaqueous group = 0.76 foals/F, control = 0.97 foals/F), and we judged that the BLM would not find the small reduction in foaling due to contraception to be of interest. Having no reason to suspect that drug performance would improve in subsequent years and not wanting to contribute foals unnecessarily to the off-range population of BLM horses, we removed the nonaqueous group from the experiment at the end of the second foaling season. The fact that few treated animals were fertile in the first year indicates that the time we allowed between vaccination and introduction of the males (~2 months) was sufficient for contraceptive efficacy to develop.

We used logistic regression to model probability of foaling in the aqueous and nonaqueous treatment groups in 2012 and 2013. Our initial model contained the potential explanatory variables treatment, year, and a treatment by year interaction. Of these, only year was significant (*z* = 2.675, *P* = 0.007), consistent with the fact that the fertility rate increased in both groups in 2013. Addition of body condition at the time of treatment did not improve the model (Δ AIC = 0.96).

Mean recorded PZP antibody titers reached a maximum more quickly for the nonaqueous formulation, but also declined more quickly and were consistently lower than means for the aqueous formulation after 6 July 2011 (Fig. 1). A similar pattern was apparent in the number of females that exhibited their maximum recorded titer on each sampling date (Table 2). Greater than half of the 30 females treated with the nonaqueous formulation exhibited their maximum recorded titer on 3 May 2011, and 29 of the 30 did so by 6 July 2011. In contrast, individual females treated with the aqueous formulation exhibited their maximum titers over a longer period of time. Across 11 sampling dates postvaccination, mean PZP titers were 33.7–91.9% greater in nonpregnant females than in pregnant females in the aqueous treatment group (Fig. 2A) and 7.8–82.8% greater in the nonaqueous group (Fig. 2B). The relationship

between antibody titer and fertility in individual females was less clear (Table 3). Female number 609, for example, had a maximum titer of 60.11%, was not one of the females that ceased cycling postvaccination, and did not become pregnant in any of the 3 years of our study, whereas several females with greater maximum titers did become pregnant.

None of the 6 females that received saline control inoculations exhibited any reaction at the injection site (Table 4). Of the 84 animals that received either SpayVac or the liposome+adjuvant control doses, 25 (29.8%) exhibited a reaction of some kind (swelling, lump, or abscess). Abscesses were present in 5 (6.0%) of the 84 during the year in which we monitored the injection sites; all 5 occurred in animals that received either the nonaqueous vaccine or the nonaqueous control injection. Abscesses appeared as early as 2 months postinoculation and as late as 5 months; some healed only to reappear at a later date. On female 3425, for example, an abscess was first recorded on 7 September 2011, was recorded again on 5 October, had disappeared by 14 December, and reappeared by 7 March 2012. In no case,

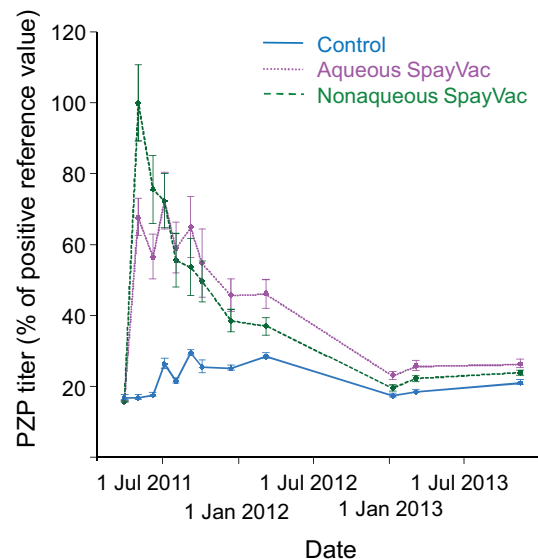


Figure 1. Mean porcine zona pellucida antibody titers, expressed as % of a positive reference, in adult female feral horses enrolled in a captive breeding trial of the immunocontraceptive SpayVac at Pauls Valley, Oklahoma, USA, 2012–2014. Aqueous and nonaqueous refer to 2 different formulations of SpayVac. Vertical bars represent ± 1 standard error.

Table 2. Number of adult female feral horses for which maximum antibody titers were recorded on each sampling date in a captive breeding trial of the immunocontraceptive SpayVac at Pauls Valley, Oklahoma, USA, 2012–2014. Females were treated with either aqueous ($n=30$) or nonaqueous ($n=30$) SpayVac on 30 March 2011.

