THESIS

IMMUNOGENICITY AGAINST A VACCINIA VIRUS-VECTORED ORAL PLAGUE VACCINE IN BLACK-FOOTED FERRETS (*MUSTELA NIGRIPES*)

Submitted by

Deborah Grossblat McCuen

Department of Biology

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Master's Committee:

Advisor: Michael F. Antolin
Co-Advisor: Michael W. Miller

Lisa M. Angeloni
Alan R. Schenkel
Tony A. Schountz

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ABSTRACT

IMMUNOGENICITY AGAINST A VACCINIA VIRUS-VECTORED ORAL PLAGUE VACCINE IN BLACK-FOOTED FERRETS (*MUSTELA NIGRIPES*)

Black-footed ferrets (*Mustela nigripes*) are one of the most endangered mammals in North America, in part because they suffer high mortality when infected by plague, caused by the bacterium *Yersinia pestis*. An injectable plague vaccine is effective in black-footed ferrets but protecting wild ferrets is laborious because of the need to capture and handle these animals. In contrast, an orally delivered vaccine could be more easily distributed, as is the case with the Yersinia Pestis Vaccine that effectively protects the ferrets' main prey, prairie dogs. We evaluated in black-footed ferrets the immunogenicity of an oral vaccinia virus vectored plague vaccine, previously shown to be protective in laboratory mice. We compared antibody response of the oral vaccine to the injectable plague vaccine. Although the oral vaccine appears to be safe in ferrets, lateral flow results indicated an absence of measurable antibody response to plague antigens. Furthermore, a plaque reduction neutralization test revealed that black-footed ferrets have some ability to neutralize vaccinia virus, even without delivery of the oral vaccine or any known exposure to orthopoxvirus. We also investigated if maternal antibody to plague antigens could be detected in ferret kits aged between 39 and 50 days, but results were largely negative. Blood samples of sufficient volume were difficult to obtain from kits and may have contributed to negative results. Based on our findings, it is unlikely that orthopoxvirus-

based vaccines will protect captive black-footed ferrets, and other oral plague vaccines should be considered in future research.

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Immunogenicity Against a Vaccinia Virus-Vectored Oral Plague Vaccine in Black-footed Ferrets (*Mustela nigripes*)

Introduction

Protecting black-footed ferrets from plague is the foundation for successfully reintroducing and ultimately delisting this endangered species [1-3]. Supporting self-sustaining, free-ranging ferret populations requires protecting both ferrets and their primary prey, prairie dogs (*Cynomys spp*.), from plague, caused by the bacterium *Yersinia pestis* [1, 4-6]. Vaccination is one strategy for protecting wildlife from infectious diseases like plague [3, 7], but the current injection-based approach for vaccinating ferrets against plague is not practical for field use because of the need to capture these secretive nocturnal animals and then later recapture them for a second vaccination dose. An effective, orally delivered plague vaccine could enhance the success of ferret reintroduction and accelerate wild ferret population recovery.

Plague originated in Asia and is the pathogen responsible for at least three human pandemics [8]. It was introduced to the western U.S. in 1899 by flea-infested rats on ships traveling from Asia and became established in wild ground dwelling rodents throughout western North America by the 1940's [8-9]. Rodents worldwide continue to serve as primary hosts for this pathogen [10-11], with some spillover to humans. Sylvatic plague, as it is called when infecting wildlife, is managed on a landscape scale in part with mass oral vaccination and insecticides applied to burrows to control flea vectors [5, 12]. The sylvatic plague cycle is not completely understood but is characterized by sporadic high-mortality epizootics and intervals when the pathogen becomes nearly undetectable [1, 11]. Sylvatic plague causes reductions in abundance in both black-footed ferret and prairie dog

populations across the Great Plains region, even with management efforts [13]. Consequently, controlling plague is critical for restoring wild black-footed ferret populations [1, 14].

The development of plague vaccines has focused on two antigens F1 (Caf1) and V (LcrV), that together provide the best protection in current plague vaccines because they are highly immunogenic [15]. The F1 protein is part of the *Y. pestis* capsule that helps bacteria resist phagocytosis in mammalian hosts. The virulence antigen V is a cell surface protein involved in the Type III secretion system of *Y. pestis*, which assembles the injectisome and delivers virulence factors to host cells [15]. Both F1 and V activate strong antibody responses by a host immune system after infection or vaccination [16]. Although effective, the one available vaccine for ferrets, an adjuvanted F1-V fusion protein, requires at least two injections to stimulate protective immunity [3]. Injected vaccines are feasible for use in captive settings but are unrealistic for long-term protection of free-ranging populations. Trapping and hand injecting wild ferrets is labor intensive, requires considerable resources, and only benefits the older animals that are more likely to be trapped [17]. Young kits may remain unvaccinated and thus susceptible to plague.

In contrast, an orally delivered plague vaccine for wild ferrets would offer the potential for landscape-level application and a higher magnitude of protection similar to the conditionally licensed oral plague vaccine, Yersinia Pestis Vaccine (Colorado Serum Company, Denver, CO), currently used in prairie dogs [5, 12]. It would have been most convenient for the Yersinia Pestis Vaccine (YPV) to be broadly beneficial in other species but we were discouraged by an unpublished report of the raccoon poxvirus vector used in YPV not being effective in ferrets [18].

We explored an alternative oral plague vaccine vector for use in ferrets. An oral recombinant vaccinia virus vector has been used successfully in orally immunizing mice against intranasal plague challenge with a single vaccine dose [19]. This same vaccinia virus vectors an oral rabies vaccine that has an excellent safety record in multiple wildlife species for the oral rabies bait program in the U.S., Canada, and parts of Europe [20, 21], and has been shown to stimulate humoral or antibody immunity in raccoons [22]. The vaccinia vectored oral plague vaccine (VV-F1-V) uses a recombinant F1-V fusion protein that is antigenically similar to the F1-V fusion protein used in the current injectable plague vaccine for ferrets.

Another key consideration for the development of an effective vaccine-based management plan is timing of inoculation. The current black-footed ferret vaccination schedule is based on a generalized understanding of maternal antibody studies in other species, but these assumptions have not been validated in black-footed ferrets. Understanding whether passive antibody transfer from plague-vaccinated dams to kits occurs, and assessing at what age kits potentially lose passive immunity to plague, could inform when delivery of oral vaccine baits would be most effective.

The objective in this study was to evaluate the safety and immunogenicity of the vaccinia virus oral plague vaccine in black-footed ferrets. We compared the vaccine's antibody response to the current injectable plague vaccine. We also investigated whether kits born to injectable plague-vaccinated dams exhibit measurable maternal antibody levels to *Y. pestis* antigen V.

