

WH&B ANNUAL PROGRESS REPORT
BLM Wild Horse and Burro Program

Study I.D. Number: Second SpayVac® Pasture Trial, a study conducted under BLM Reimbursable Agreement 4500065781

Institution: USGS Fort Collins Science Center, Fort Collins, CO

Date of report: 2 November 2015, covering FY 14 and 15 (1 January 2014 – 30 September 2015)

Title of project: Second captive breeding trial to evaluate the efficacy of SpayVac® as a wild horse contraceptive

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Start date: 1 March 2014

End date: 30 September 2019

Executive Summary: We conducted a pasture breeding trial to assess the efficacy of two new combinations of SpayVac (PZP in multilamellar liposomes) and adjuvant in reducing fertility in wild horse mares. On 1 April 2014, mares received one of three treatments: 400µg PZP in liposomes + Freund's Complete Adjuvant, 400 µg PZP in liposomes + Freund's Modified Adjuvant, or a sham injection ($N = 30$ for each treatment). Mares were assigned to one of three pastures on a stratified random basis such that each treatment was equally represented in each pasture. On 3 June 2014, a single stallion randomly selected from eight available was introduced into each pasture. Stallions were rotated in a randomly selected order every 3 weeks.

Summary of progress to date as related to each of the original objectives

The study described herein was designed to assess the effectiveness of a higher dose of SpayVac (400 µg PZP as opposed to 200 µg in our first study) and, for one treatment group, a more powerful adjuvant (Freund's Complete Adjuvant). We initiated the study on 1 April 2014 by treating three groups of mares as described in Table 1.

Group	Treatment	Rationale
1	Control group treated with adjuvant + liposomes or saline (12 mares with Freund's Complete Adjuvant, 12 with Freund's Modified Adjuvant, 6 with saline only)	Provide the basis for statistical comparison with treated animals. By randomly selecting the control group and treatment groups from the same set of animals, we will ensure a similar distribution of age and body condition in each group.
2	400 µg PZP in liposomes, aqueous emulsion, Freund's	400-µg dose appeared to cause long-term infertility in the study by Killian et al. (2008). Aqueous emulsion

	Complete Adjuvant	formulation worked best in our previous trial, and Freund's Complete is widely considered to be the "gold standard" of adjuvants.
3	400 µg PZP in liposomes, aqueous emulsion, Freund's Modified Adjuvant	Freund's Modified is preferable if it produces infertility similar to that produced by Freund's Complete, as the modified adjuvant is thought to result in fewer and less severe reactions at the injection site.

Table 1. Treatment groups in the second SpayVac study and the rationale for choosing them. Thirty mares were enrolled in each group.

Mares were randomly assigned to one of three pastures in a stratified random fashion such that each treatment was equally represented in each pasture ($N = 30$ mares per pasture). On 3 June 2014 a single stallion, randomly selected from a group of eight available, was introduced into each pasture. Stallions were changed every 3-weeks in a randomly selected rotation, with each stallion having a least a 3-week rest period between pasture assignments. Stallions were removed from all of the pastures on 30 September 2014.

Specific objectives of our study were to:

- 1) Quantify the effectiveness of three new combinations of SpayVac + adjuvant (Table 1) in reducing mare fertility over 5 breeding seasons post-injection when mares are exposed to stallions in a pasture setting throughout the breeding season.
- 2) Determine the relationship between PZP antibody titers and pregnancy fertility.
- 3) Estimate the incidence of injection related and generalized side effects, if any, of SpayVac + adjuvant or sham injections (controls) following intramuscular (i.m.) injection by hand.
- 4) Determine whether mares treated with SpayVac continue to exhibit estrous cycles.

Progress toward each of these objectives was as follows.

Objective 1. Efficacy of SpayVac

On 3 February 2015 we drew blood from each mare, extracted serum, and shipped the serum frozen to the Endocrinology Laboratory, Animal Health Diagnostic Center, Cornell University, for evaluation of pregnancy status by estrone sulfate (E1SO₄) assay. Four samples were apparently mislabeled during processing, so 86 mares were identified as pregnant, open, or inconclusive. On 9–10 April 2015, in part because the results of the E1SO₄ assays showed higher than expected pregnancy rates, we also engaged Dr. Irwin Liu to evaluate pregnancy status in 89 mares by palpation and ultrasound; one mare was not evaluated because of behavioral issues. In addition, a technician who was blind to the treatment status of individual mares made direct observations of foals, spending approximately 1 hour per day, 5 days per week, in each of the three pastures from 1 April 2015 to 30 September 2015. For our analyses, a

mare was considered fertile if any of our three measures (hormone assay, palpation, foal observation) was positive. Results of these analyses are shown in Table 2.