Sampling date	Aqueous SpayVac	Nonaqueous SpayVac
3 May 2011	8	17
7 Jun 2011	6	4
6 Jul 2011	7	8
2 Aug 2011	2	0
7 Sep 2011	5	0
5 Oct 2011	1	1
14 Dec 2011	1	0

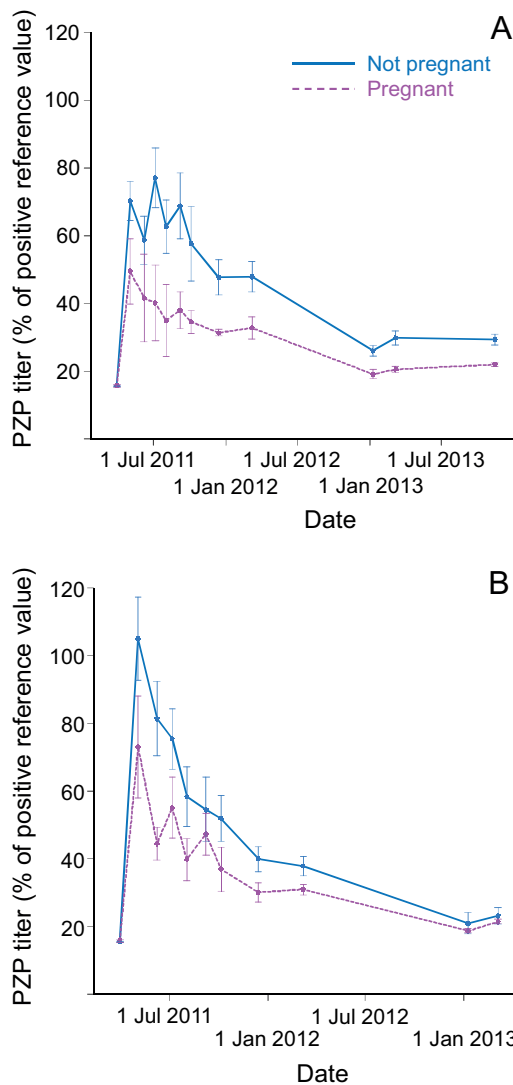


Figure 2. Mean porcine zona pellucida antibody titers, expressed as % of a positive reference, in pregnant and nonpregnant adult female feral horses enrolled in a captive breeding trial of the immunocontraceptive SpayVac at Pauls Valley, Oklahoma, USA, 2012–2014. Aqueous (A) and nonaqueous (B) refer to 2 different formulations of SpayVac. Vertical bars represent ± 1 standard error.

however, did we record a severity score >1 (reaction present, but no pain or lameness noticeable).

Neither Fisher's exact test ($P=0.28$) nor logistic regression analysis (deviance = 2.648, $df=2$, $P=0.27$) revealed an effect of treatment (control, aqueous vaccine, or nonaqueous vaccine) on the probability of a female exhibiting a reaction at the injection site. However, combining control and treated animals by formulation (aqueous or nonaqueous) in a logistic regression model revealed a greater ($z=2.183$, $P=0.03$) incidence of injection-site reactions in animals receiving the nonaqueous formulation (41.5%) than in those receiving the aqueous formulation (19.0%). Interestingly, adding a variable for presence or absence of PZP did not improve the model ($\Delta AIC = -1.52$); that is, reactions were as likely to occur in animals receiving liposomes+adjuvant as in animals that received the vaccine with antigen. Addition of body condition also failed to improve the model ($\Delta AIC = -1.91$).

On 9 and 10 January 2013, we rectally palpated 59 treated females (1 F in the nonaqueous treatment group died of torsion of the small intestine unrelated to the study in Feb 2012). Of those, 25 were open and only 1, treated with the aqueous vaccine, exhibited slight uterine edema. On 13 and 14 November 2013, we rectally palpated 30 animals treated with the aqueous vaccine. Of the 17 females that were open, 2, 2, and 1 showed slight, moderate, and extensive uterine edema, respectively.

Of the 30 females treated with aqueous SpayVac, 5 (numbers 461, 3763, 3904, 4305, and 4330) had progesterone levels consistently <0.5 ng/mL and were thus judged to be not cycling. None of the 5 was fertile in any of the first 3 years of the study, but they were not the females with the highest maximum antibody titers (Table 3).

DISCUSSION

A single-injection contraceptive having multiyear efficacy in horses would be of great utility to the BLM and other agencies that manage feral horses. In the only other trial of SpayVac in horses where pregnancy or foaling was an endpoint, Killian et al. (2008) reported pregnancy rates in 12 treated adult females of 0% in the first year after vaccination and 17% in the next 3 years, as compared with pregnancy rates of 75%, 75%, 88%, and 100% in 8 control females. In addition, although breeding was not a part of their study, Bechert et al. (2013) found that 93% of 14 adult females treated with SpayVac ceased estrous cycling within 3–4 months of vaccination. Thus, both of these earlier studies offered hope that SpayVac might provide the single-injection, multiyear efficacy desired by the BLM for management of wild horses. Although we received all vaccines frozen, the nonaqueous vaccine was of particular interest because the lyophilized material could be stored unfrozen and easily reconstituted with adjuvant in the field, rather than having to create an emulsion as with the aqueous version.