Methods

Experimental animals and vaccines

This study was reviewed and approved by the USFWS (U.S. Fish and Wildlife Service), NBFFCC (National Black-footed Ferret Conservation Center) Animal Care and Use Committee (File #16-01, June 22, 2016) and all live animal work was conducted at the NBFFCC. Study animals were born in captivity and housed indoors individually except for dams with kits. Captive ferrets were fed a diet derived from wild-caught prairie dogs, lab raised rats, and/or a raw horsemeat-based commercially prepared diet (Toronto Zoo Small Carnivore Mix Diet prepared by Milliken Meat Products), and Syrian hamsters raised in quarantined facilities on site. We use the same studbook (SB) numbers that identify animals in the captive breeding program.

For the oral vaccinia vectored plague vaccine safety trial, we used five ferrets that had been previously excluded from breeding because of age (> four years-old). For the duration of the safety trial, these animals were housed individually in isolation cages in a separate building than the one where ferret breeding occurs at NBFFCC. For the vaccine immunogenicity trial, we used 18 ferret kits, initiating the study at approximately two months of age (range 60-68 days) until approximately five months of age (range 132-161 days). For the duration of the immunogenicity trial, kits remained housed with their respective dams and littermates within the breeding building at NBFFCC. Immunogenicity trial kits were vaccinated only against plague, either with the injectable plague vaccine (F1-V) or the experimental oral vaccinia plague vaccine (VV-F1-V).

Sera acquired from captive adult ferrets and wild-caught ferrets were also examined in our experiment. Captive adult sera used were drawn from ferrets that had never been

vaccinated against plague. These animals had been vaccinated against rabies with a killed rabies virus vaccine (Imrab® 3TF, Merial) and against canine distemper using a recombinant live canarypox vectored vaccine (PureVax® Ferret Distemper, Merial). Free-ranging ferret sera were drawn from wild-born animals at approximately one year of age. These animals were vaccinated against only canine distemper virus using the same canarypox vectored vaccine as captive ferrets.

The injectable F1-V vaccine is a recombinant vaccine containing a fusion protein of the F1 and V antigens combined with an adjuvant, which has been previously described [23] and modified for animal use [24], and is currently used by USFWS to vaccinate ferrets against plague. The vaccine is administered by subcutaneous (SC) injection at approximately 60 days of age, with a second dose given one month later. The alternative oral vaccine, VV-F1-V, was designed to use a recombinant vaccinia virus expressing the F1 and V fusion protein. The virus-vectored vaccine demonstrated a dose-dependent antibody response in mice after oral administration as well as protection against intranasal plague challenge [19].

Vaccine preparation

Injectable F1-V vaccine was prepared by veterinary staff at NBFFCC by combining the F1-V fusion protein, recombinant from *Escherichia coli* (BEI Resources, NR-4524), with DMEM (Dulbecco's modification of minimal essential medium) to achieve a protein concentration of 160 μg/mL. This solution was mixed with equal parts of the adjuvant, 0.2% Alhydrogel solution diluted with DMEM. This solution was rocked gently 12-15 hours at 4 C. Individual doses were prepared to contain 40 μg of F1-V fusion protein in a volume of 0.5 ml for the initial dose and 20 μg in a volume of 0.25 ml for the booster dose.

The oral vaccine VV-F1-V was prepared using a recombinant vaccinia virus (VV) constructed from strain vRB12 with gene insertions for expression of a F1-V fusion protein acquired from the Linden Hu laboratory at the Division of Geographic Medicine and Infectious Diseases at Tufts Medical Center. Crude stock VV-F1-V was produced in the Hu lab using BS-C-1 cells, then maintained in HeLa cells with viral titers determined using 0.1% crystal violet staining at 5 x 10^9 pfu/mL (plaque forming units per milliliter) prior to shipment to Colorado Parks and Wildlife/Wildlife Health Lab (CPW WHL) [19, 25]. We finished preparing the vaccine by trypsinizing crude VV-F1-V stock mixing equal parts VV-F1-V and 0.25 mg/mL trypsin with EDTA (ethylenediamine tetraacetic acid) and vortexed vigorously. The virus/trypsin solution was incubated 30 minutes in a 37° C water bath, vortexing at five to ten minute intervals. Trypsinized virus was mixed with MEM (minimal essential medium) to achieve individual dose volumes of 0.25 mL at a concentration of 1 x 10^8 pfu. The solution was immediately transported on ice to NBFFCC where it was centrifuged at 2000 RCF (relative centrifugal force) to minimize the chance of pipetting cell debris into individual doses, and kept on ice prior to administration.

Vaccine safety trial

A complete blood count and chemistry blood panel was performed for each of the five ferrets 30 days prior to starting the safety trial to confirm all animals were healthy. After allowing one week for ferrets to adapt to new cages, we vaccinated ferrets by spraying the oral vaccinia vectored plague vaccine into the inner cheek from a syringe at a vaccinia virus concentration of 1 x 10^8 pfu in a 0.25 mL volume. This dose was selected based on an effective vaccine dosage given to mice [19]. Booster vaccinations were given in the same manner and dosed 30 days after the initial vaccinations. We monitored animals

daily for any signs of illness or oral disease for six weeks. During this time, buccal swabs were collected from each animal on Day 1, 2, and 7 post initial vaccination, and Day 1, 5, and 8 post booster vaccinations. Two swabs were taken in succession for each animal at each sampling event. Buccal swabs were placed in 2.5% FBS (fetal bovine serum) in MEM and stored individually at -70° C for future analysis.

Presence of vaccinia virus: virus isolation of buccal swabs. Virus isolation was performed on the buccal swabs to determine if study animals were shedding vaccinia virus after vaccination. Twenty-four well plates were seeded with African green monkey kidney epithelial (Vero) cells and DMEM plus 5% FBS and antibiotics (penicillin and streptomycin). Plates were incubated at 37° C for two days for Vero confluency. Buccal samples were thawed, vortexed, and centrifuged. The media was removed from the 24-well plate with confluent Vero cells and 100 µL BA-1 medium (MEM salts, 1% bovine serum albumin, sodium bicarbonate, Tris buffer, pH 7.6) was added to the wells. To minimize bacterial and fungal growth, 50 µL of combined gentamycin, amphotericin B, polymixin B, nystatin, and penicillin streptomycin added to the media in each well. Two hundred µlL from each buccal swab sample was added to respective prepared wells and incubated 30 minutes at 37° C. Negative and positive control wells were prepared with DMEM and antibiotics only and serial dilutions of trypsinized VV-F1-V in 2.5% FBS in DMEM, was added to the positive control wells. After incubation, 500 µL DMEM + 5% FBS and penicillin streptomycin, gentamycin, and amphotericin B were added to wells. Plates were incubated at 37° C for seven days. Supernatant was collected from wells and stored at -70° C and plates were fixed and stained with 70% methanol and crystal violet before reviewing for evidence of plaque formation.