Treatment	Fertility Rate (<i>N</i>)
SpayVac + Freund's Complete Adjuvant	0.53 (30)
SpayVac + Freund's Modified Adjuvant	0.70 (30)
Control	0.83 (30)

Table 2. Fertility rate for three groups of mares exposed to stallions in a pasture setting at Pauls Valley, Oklahoma. A mare was considered fertile if any of three measures of pregnancy or foaling (hormone assay, palpation, foal observation) was positive.

The EISO4 assay was inconclusive for five mares, all of which were open on palpation. For the remaining 81 mares for which data are available from both the EISO4 assay and palpation, there was perfect correspondence between the two measures. Direct observation added only a single mare to the composite fertility measure, a mare that was classified as open by both palpation and hormone assay. This may be a case of assigning the foal to the wrong mare.

Objective 2. Antibody Titers and Fertility

We drew blood for antibody titer analyses on 1 April, 6 May, 3 June, 8 July, 5 August, 3 September, and 30 September 2014, and on 3 February 2015. We extracted serum from each sample and shipped the serum frozen to ImmunoVaccine Technologies Inc. (IVT; Halifax, Nova Scotia, Canada), the manufacturer of SpayVac, for analysis by enzyme-linked immunosorbent assay (ELISA).

Mean titers for each of the treatment groups are shown in Figure 1. One mare (#0766) in the group that receive Freund's Modified Adjuvant was eliminated from the analysis because on 3 September 2014 her titer increased to an order of magnitude greater than the group mean; inclusion of her titers obscured an otherwise clear pattern. Antibody "titers" in the control group are a result of background optical density of the samples. While titers in the two groups that received SpayVac were higher than those in the control group, the distinct peak at 2–3 months that we saw in our first experiment was not apparent. Also of note is the fact that Freund's Complete Adjuvant did not produce higher titers than did Freund's Modified Adjuvant. Finally, we have no explanation for the apparent increase in titers between 30 September 2014 and 3 February 2015. (The same sort of increase was also observed in mares remaining in our first experiment.) Personnel at IVT also could not explain this increase, and they are planning to

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reanalyze the samples to see if results are similar.

