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VACCINATION WITH F1-V FUSION PROTEIN PROTECTS BLACK-FOOTED FERRETS (*MUSTELA NIGRIPES*) AGAINST PLAGUE UPON ORAL CHALLENGE WITH *YERSINIA PESTIS*

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ABSTRACT: Previous studies have established that vaccination of black-footed ferrets (*Mustela nigripes*) with F1-V fusion protein by subcutaneous (SC) injection protects the animals against plague upon injection of the bacterium *Yersinia pestis*. This study demonstrates that the F1-V antigen can also protect ferrets against plague contracted via ingestion of a *Y. pestis*-infected mouse, a probable route for natural infection. Eight black-footed ferret kits were vaccinated with F1-V protein by SC injection at approximately 60 days-of-age. A booster vaccination was administered 3 mo later via SC injection. Four additional ferret kits received placebos. The animals were challenged 6 wk after the boost by feeding each one a *Y. pestis*-infected mouse. All eight vaccinates survived challenge, while the four controls succumbed to plague within 3 days after exposure. To determine the duration of antibody postvaccination, 18 additional black-footed ferret kits were vaccinated and boosted with F1-V by SC injection at 60 and 120 days-of-age. High titers to both F1 and V (mean reciprocal titers of 18,552 and 99,862, respectively) were found in all vaccinates up to 2 yr postvaccination, whereas seven control animals remained antibody negative throughout the same time period.

Key words: Black-footed ferrets, F1-V protein, sylvatic plague, vaccine, *Yersinia pestis*.

INTRODUCTION

Plague, caused by the gram-negative bacterium, *Yersinia pestis*, is a natural threat to black-footed ferrets (*Mustela nigripes*), and it has severely hindered recent efforts to restore this endangered species to its historic range (Barnes, 1993). The disease occurs regularly in rodents throughout the western USA and is particularly devastating for prairie dogs (*Cynomys* spp.). Ferrets, which feed exclusively on prairie dogs, are likely exposed to *Y. pestis* by direct and indirect association with their prey. They may be bitten by *Y. pestis*-infected fleas as they hunt and kill prairie dogs in their burrows. Alternatively, and probably more importantly, ferrets may contract plague through direct contact or inhalation of infectious aerosols as they feed on ill or dead prey

(Williams et al., 1994; Castle et al., 2001; Roche et al., 2006).

In recent studies conducted at the US Geological Survey, National Wildlife Health Center (NWHC), we demonstrated that a majority of ferrets could be protected against subcutaneous (SC) *Y. pestis* challenge by vaccination with the F1-V protein (Roche et al., 2004, 2006), a recombinant fusion protein that consists of two known protective antigens expressed by *Y. pestis* (Powell et al., 2005). Vaccinated animals that survived an initial SC challenge with *Y. pestis* were completely resistant to a secondary exposure via consumption of a *Y. pestis*-infected mouse (Roche et al., 2006), and a few vaccinated animals even survived primary oral challenge (Roche, unpubl. data). Although these results were considered promising, both of these studies were

conducted in older animals that represented less-than-ideal candidates for vaccination (Rocke et al., 2006) because they were 4- to 6-yr-old spent breeders nearing the end of their normal life span. Ferret kits that are bred in captivity for field release are typically handled only twice: when they are placed in conditioning pens at 60 days-of-age and just before release at approximately 120 days-of-age. This timing may align with a practical two-dose schedule for immunization, if vaccines administered at those age and time intervals were shown to elicit protective immunity. Moreover, because ingestion and/or inhalation are probably important transmission routes for *Y. pestis* in ferrets, an effective vaccine for field use must demonstrate protection against plague after consumption of infected prey.

Here, we establish that vaccination of ferret kits with F1-V protein confers protection against primary oral challenge via consumption of a *Y. pestis*-infected mouse. Furthermore, we demonstrate the longevity of antigen-specific antibody titers in immunized ferrets, which remain high up to 2 yr postvaccination (PV).

MATERIALS AND METHODS

Experimental animals

Thirty-seven black-footed ferret kits were selected for this study at the US Fish and Wildlife Service, National Black-Footed Ferret Conservation Center (NBFCC), Wheatland, Wyoming. One group of 12 kits (all males) was designated for the vaccine efficacy trial. The second group of 25 kits (mixed sex) was used to determine the duration of antibody after vaccination. All animals were 60 days-of-age and were marked individually with SC embedded microchips (AVID, 78294 Oak Ridge Road, Folsom, Louisiana 70437, USA). At NBFCC, the kits were housed with dam and littermates until they reached 120 days-of-age. At NWHC, the animals were housed individually as described previously (Rocke et al., 2004). After the initial vaccination (described later), the group of 12 animals was transported to the NWHC where they were placed in a biosafety level-three animal-holding facility; the other group of 25 remained at the NBFCC. Upon arrival at

NWHC, the animals were treated prophylactically for coccidiosis and housed individually in stainless-steel cages as described previously (Rocke et al., 2004). The animals were fed a diet of raw horse meat (Toronto Small Carnivore Diet, Meat Products, 3347 Kennedy Road #1, Scarborough, Ontario M1V 3P1, Canada), periodically supplemented with mice (NWHC) and prairie dog meat (NBFCC).

This study was reviewed and approved by NWHC's Animal Care and Use Committee and Biosafety Committee. All personnel handling *Y. pestis*-infected animals or carcasses were required to wear high efficiency particulate air-filtered respirators with full-face shields, rubber aprons and boots, and double surgical gloves. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals.