Presence of vaccinia virus: DNA extraction and PCR of buccal swabs. Extraction of DNA was performed from two sources for each animal, with buccal swab media and supernatant collected from virus isolation plates. In both instances, we used a DNA purification kit (Qiagen DNeasy kit) and followed protocol for the spin-column method for cultured cells. Buccal swab samples containing the original swabs were thawed and vortexed. Supernatant was thawed and centrifuged. Either 200 µL of swab media or supernatant was added to 200 µL phosphate-buffered saline (PBS) and 20 µL proteinase K in an Eppendorf tube and procedure was followed as described by the manufacturer. Our positive vaccinia virus control was prepared in a similar fashion using 100 µL VV-F1-V as previously described and added to 2.5% FBS in MEM and combined with 100 µL PBS and 20 µL proteinase K. We eluted the DNA in 100 µL AE buffer.

We derived primers from Vaccinia A5L sequence; (5' to 3') forward CTT CTT TAA CAA GTT CTC ACA G and reverse AGC CTC ATT TTA ATA TCC (Gen Bank accession AY243312.1). The expected target product is 865 base pairs [26]. We combined 2 µL of extracted DNA with 22.5 µL of Invitrogen PCR SuperMix and 0.25 µL each vaccinia primer. PCR amplification was achieved by a hot start step at 94° C for two minutes, 40 cycles of denaturation for 30 seconds at 94° C, annealing at 42° C for 60 seconds, and extension at 72° C for 30 seconds, and a final single extension step at 72° C for ten minutes. The PCR reaction was carried out in an Eppendorf Mastercycler gradient.

To ensure that a fragment of expected size was amplified, the PCR products were separated by electrophoresis on 1% agarose gels (one gram agarose dissolved in 100 mL Tris-Acetate EDTA buffer). Ten µL of one Kb plus DNA molecular weight marker (Invitrogen) elucidated fragment size.

Vaccine immunogenicity trial

The injectable F1-V treatment and the oral VV-F1-V treatment were each tested in nine randomly assigned ferret kits. Vaccine treatments were administered as described above beginning at approximately 60 days of age (range 61-69 days) and repeated at >90 days of age (range 97–108 days). Kits were anesthetized with isoflurane gas within a transparent acrylic induction box with anesthesia maintained via a mask. Blood was drawn from the cranial vena cava with some whole blood applied to Nobuto filter-paper strips and stored at -70° C with desiccants [27] and the remainder processed to collect plasma and serum and stored separately at -70° C for future analysis. F1-V was administered SC to kits under anesthesia whereas oral vaccinia vaccinations were administered once kits were fully recovered from anesthesia. In addition to assigned treatments, all kits received a passive integrated transponder (AVID® PIT tag) SC and preventative penicillin 0.2 mL SC. No other vaccinations were administered to the immunogenicity trial ferrets during the study. An additional blood sample was collected and stored in the same manner on all study animals at >120 days of age (range 132-161 days).

IgG antibody response to V: lateral flow assay. We followed the lateral flow strip procedure as described by Abbott, et al. [28] for plasma samples from all time points in the trial. Lateral flow assay strips (IDxDI, Carlsbad, CA) use a Protein A capture and specific V antigen-coated particles applied to the strip. Ferret IgG antibodies specific to V, if present in the tested sample, will bind to the V antigen-coated particles forming an antibody-particle complex which will migrate up to the Protein A impregnated area on the strip. The Protein A will then capture the Fc region of the antibody on the antibody-particle complex thus forming a colored test line on the assay strip. If no specific V antibody is present in the

sample, then no complex is formed, and no line will appear on the test strip. All assay materials including plasma samples were brought to room temperature. Plasma samples were vortexed for five seconds and centrifuged for 30 seconds at 8000 revolutions per minute (RPM). We added 120 µL of assay diluent to wells in an ELISA plate and pipetted one µL of each sample to individual wells and flushed well with the pipette tip. Lateral flow test strips were placed in their respective wells and allowed to sit for 15 minutes before removing strips and reading results. Reactions were scored and categorized based on the color intensity of the test line.

Presence of neutralizing antibody to vaccinia virus: plaque reduction neutralization test (PRNT). We initially carried out a PRNT to determine whether serum from VV-F1-V vaccinated ferrets had neutralizing antibodies to VV indicating the vaccinia virus caused a transient infection and was an effective vector. We tested nine VV-F1-V vaccinated ferrets and one F1-V vaccinated ferret as an assumed control, but the PRNT result of the F1-V vaccinated ferret unexpectedly returned positive for vaccinia virus neutralizing antibodies. Subsequently, we tested the eight remaining F1-V vaccinated ferrets, plus three captive and five wild-caught ferrets with no previous plague vaccination history to further investigate this unanticipated result.

Cell culture plates were prepared with confluent *Cercopithecus aethiops* fibroblast cells (CV-1). Cells were grown at 37°C, 5% carbon dioxide, and 100% humidity in minimum essential medium with Earle's salts and 2.0 millimolar (mM) l-glutamine supplemented with 2 mM l-glutamine, 50 U/mL penicillin G, 50 µg/mL streptomycin, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids (MEM-C), with 10% defined fetal bovine serum (FBS). Sodium pyruvate and nonessential amino acids were from Corning

Incorporated (Corning, NY, USA). All other cell culture reagents were from GE Healthcare UK Ltd (Little Chalfont, UK).

We used serum samples collected from captive VV-F1-V vaccinated and F1-V vaccinated ferret kits at approximately 30 to 60 days post-booster vaccination (132-161 days of age), as well as sera from captive (three to four years of age) and wild caught adults (one year-olds). We chose this time point for the captive study ferrets in order to maximize vaccinia virus exposure and visualization of antibodies to VV as evidence of viral infection. Captive adults had been previously vaccinated against distemper and rabies viruses, and wild-caught adults had been previously vaccinated as kits against distemper only, as previously described.

Sera were thawed and aliquots were heat-inactivated (HI) at 56° C for 30 minutes which should deactivate complement in the HI treatment. We compared the sera heat-inactivated sera (HI) to remove complement with sera that were not heated (non-HI) which should leave complement intact. Complement associated with the innate immune response is an integral part of host defense against initial infections and can be activated independently from antibody [29]. Two-fold serial dilutions of both heat-inactivated and non-heat-inactivated samples were prepared in MEM-C. Vaccinia virus (VV) concentration was diluted to a multiplicity of infection of 1 (MOI = 1), where the ratio of one VV particle to one target cell would be achieved with each combined sample. Cells were inoculated with 75 µL of each serum sample dilution combined with 75 µL of diluted VV or with control samples. Negative controls contained 150 µL of MEM-C and positive controls contained 75 µL of diluted VV combined with 75 µL MEM-C. Plates were incubated for 30 minutes at 37° C before adding 500 µL MEM-C with 10% FBS. After incubation for 24 hours at 37° C, media

and cells were removed and frozen at -80° C. Samples later were exposed to three rounds of freeze-thawing and were vortexed before diluting each sample 1:1000 in MEM-C. Confluent CV-1 cells were inoculated with 400 µL of diluted sample and incubated for at 37° C for one hour. 4 mL of a mixture of one part 2X MEM-C with 20% FBS and one part 1% agarose was added to each well. Plates incubated at 37° C for three to five days until viral plaques were observed. Viral plaques were counted for each well. Plaque forming units per mL were calculated versus the sample dilution factor using Microsoft Excel® 2010 (Microsoft Corporation, Redmond, WA, USA). This calculation was converted to a percentage of viral plaques relative to the positive control at 100%. Human serum from a VV vaccinated researcher (ALM) was used as a positive control for the presence of neutralizing antibodies.