We were unable to attain contraceptive efficacy as great as that reported by Killian et al. (2008). Individual variation in response to the vaccine may account for some of the difference between our results and those of Killian et al.

Table 3. Number of pregnancies and maximum recorded porcine zona pellucida (PZP) antibody titer, expressed as percent of a positive reference, in adult female feral horses treated with the aqueous formulation of the immunocontraceptive SpayVac in a captive breeding trial at Pauls Valley, Oklahoma, USA, 2012–2014. Females are listed in order of increasing maximum PZP antibody titer.

Female ID number	Max. PZP titer ^a	No. of pregnancies ^b	Female ID number	Max. PZP titer ^a	No. of pregnancies ^b
7802	31.19	3	4305	85.00	0
3908	37.94	2	3904	85.40	0
1688	39.66	3	8816	86.38	2
500	41.90	2	4257	92.69	0
4320	44.31	2	504	96.88	0
9186	46.33	1	3763	99.12	0
9655	51.33	2	3724	107.74	0
6105	56.23	2	4301	111.07	0
609	60.11	0	4330	111.39	0
8789	60.38	2	4253	134.79	1
3901	69.62	3	4133	135.26	0
394	70.60	1	3795	137.72	0
4124	72.98	3	3815	176.06	0
461	73.98	0	1029	196.89	0
8667	78.12	2	4319	297.25	0

^a PZP antibody titer is expressed as a percentage of a positive reference, represented by a female vaccinated in a previous study and judged to have a strong, PZP-specific antibody response. The positive reference sample was considered to have a titer of 100%.

^b Pregnancy was determined through hormone assay (pregnant mare serum gonadotropin and estrone sulfate), rectal palpation, and direct observation of foals. A female was considered pregnant if any of the 3 measures was positive.

(2008); other factors that may be important include dose, adjuvant, and injection site. Henderson et al. (1988) showed that dose (amount of PZP administered) can affect both estrous cyclicity and duration of infertility. In the study by Killian et al. (2008), each dose contained 400 µg of PZP, whereas our vaccines and those used by Bechert et al. (2013) contained only 200 µg of PZP/dose. This change was first instituted by the manufacturer in the vaccine provided to Bechert et al. (2013) based on the fact that a primer dose of 65 µg of PZP emulsified with Freund's Complete Adjuvant (FCA) followed by a booster dose of 65 µg of PZP emulsified with Freund's Incomplete Adjuvant was sufficient to contracept horses on Assateague Island, Virginia, USA (Kirkpatrick et al. 1990, 1995). The manufacturer thus expected that 200 µg of PZP in the liposome formulation used in SpayVac would be more than sufficient for contraception. The fact that 93% of females treated by Bechert et al. (2013) stopped cycling seemed to support that expectation.

Our choice of adjuvant was influenced by several factors. First, we were uncertain that AdjuVacTM, which was used by Killian et al. (2008), would be readily available in the future. The various versions of Freund's adjuvants, on the other

hand, are available from several suppliers. Second, MFA is thought to be less likely to cause injection-site reactions than FCA. Lyda et al. (2005) showed that antibody titers in adult females treated with MFA+PZP were greater than those in females treated with FCA+PZP, although this finding was not statistically significant. Finally, Bechert et al. (2013) achieved what appeared to be positive results with SpayVac formulated with MFA. All of these factors influenced our choice of MFA as the adjuvant.

In both previous studies of SpayVac in horses, injections were given in the neck (Killian et al. 2008, Bechert et al. 2013). Although no data were presented, several authors suggested that reactions to injections given in the rump were less common and perhaps less severe than to injections given in the neck, which influenced our decision on injection site (Lyda et al. 2005, Kirkpatrick et al. 2011, Bechert et al. 2013). Additional work to clarify the relationship between injection location and reactions may be warranted.

Antibody titers in response to vaccination with PZP have been shown to vary considerably within a species even when dose and adjuvant are the same (Kirkpatrick et al. 2011). Differences in response may to some extent depend on an animal's physiological state, but we saw no effect of body condition on probability of foaling. This is perhaps not surprising in that body condition ranged only from 4.0 to 6.0 (\bar{x} = 4.9 for all 90 females) when vaccinations were given in March of 2011, and improved substantially by March of 2012 (\bar{x} = 6.6, range = 5.0–8.0).

Comparisons between antibody titers measured in our study and those reported by Killian et al. (2008) and Bechert et al. (2013) are difficult because of differences in measurement scale and units in which titers were quantified. In general, however, it appears that, for unknown reasons, high titers were sustained longer in those 2 previous studies than in ours. Other studies have shown a correlation between antibody titers and contraception in females, with antibody

Table 4. Percent of treated and control adult female feral horses exhibiting an injection-site reaction in a captive breeding trial of the immunocontraceptive SpayVac at Pauls Valley, Oklahoma, USA, 2012–2014.