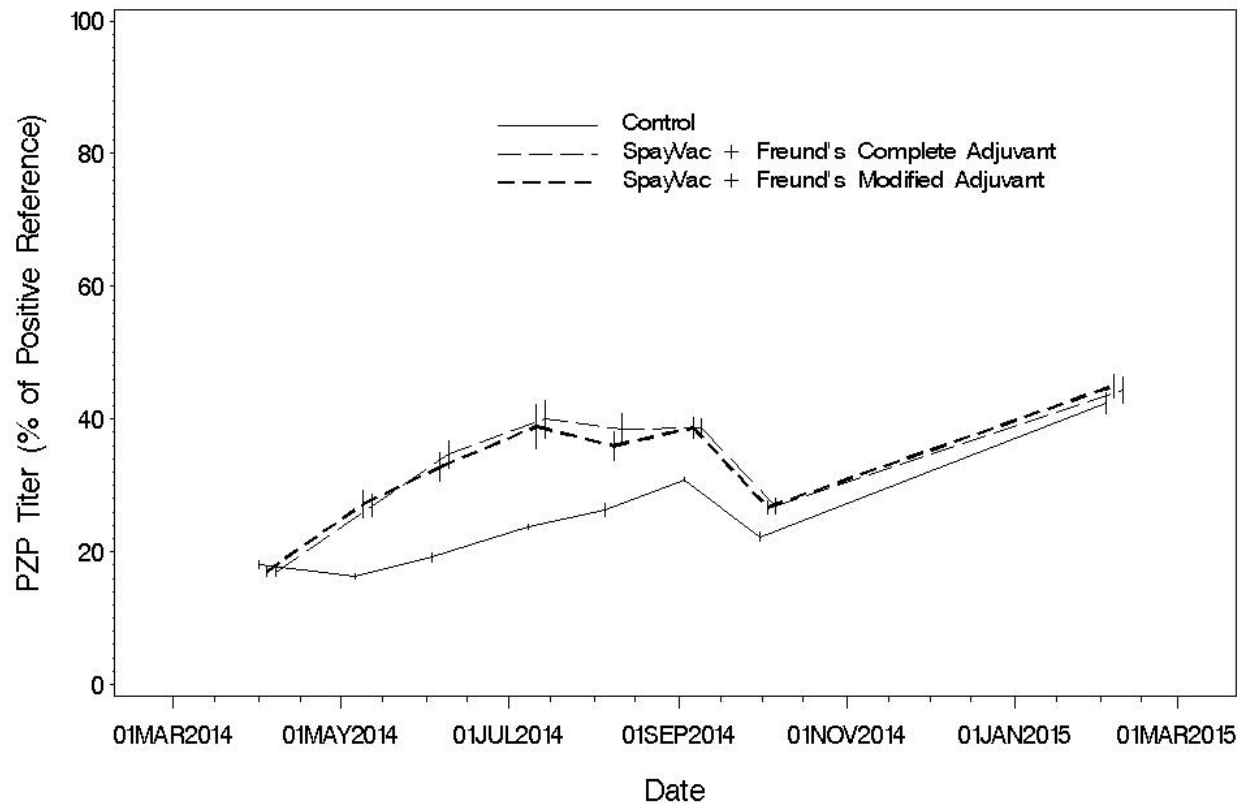


Figure 1. Mean PZP antibody titers, expressed as percent of a positive reference standard, in mares treated with SpayVac and exposed to stallions in a pasture breeding trial at Pauls Valley, Oklahoma. Vertical bars represent one standard error.

Mean PZP antibody titers were higher in open mares than in pregnant mares for mares treated with (A) SpayVac + Freund's complete Adjuvant and (B) SpayVac + Freund's Modified Adjuvant (Figure 2). However, in neither treatment group was there a clear cut-off value above which a mare would certainly be infertile (Table 3). We chose 8 July 2014 as the date for this comparison because mean titers were at a maximum at that time. A similar pattern would be apparent on any other date.