Maternal antibody presence

We tested for the presence of maternal IgG antibodies in young kits age 39-50 days old. Because of their age and size, direct venous blood draws were not possible so instead we clipped kits' claws to collect blood onto Nobuto filter papers (Advantec®, Japan). Kits were born to dams that had been previously vaccinated against plague using the injectable F1-V at a minimum of six months prior to blood collection. Kits belonging to one litter were pooled on each filter paper. Kits were pooled since toe clips from individual kits did not provide enough blood to saturate a Nobuto paper. Filter papers were stored at -70° C with desiccants until evaluation.

We followed the lateral flow strip procedure as described by Abbott, et al. [28]. All materials were brought to room temperature. Nobuto filter-paper samples were cut into five pieces and placed into 1.5mL centrifuge tubes. We added 400 µL PBS (phosphate-

buffered saline) to each tube, vortexed and rocked tubes overnight at 4° C for elution. Tubes were vortexed again and eluate samples were pulled and stored in 1 mL microcentrifuge tubes at -70° C until analysis. Using an ELISA plate, 100 µL of assay diluent was pipetted into each well. We pipetted 5 µL eluate into individual wells before dipping the lateral flow test strip into each well. After 15 minutes, reactions were scored and categorized based on the color intensity of the test line.

Statistical analyses

All immunogenicity trial data were analyzed using R software (Version 3.2.2, R Development Core Team 2015). Because individual sera were tested in different treatments (HI and non-HI), we were able to employ paired t-tests and two-way ANOVAs, including "individual" as a factor. We did not find the effect of individuals was significant in any test we performed, and these results are not reported. We defined our significance probability or p-value at less than 0.05. We calculated confidence intervals (CIs) using the Student's t distribution values from R.

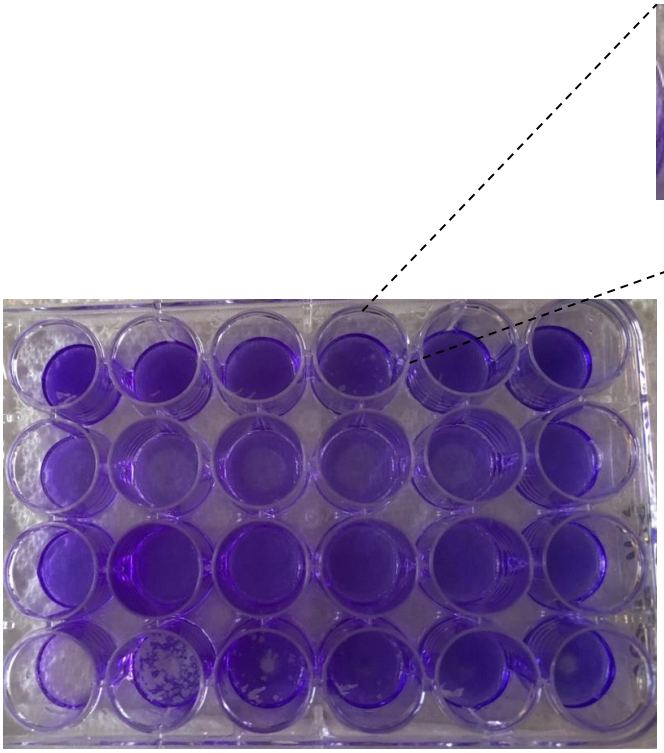
Results

Vaccine safety trial

In the five adult ferrets exposed to the oral vaccinia virus vaccine, no adverse effects were observed during the six-week course of the safety trial. No vomiting, lethargy, inappetence, diarrhea, or oral lesions were noted at any time during the six-week period.

Presence of vaccinia virus: virus isolation of buccal swabs. Of the five ferrets, vaccinia virus was only isolated from one ferret (#7163) from a single buccal swab on day 1 post re-vaccination (Figure 1). The second swab from this ferret on this sampling day was negative,

and all swabs from subsequent sampling days were negative. Live virus was not isolated from any swabs from any other ferrets at any time during this trial.



#7163, L to R: 1 d PV, 2 d PV, 9 d PV, 1 d PRV, 5 d PRV, 8 d PRV

No sample

Negative control

Positive control dilutions, L to R: 5×10^7, 5×10^6, 5×10^5, 5×10^4, 5×10^3, 5×10^2

Figure 1: Virus isolation plate of buccal swabs with positive #7163 highlighted. Arrow is pointing to a viral plaque in positive #7163 well. Sample handling artifacts such as pipette tip markings are seen in some wells. PV = post vaccination, PRV = post re-vaccination, d = day(s).

Presence of vaccinia virus: PCR of buccal swabs. PCR of all buccal swab media was negative for all animals on all sampling days. Although PCR of the buccal swab media of #7163 was negative, we obtained a PCR-positive result for presence of vaccinia virus from the virus isolation supernatant of this sample (Figure 2).