Treatment	n	Females exhibiting a reaction	
		No.	%
Saline control	6	0	0
Aqueous control	12	1	8.3
Nonaqueous control	12	5	41.7
Aqueous SpayVac	30	7	23.3
Nonaqueous SpayVac	30	12	40.0

titers 50–60% of a positive reference serum being sufficient to render 90–95% of adult females infertile (Liu et al. 1989, 2005; Turner et al. 1997). However, none of these studies were >1 year in duration. Our data suggest that a similar threshold may exist for multiple-year efficacy in horses treated with SpayVac. Only a single foal was produced (female 4253) in 3 years following inoculation in females having a maximum titer greater than approximately 90% of the positive reference; we believe that datum may be an error in foal assignment to a dam, because the female tested inconclusive for pregnancy by hormone analysis and negative for pregnancy by palpation in that year. Despite our careful efforts to associate foals with dams, an occasional error is possible because many of our females, particularly those with bay coloration, were very similar in appearance. Titers reported as a percentage of a positive reference compare serum taken from a successfully contracepted female (the positive reference) with sera from other treated and control females. The lower values reported here, for example in the control females, were probably below the limit of detection for the assay and likely reflect background issues, which are not uncommon when testing for polyclonal antibodies. As such, most values below approximately 30% of the reference serum likely do not represent true immune responses, which may explain the fluctuations in control values through time.

Our study and that of Bechert et al. (2013) clearly showed that reactions at the injection site were fairly common even with MFA as adjuvant and, contrary to a previous report (Roelle and Ransom 2009), even when injections are delivered by hand rather than by dart. Both studies reported that reactions can resolve only to reappear at a later time, and both studies provided some evidence that reactions at the injection site are attributable to the adjuvant rather than the immunogen. Increased rates of injection-site reactions in these 2 studies are most likely the result of more frequent and closer examination, both visually and by palpation. Interestingly, however, even such close scrutiny does not detect all reactions. In our study, necropsy of female 740, which died of torsion of the small intestine, revealed a sterile abscess that had gone undetected in 7 examinations (visual and by palpation) over a period of 7 months. It thus seems highly likely that studies based on field observation alone under report injection-site reactions. Bechert et al. (2013) found injection-site reactions to be more frequent for the aqueous formulation of SpayVac (5/7 = 71.4%) than for the nonaqueous formulation (3/7 = 42.9%), whereas the reverse was true in our study. Reasons for this difference are unclear, but the small sample sizes ($n = 7$ F/group) in the study by Bechert et al. (2013) may be a contributing factor. Despite the fact that injection-site reactions were fairly common in our study, perhaps the most striking and important observation was that none of the reactions appeared to be painful or caused any noticeable change in mobility or gait.

Unlike Killian et al. (2008), we found uterine edema in only a small proportion of open females in January of 2013, likely because most of the females were in anestrus. In November of 2013, however, we found uterine edema in a proportion of females similar to the expected normal frequency (~25–30%)

suggested by Killian et al. (2008) assuming a 21-day estrous cycle and 5–7 days of the cycle actually in estrus. Our November examinations should be roughly comparable to those done by Killian et al. (2008) in mid to late October, so the reasons that they found much higher rates of uterine edema remain unclear. We reiterate, however, that we view the frequencies of uterine edema observed here as normal and not a contraindication to the use of SpayVac.

Bechert et al. (2013) found that 93% of females treated with SpayVac ceased cycling 3–4 months after vaccination. Unfortunately, they were unable to follow the females to see whether normal ovarian function was restored over time. We found that 5 of 30 females treated with the aqueous formulation of SpayVac were not cycling >3 years after vaccination, which may suggest that loss of ovarian function is permanent when it occurs. As would be expected, none of these 5 females was fertile in the first 3 years of our study. However, an additional 10 females that appeared to be cycling were also infertile in the first 3 years of our study, which suggests that some mechanism other than disruption of ovarian function is responsible for infertility. One possibility is that antibody titers, even though they declined significantly following vaccination, were still high enough to prevent sperm binding to ova in these 10 females.

MANAGEMENT IMPLICATIONS

We did not find SpayVac to be as efficacious as other investigators have. However, both modeling (Ballou et al. 2008) and field studies (Kirkpatrick and Turner 2008) have shown that significant reductions in fertility can be achieved with <100% contraceptive efficacy. If even the modest results that we observed could be duplicated on a large scale, which would require registration of SpayVac by the U.S. Environmental Protection Agency, the BLM and other agencies charged with managing feral horses would have a more effective fertility control tool than has heretofore been available, especially given the fact that no booster inoculation is required with SpayVac. Concerns about reactions at the injection site and uterine edema appear to be minimal and should not deter BLM or others from using SpayVac.

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