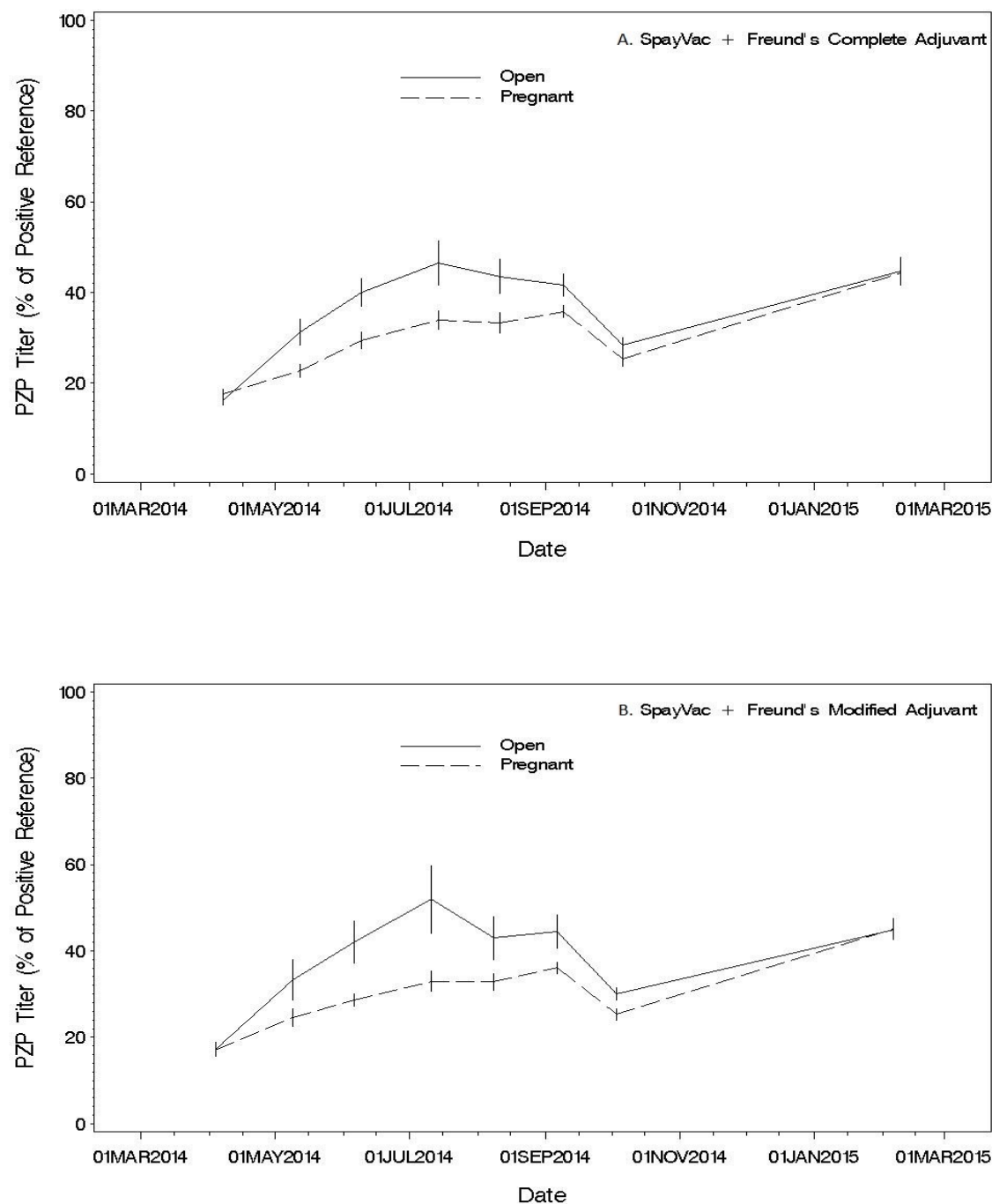


Figure 2. Mean PZP antibody titers, expressed as percent of a positive reference standard, in pregnant and open mares treated with SpayVac and exposed to stallions in a pasture breeding trial at Pauls Valley, Oklahoma. Vertical bars represent one standard error.

Freund's Complete Adjuvant			Freund's Modified Adjuvant	
Titer on 8-Jul	Status		Titer on 8-Jul	Status
23.63	O		20.13	P
24.37	O		21.99	P
24.52	P		24.67	P
24.61	P		26.67	P
25.48	O		27.56	P
25.71	P		27.56	O
25.85	P		28.60	P
28.83	P		28.68	P
30.16	P		29.00	O
31.35	P		29.00	P
31.74	P		29.74	P
35.25	P		30.33	P
36.48	P		30.68	P
36.66	O		30.85	P
38.22	P		33.28	P
38.41	O		34.38	P
38.61	O		36.83	P
38.94	P		37.53	P
39.85	O		37.68	P
41.10	P		38.85	P
42.20	O		41.01	P
44.69	P		41.43	O
47.39	O		43.74	O
51.62	O		44.73	P
51.73	P		45.86	O
65.11	O		51.17	O
65.25	O		58.32	O
66.37	O		64.95	O
86.06	O		70.54	P
.	O		105.49	O

Table 3. Pregnancy status (O = open, P = pregnant, dot = missing data) of individual mares treated with SpayVac compared to their PZP antibody titer on 8 July 2014. For each treatment group, mares are arrayed in order of increasing titer.

Objective 3. Injection-site Reactions

Beginning on 6 May 2014, on each occasion when we drew blood for antibody titer assays and pregnancy tests, a veterinarian evaluated each mare for pain and lameness and palpated the area surrounding each injection site while the mare was restrained in a hydraulic squeeze chute. Injection-site reactions and pain/lameness were scored as indicated in Table 4.

Reaction Type	Pain or Lameness
0 = None	0 = no pain detected on palpation; no stiffness or lameness observed
1 = Swelling – a diffuse raised area, often irregular in shape; may be firm, but does not appear or feel hard	1 = no pain detected on palpation, but stiffness observed
2 = Lump – a distinct, roughly circular nodule; looks or feels hard	2 = mild to moderate reaction to palpation or mild to moderate lameness observed
3 = Abscess – a sore or pocket with palpable or draining fluid or pus; drainage may be either natural or induced by veterinary treatment	3 = strong reaction to palpation or severe lameness observed

Table 4. Scoring system for injection-site reactions and resulting pain or lameness.