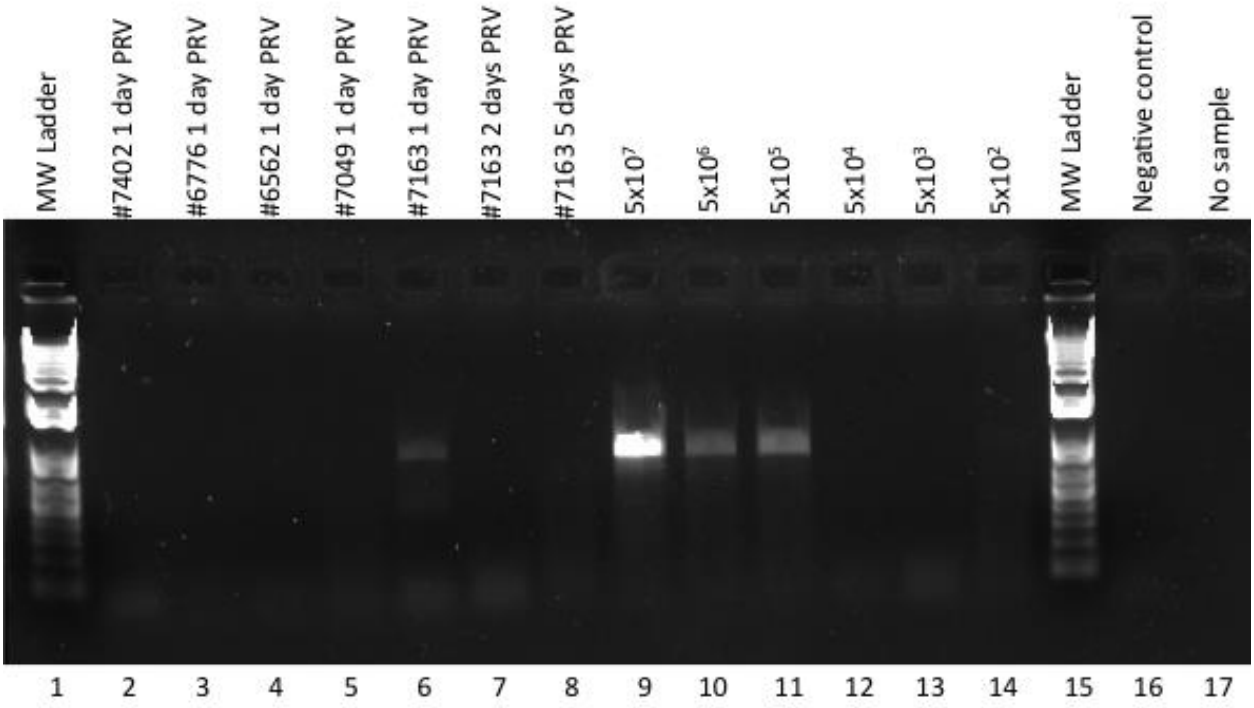


Figure 2: Gel electrophoresis of conventional PCR of virus isolation supernatant. Lanes 9-14 are positive control dilutions. Note detection of vaccinia virus DNA in the supernatant from virus isolation positive ferret (#7163) in lane 6.

Vaccine immunogenicity trial

IgG antibody response to V: lateral flow assay. We hypothesized that both vaccinia oral and F1-V injectable vaccine groups would demonstrate similar V antibody responses. The lateral flow assay demonstrated some measurable V antibody response by 120+ days of age in ferrets vaccinated with the injectable F1-V. Immunogenicity trial data are shown in Table 1. Examples of how lateral flow V antibody strips were scored are shown in Figure 3. Lateral flow results for three ferrets showed a reaction score of "1" prior to vaccination at blood draw 1 (8746, 8788, and 8748), which was assumed to indicate maternal antibody in these animals. No ferrets in the vaccinia oral vaccine group demonstrated an antibody response after vaccination. Antibody response in the F1-V group differed from antibody

response in the vaccinia group at blood draws 2 and 3 (p=0.0004 and p=0.00004, Fisher's Exact Tests).

Table 1: Lateral flow V antibody results for immunogenicity trial ferrets, grouped by vaccine type. Columns 7-9 show antibody response scores at blood draw points 2 (BD2 V) and 3 (BD3 V). Blood draw 1 (BD1 V) reflects pre-vaccination baseline antibody levels. BD2 V reflects 30+ days post-vaccination and when booster vaccination occurs. BD3 indicates 30+ days post-booster vaccination. Age is in days. Lateral flow results are categorized between 0-4, with 0 indicating a negative result, and 1-4 indicating positive V antibody levels with increasing color band intensity corresponding to an increasing antibody level, as seen below in Figure 3.

Animal ID	Sex	Vaccine type	Age at BD1	Age at BD2	Age at BD3	BD1 V	BD2 V	BD3 V
8720	M	F1-V	65	99	132	0	1	4
8721	M	F1-V	65	99	132	0	1	2
8786	F	F1-V	65	108	156	0	3	3
8796	M	F1-V	63	98	154	0	1	2
8798	M	F1-V	63	98	154	0	3	3
8799	M	F1-V	63	98	154	0	3	4
8747	M	F1-V	69	105	161	0	3	3
8745	M	F1-V	69	105	141	0	2	4
8746	F	F1-V	69	105	141	1	0	1
8787	F	Vaccinia	65	108	156	0	0	0
8788	F	Vaccinia	65	108	156	1	0	0
8797	M	Vaccinia	63	98	154	0	0	0
8831	F	Vaccinia	61	97	145	0	0	0
8748	M	Vaccinia	69	105	161	1	0	0
8749	M	Vaccinia	69	105	161	0	0	0
8826	M	Vaccinia	62	98	146	0	0	0
8743	M	Vaccinia	69	105	141	0	0	0
8744	M	Vaccinia	69	105	141	0	0	0

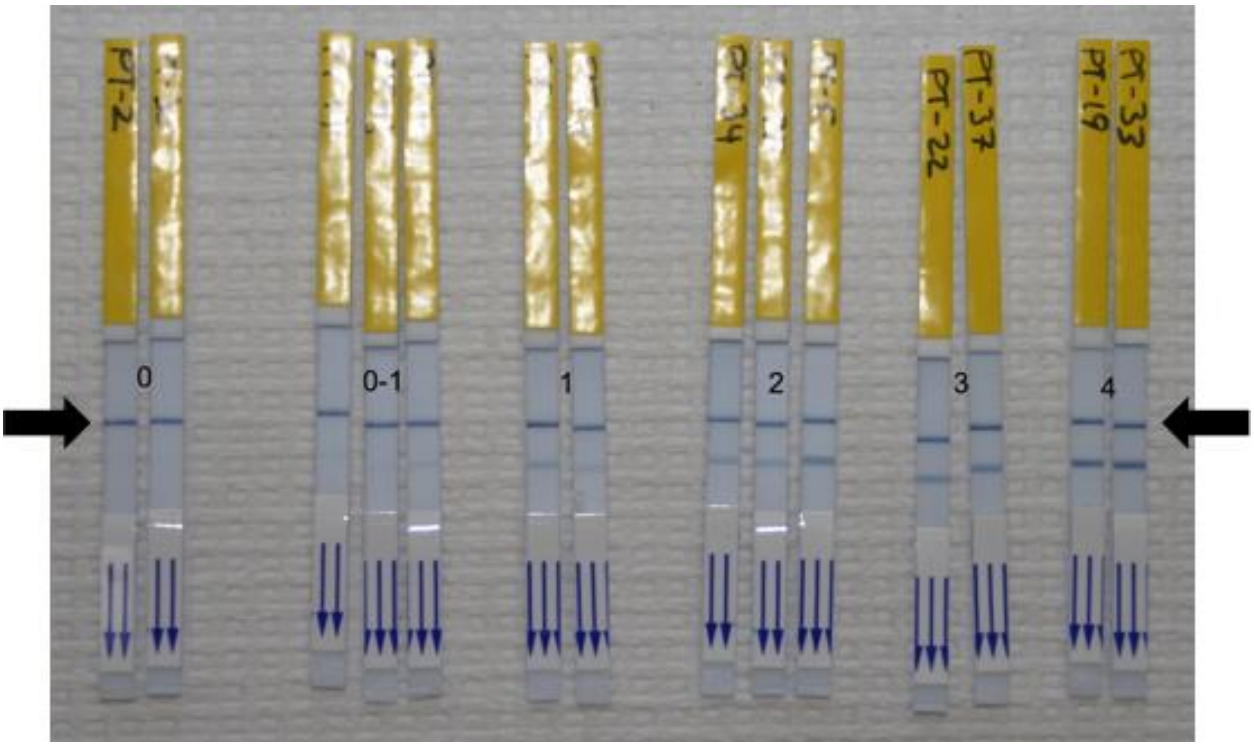


Figure 3: Examples of lateral flow V antibody strips and categorized scores based on color intensity of the bottom band corresponding to qualitative V antibody level. The uniform dark bands above (black arrows) are control lines indicating the assay is working properly. Photo courtesy of Elsa Cardenas, Colorado Parks and Wildlife.

Presence of neutralizing antibody to vaccinia virus: PRNT. We carried out a PRNT to test whether serum from VV-F1-V vaccinated ferrets had antibodies to VV that could indicate that the vaccinia virus vector was effective in replicating in ferrets. Additionally, it could help explain why VV-F1-V didn't induce an antibody response to the V antigen in the lateral flow assay.

We hypothesized that the oral VV-F1-V ferrets would have a lower number of vaccinia viral plaques (lower % of viral growth) than the injectable F1-V ferrets and the positive control (100%). We expected an increase in neutralizing antibody to vaccinia virus from the oral vaccine vector. Additionally, we compared sera that were heat-inactivated (HI) to remove complement with sera that were not heated (non-HI) with intact complement. We hypothesized that non-HI treated sera would have a lower number of

viral plaques (lower % of viral growth) than the HI treated sera: the smaller the vaccinia viral plaque percentage, the greater neutralization to vaccinia virus in the serum sample, and vice versa.

Sera from all vaccinia-vaccinated (VV-F1-V) and non-vaccinia vaccinated (F1-V) ferrets demonstrated some capacity to neutralize vaccinia virus (Table 2). Average viral plaque percentages in the F1-V and VV-F1-V groups did not differ from each other in either HI or non-HI sera ($p = 0.58$ and $p = 0.090$, respectively, Welch two sample t-test). Average viral plaque percentages were higher in HI than non-HI in both F1-V and vaccinia groups ($p = 0.00039$ and $p = 0.023$, respectively, paired t-tests). In addition, heat inactivated sera had overall higher viral plaque percentages across both vaccine groups than non-heat inactivated sera. We conducted a two-way ANOVA to determine if the HI treatment was similarly effective in both vaccine groups. No statistically significant interaction between HI treatment and vaccine type was detected ($p = 0.58$, $F = 0.33$), so we refit the model without the interaction. Sera heat treatment (HI vs. non-HI) was still significantly different ($p = 0.000039$, Figure 4) but vaccine type was not ($p = 0.097$, Figure 5).

Table 2: Summary of mean vaccinia viral plaque percentages +/- standard error of mean (SEM) and 95% confidence interval (CI) based on PRNT results. Data are grouped by vaccine type and sera treatment.

	Heat Inactivated (HI)	Non-heat Inactivated (Non-HI)
F1-V	33.3 +/- 3.34 (25.6, 41.02)	5.59 +/- 2.87 (-1.04, 12.22)
Vaccinia	38.66 +/- 8.21 (19.69, 57.63)	16.26 +/- 4.69 (5.43, 27.09)
Overall HI vs. Non-HI	35.99 +/- 4.61 (26.26, 45.71)	10.93 +/- 3.11 (4.37, 17.49)

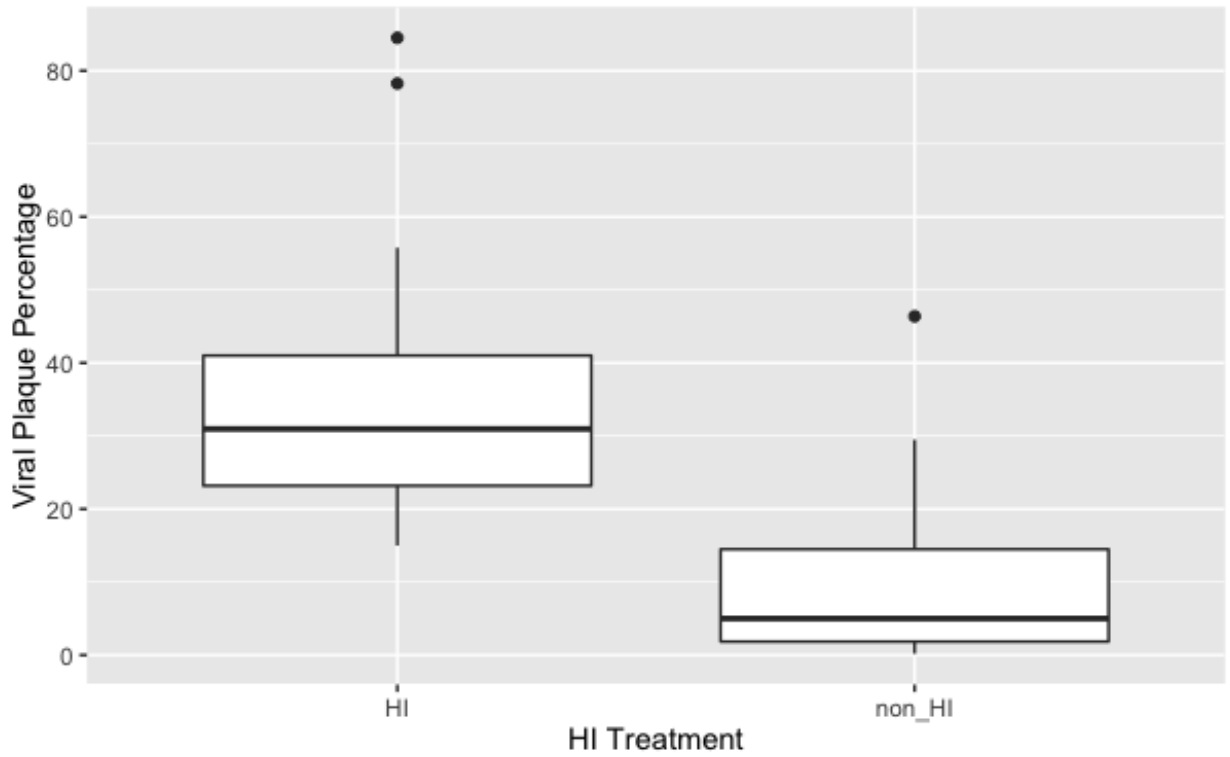


Figure 4: Box plot with error bars and outliers (individual points) of mean viral plaque percentages for sera exposed to heat inactivation (HI) or not exposed to a heat treatment (non-HI). Heat inactivation is expected to inhibit innate immunity in serum (complement), and lead to more viral growth in cell culture. Values are expressed as a percentage of the maximum.