Two of the six mares injected with saline developed minor swelling at the injection site (Table 5), but in both cases the reaction was present for less than 1 month. Of the 42 mares that received Freund's Complete Adjuvant, 39 (92.9%) exhibited a reaction of some type, and 8 (19.0%) developed an abscess. In contrast, 30 (71.4%) of the 42 mares that received Freund's Modified Adjuvant exhibited a reaction, and only 4 (9.5%) developed an abscess. Although we have not analyzed this information statistically, it appears to support the notion that Freund's Complete Adjuvant causes more frequent and severe reactions at the injection site than does Freund's Modified Adjuvant. In our previous experiment, only 7 (19.0%) of 30 mares treated with 200 µg PZP + Freund's Modified Adjuvant exhibited a reaction, as compared to 23 (76.7%) of 30 mares in the current experiment treated with 400 µg PZP + Freund's Modified Adjuvant. Frequency of injection site reactions may thus be related to amount of PZP delivered as well as to adjuvant. It is also worth noting that no mare was ever scored as a 3 on the pain or lameness scale, and only 10 mares received a score of 2 on any sampling occasion. Additionally, at the time of our last blood draw (3 February 2015), only three mares still exhibited a reaction. All of these were lumps (reaction type = 2), and in none of the three cases was pain or lameness detected.

Treatment	Reaction Type				Total Mares
	None	Swelling	Lump	Abscess	
Liposomes + Freund's Complete Adjuvant	0 (0.0)	4 (33.3)	7 (58.3)	1 (8.3)	12
Liposomes + Freund's Modified Adjuvant	5 (41.7)	3 (25.0)	4 (33.3)	0 (0.0)	12
Saline	4 (66.7)	2 (33.3)	0 (0.0)	0 (0.0)	6
SpayVac + Freund's Complete Adjuvant	3 (10.0)	7 (23.3)	13 (43.3)	7 (23.3)	30
SpayVac + Freund's Modified Adjuvant	7 (23.3)	5 (16.7)	14 (46.7)	4 (13.3)	30
Total Reactions	19	21	38	12	90

Table 5. Number and percentage of mares exhibiting various types of injection-site reactions.

Objective 4. Estrous Cyclicity

In a cycling mare, serum progesterone levels are elevated for about 10 of the 22 days in the cycle. We drew blood at weekly intervals on 5 occasions between 3 June and 1 July 2014. We extracted serum from the samples and sent it to the Endocrinology Laboratory, Animal Health Diagnostic Center, Cornell University, for progesterone analysis. We reasoned that if a mare was cycling, at least one of the five samples should show elevated progesterone (>0.50 ng/mL). By this criterion all but one of the mares were judged to be cycling, and the mare that was not cycling was in the control group. This is in stark contrast to the result reported by Bechert et al., who found that 93% of 14 mares treated with SpayVac ceased cycling within 3 to 4 months of vaccination.

Describe any changes in scientific goals/methodology from that outlined in original proposal. Schedule of unfinished experiments; completion dates.

We initiated this second pasture breeding trial with the expectation that a larger dose of SpayVac and, for half of the treated horses, a more powerful adjuvant, would result in greater efficacy of the vaccine. In fact, efficacy was much poorer for reasons that remain unclear. Lack of a distinct peak in antibody titers also indicates that the vaccine simply did not work very well. Therefore, in consultation with BLM, we decided not to continue the experiment past the first foaling season.

The original study design also called for a group of 30 mares to be treated with a dose of 600 μ g PZP and Freund's Complete Adjuvant. At the outset of the study, insufficient PZP was on hand at IVT to produce both the 600- μ g and 400- μ g doses, so we deferred initiation of the 600- μ g trial, intending to start it at some future time. However, we also dropped that plan when it became apparent that the 400- μ g vaccine did not perform as well as the 200- μ g vaccine used in our first pasture trial.

Literature Cited

- Bechert, U., J. Bartell, M. Kutzler, A. Menino, Jr., R. Bildfell, M. Anderson, and M. Fraker. 2013. Effects of two porcine zona pellucida immunocontraceptive vaccines on ovarian activity in horses. *Journal of Wildlife Management* 77:1386–1400.
- Killian, G., D. Thain, N.K. Diehl, J. Rhyen, and L. Miller. 2008. Four-year contraception rates of mares treated with single-injection porcine zona pellucida and GnRH vaccines and intrauterine devices. *Wildlife Research* 35:531–539.