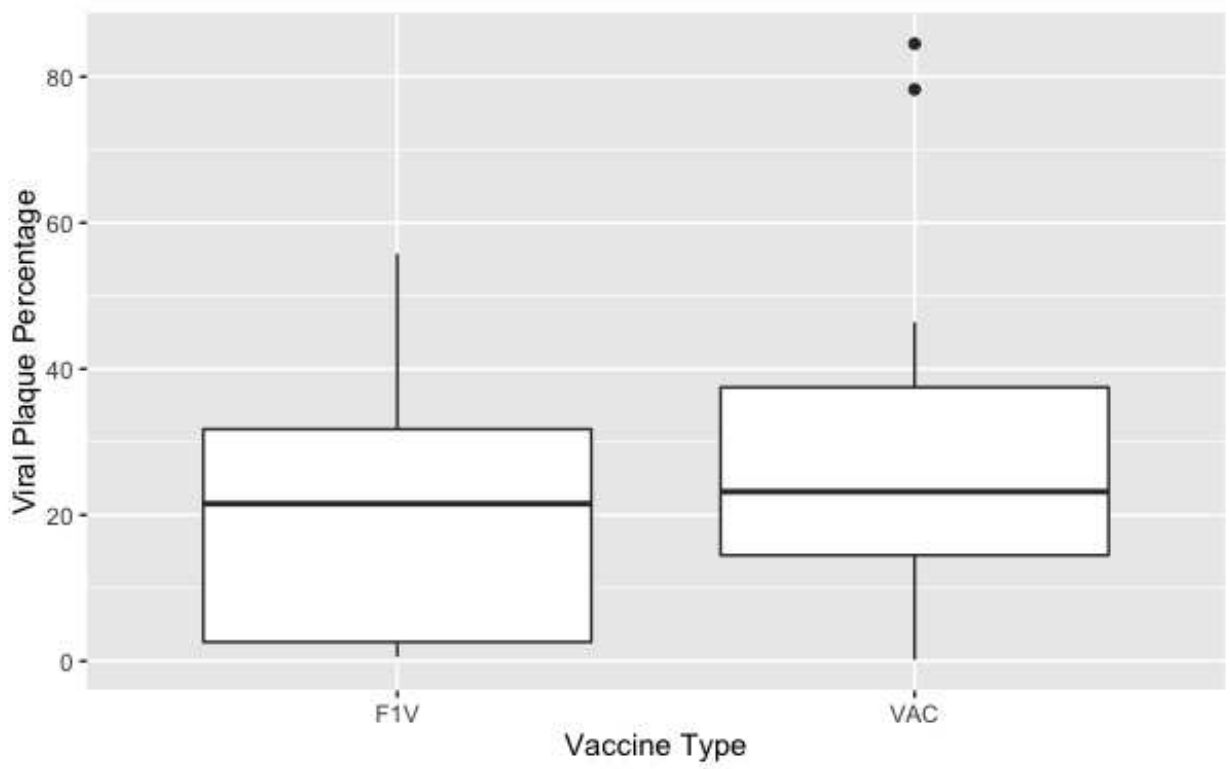


Figure 5: Box plot with error bars and outliers (individual pointes) of mean viral plaque percentages for sera from animals exposed to each vaccine type. Values are expressed as a percentage of the maximum. F1V = injectable F1-V plague vaccine, VAC = oral vaccinia virus vectored plague vaccine.

Additionally, we tested serum from wild-caught ferrets (N = 5) vaccinated only against canine distemper virus and captive ferrets vaccinated against rabies and canine distemper virus. Wild animals had vaccinia virus neutralizing capabilities in both HI and non-HI groups with average viral plaque percentages of 53.49% (SEM +/- 0.133, range of 38.1-71.0%) and 58.78% (SEM +/- 0.186, range of 41.3-88.9%), respectively. A paired t-test on the wild ferret sera showed no significant difference between HI and non-HI treated sera ($p = 0.74$). Non-plague vaccinated captive ferrets (N = 3) showed an even greater capacity to neutralize vaccinia virus in both HI and non-HI groups with average vaccinia viral plaque percentages of 13.33% and 0.17%, respectively.

Maternal antibody presence

Because all mothers of tested kits had been previously vaccinated against plague with injectable F1-V, we used lateral flow assays to investigate the presence of maternal V antibody levels in their kits aged between 39 and 49 days of age. Only four out of fifteen pooled litters demonstrated measurable V antibody responses as detected by lateral flow assay, and only at the lowest positive level (1 on a scale of 1-4, cf. Figure 3). All maternal antibody data are shown in Table 3.

Table 3: Lateral flow V antibody results of pooled kits from dams vaccinated with F1-V injectable plague vaccine. Nobuto sat level refers to how well the Nobuto filter paper was saturated with whole blood sample.

Dam SB #	# kits sampled	Age (days)	Lateral flow V	Nobuto sat level
8504	4	43	0	fair
8326	6	43	1	fair
8256	4	44	0	fair
7747	4	43	1	fair
8254	3	43	0	fair
8243	6	49	0	good
8303	4	44	0	fair
8831	4	40	0	good
8432	4	49	0	poor
8820	3	45	0	poor
8844	4	43	0	good
8253	4	45	0	fair
8130	4	42	0	good
8810	3	40	1	good
8699	4	39	1	good

Discussion

Our experiment was designed to assess a candidate oral plague vaccine for black-footed ferrets that was based on a vaccinia virus vector. This vaccine has been previously shown to stimulate effective immunity in laboratory mice (intra-nasal challenge) [19]. We did not conduct a challenge study for our research because of the highly endangered status

of the black-footed ferret and the limited number of animals available for testing this species. Furthermore, domestic ferrets are not appropriate animal models for black-footed ferrets because of their innate resistance to plague infection [30].

Our results suggest that the oral vaccinia virus-vectored plague vaccine will likely not be effective in black-footed ferrets in either captive or field settings at the given dose in our study. Although we did not observe ill effects of this vaccine, this was probably because the virus was neutralized before cell entry and failed to replicate in ferret cells. A single positive virus isolation result from a buccal swab (Figure 1) suggested the vaccinia virus is viable, although transiently, in ferrets. It is more likely that we swabbed food remnants lingering in the mouth that had been contaminated with oral vaccine from the previous day.

We found vaccinia virus neutralizing capability, based on PRNT results in all ferrets, from both vaccine treatment groups, from a sample of ferrets from the NBFFCC breeding facility and from sera of wild-caught ferrets. The heat treatment in the PRNT analysis demonstrated that complement contributes to virus neutralization, particularly in captive ferrets. The complement cascade is one of the first host defenses against pathogens and a crucial part of the innate immune system [31, 32]. Complement-mediated protection in serum is disabled by heat-inactivation, so we expected higher viral plaque percentages in the PRNT from serum that was heat-inactivated. In general, this was confirmed in captive ferrets, with heat-inactivated serum having higher viral plaque percentages than untreated serum. Substantial complement contribution was not as prevalent in wild-caught ferrets, as both sera groups had similar plaque percentages. This difference in overall vaccinia virus neutralization between wild and captive ferrets may suggest an artifact of captive breeding.

Although innate immunity may have been partially responsible for preventing vaccinia virus replication [33], the possibility remains that vaccinia failed to replicate because of cross-neutralization from previous exposure and antibody generation in response to other poxviruses.

Captive ferrets could be inadvertently exposed to a poxvirus from their horsemeat- and rodent-based diet, therefore producing neutralizing antibodies that prevent vaccinia virus infection. Antigenic cross-reactivity between the numerous species within the genus *Orthopoxvirus*, which includes vaccinia virus, is often observed (34-37], and is the basis of smallpox immunization, using the vaccinia virus rather than the highly pathogenic variola virus [38]. This cross-reactivity has not been observed in other genera within the *Poxviridae* family, such as *Avipoxvirus,* which includes the canarypox virus used as a vector for the effective canine distemper virus vaccine administered to ferrets. The *Orthopoxvirus* genus also includes raccoon poxvirus and horsepox virus, with anecdotal evidence suggesting vaccinia virus originated from horses [36, 39]. Raccoon poxvirus was first discovered in a raccoon [40] but its true origin is unknown, and rodents are considered the primary reservoir hosts of many orthopoxviruses [41-43]. An appropriate next step would be to analyze diet sources for presence of an orthopoxvirus. We do not recommend a diet change for captive black-footed ferrets, but it would be of interest to determine whether avirulent exposure to orthopoxvirus from food would preclude vaccinia-vectored or closely related virus-vectored vaccines for captive black-footed ferrets. Testing captive animals at different breeding facilities may also be of interest as each facility may have diverse exposures based on the sources of diet.

Wild ferrets primarily eat prairie dogs and other rodents [44], and if wild prey are naturally infected with orthopoxvirus that may explain why wild ferrets show vaccinia virus neutralizing capability. In our study, wild caught ferret samples were collected prior to the experimental release of YPV into prairie dogs and had generally lower virus neutralizing capability (higher viral plaque percentages) than captive ferrets. Anti-orthopoxvirus antibodies have been detected in many North American mammals [43, 45] and with greater landscape scale use of YPV, ferret exposure to an orthopoxvirus seems to be even more probable. Exposure could occur either by direct contact with baits or potentially from ingestion of vaccinated rodents, since raccoon poxvirus serves as the vaccine vector [46].

We encountered several issues attempting to determine whether kits gain passive immunity to the plague V antigen from their vaccinated mothers while nursing, and at what point those antibodies would start to decline. It proved difficult to collect enough blood from kits by clipping claws to saturate Nobuto strips (100 μL), even with pooling samples from entire litters. Other blood collection methods, such as jugular or retrobulbar venipuncture used in lab animals [47], would not likely succeed in ferret kits without sedation or general anesthesia. The low yield of the claw blood led to some poorly saturated Nobuto strips that simply may not have responded to lateral flow assays. Eluting antibodies from Nobuto strips requires a dilution of the sample, which would further decrease sensitivity of the assay making detection of low antibody levels even less likely. If this blood collection method were to be used again, we would consider saturating only a partial strip with blood and reducing the amount of PBS in the lateral flow assay in order to maximize our eluate concentration, or using heparinized microhematocrit tubes for blood

collection instead of Nobuto strips. Previous studies using lateral flow assays on black-footed ferret serum only detected plague antigen antibodies at 2,560 or greater titers [28], suggesting an already low sensitivity of the assay which likely contributed to poor antibody detection.

Furthermore, a circulating maternal antibody level at this age is likely quite low or potentially even non-existent. Captive-born kits nurse exclusively from their dams until 30 days of age when they begin eating semisolid food and are likely continuing to nurse intermittently until 60 days [48]. In domestic ferrets (*Mustela putorius furo*) and farmed American mink (*Neovison vison*), maternal IgG is transferred to kits through mother's colostrum and milk and is transported across the gut epithelium into the kit's bloodstream [49-50]. Extrapolating from domestic ferret and mink biology, it is likely that maternal milk IgG antibodies would begin to decline in the kits once weaning begins. We tested ferret kits between ages 39 and 50 days, which is during the weaning process, so the timing of our testing may have contributed to indeterminately low antibody levels.

Wild ferret kits are likely vulnerable to plague infection in the time after they have lost maternal plague immunity from their vaccinated mothers and before they can be vaccinated against plague. Kits remain underground for several weeks prior to consistent above ground emergence at 60-75 days of age [17] when they can be trapped for vaccination. By this age, they would have already been exposed to plague-infected prey and plague vectors by their mothers leaving kits at risk of plague infection. An oral plague vaccine in bait or natural prey form carried down by the dam could shorten the window of vulnerability for kits and reduce their risk of plague infection.

An orthopoxvirus-based plague vaccine does not appear to be the immediate solution for black-footed ferrets and further research of preexisting orthopoxvirus antibodies in ferrets should be explored. Testing pre-vaccination sera using PRNT could be beneficial to evaluate baseline neutralization of vaccinia virus and how viral plaque percentages could change as kits age and are exposed to more potential sources of orthopoxvirus. It would also be valuable to vaccinate with higher doses of VV-F1-V in a repeat immunogenicity trial to determine if a higher doses of the vaccinia virus vector could overcome neutralization. Additionally, kits should be housed separately from their littermates after vaccination with an orthopoxvirus-vectored vaccine to minimize potential virus exposure to each other during the immunogenicity trial. We considered other oral plague vaccines to compare to VV-F1-V for this project but were faced with various challenges. An attenuated *Yersinia pseudotuberculosis*-vectored plague vaccine [51-52] was not available for use at the time of our project and although we did attempt to evaluate an attenuated *Salmonella typhimurium*-vectored plague vaccine [54], we had difficulty maintaining the bacterial strain in our lab.

In the meantime, in addition to continuing to use injectable F1-V vaccination of pre-release and wild-caught ferrets, intense plague management by way of deltamethrin insecticide dusting [55] and plague vaccination of prairie dogs with YPV appears to be our current assets for minimizing plague losses in ferrets. The combination of dusting burrows with insecticides and oral vaccine will likely maximize plague control in wild prairie dogs [12] so an oral vaccine should help wild ferrets as well. Oral vaccines for both species could be delivered concurrently to minimize required resources to distribute baits. Lastly, having an effective, orally delivered plague vaccine that can also be dispensed to young kits could

improve the effectiveness of black-footed ferret reintroduction attempts and thus help catalyze species recovery in the face of plague.

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