Experimental design: Vaccine efficacy trial

At approximately 60 days-of-age, each of 12 ferret kits was anesthetized at NBFCC, and up to a 2 ml blood sample was drawn using methods described previously in Rocke et al. (2004). Eight of these ferrets received 0.5-ml F1-V vaccine-adjuvant preparation (100 µg of antigen) by SC injection between the scapulae. The F1-V fusion protein and our methods of preparing the vaccine-adjuvant mixture for SC injection have been described previously (Rocke et al., 2004; Powell et al., 2005). Briefly, the antigen was diluted in Modified Dulbecco's medium (Sigma, PO Box 14508, St. Louis, Missouri 63178, USA), mixed 1:1 with 0.2% Alhydrogel (United Vaccines, 7819 Airport Rd., Madison, Wisconsin 53562, USA), and rocked gently overnight at 4 C. Four control animals received a placebo of 0.5 ml of the adjuvant (without protein antigen) suspended in Dulbecco's medium.

Two mo later, all 12 animals were transported to NWHC. After an acclimation period of several wk, the animals were anesthetized, a 2-ml blood sample was drawn, and a booster injection of F1-V was administered. About 6 wk after the booster dose, another blood sample was drawn, and the animals were orally challenged by feeding each a single *Y. pestis*-infected mouse. Six-wk-old outbred Swiss ICR mice (Harlan Sprague Dawley, PO Box 29176, Indianapolis, Indiana 46229, USA) were inoculated with >4,000 cfu (in 0.1 ml) wild type *Y. pestis* strain CO92 (~200 50% lethal doses) by intradermal injection. Upon death 3 days after challenge, one mouse was placed in the cage of each ferret. Any carcasses or parts of carcasses not ingested by ferrets within 3–4 hr

were removed and discarded. For those ferrets that did not fully consume their mice, another *Y. pestis*-infected mouse was given to them the following day. Ferrets were monitored daily for signs of illness, and day of death was noted; severely debilitated animals were euthanized by CO₂ asphyxiation.

Any ferrets that survived challenge were bled to determine antibody titers after 3 wk and then euthanized by intracardiac injection of Euthasol (Delmarva Laboratories, Inc., 1500 Huguenot Road, Suite 106, Midlothian, Virginia 23113, USA). In both experiments, dead or euthanized ferrets were immediately necropsied. Selected tissues were collected for bacterial isolation (Rocke et al., 2004) and histologic examination.

Experimental design: Duration of antibody response

The second group of 25 ferrets was vaccinated via SC injection at approximately 60 and 120 days-of-age as already described; 18 animals received the F1-V vaccine-adjuvant mixture, and seven animals received the placebo. All of these animals remained at NBFCC throughout the duration of the study, and blood samples were drawn pre-vaccination, preboost, and ~3 mo, 6 mo, and 1 yr postvaccination (PV) for all 25 animals, and 2 yr PV for 20 animals.

Antibody determination

All blood samples were collected in sterile glass serum separator tubes. After centrifugation of blood samples, the serum was transferred to 2-ml polypropylene tubes and frozen at -20 C for future analyses. Antibodies against F1 and V antigens were measured using an enzyme-linked immunosorbent assay (ELISA) as previously described (Rocke et al., 2004), with slight modifications as follows. All ELISA's were conducted at NWHC. Coating of ELISA plates with V antigen was performed with the same concentration of protein (1 µg/ml) but in a 100-µl volume instead of 50 µl. Serum samples were serially diluted fourfold starting at 1:640. The highest dilution that was positive (defined as exceeding the mean of four negative control samples by three standard deviations) was considered to be the end point, and its reciprocal value was recorded as the titer.

Statistical analysis

Antibody titers were transformed by calculating the log₁₀ of the titer. Change in log titer was then calculated by subtracting an individual animal's pre-inoculation anti-F1 or

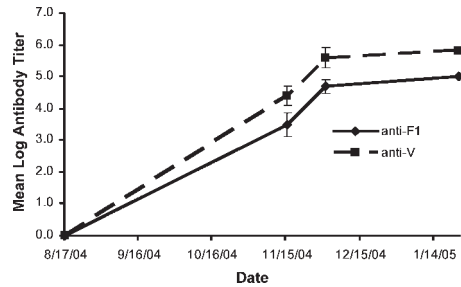


FIGURE 1. Geometric mean titers for anti-F1 antibody and anti-V antibody in black-footed ferret kits that were vaccinated with F1-V fusion protein. Animals were vaccinated via subcutaneous (SC) injection on 17 August 2004, were boosted similarly on 15 November 2004, and challenged with wild-type (CO92) *Yersinia pestis* on 1 December 2004.

anti-V log titer from the log titer of each of that same animal's subsequent blood samples. Combined (anti-F1 + anti-V) log titers were calculated by adding the log titers of individual animals for each antigen (Williamson et al., 1999). All statistical analyses were performed using SAS software (SAS Institute, Cary, North Carolina, 27513 USA). Statistical differences in change of titer between groups were tested separately at each blood sampling period using a one-tailed Wilcoxon test. For the vaccine efficacy trial, the difference in survivorship between groups was analyzed using the Fisher two-tailed exact test.

RESULTS

Vaccine efficacy

All eight vaccinated ferrets developed significant antibody titers to both F1 and V after their first vaccine dose as compared to both their prevaccination titers ($P < 0.0001$) and the titers of control-group animals ($P < 0.005$). After the second vaccine dose, all eight vaccinates had significantly increased (Fig. 1) anti-F1 and anti-V titers (at least fourfold and as much as 64-fold; $P < 0.0001$). In contrast, the titers of control animals remained negative throughout the experiment.

All eight vaccinates survived oral challenge with a *Y. pestis*-infected mouse with no signs of illness, whereas the four control animals that died succumbed to plague within 3 days of ingesting a *Y.*

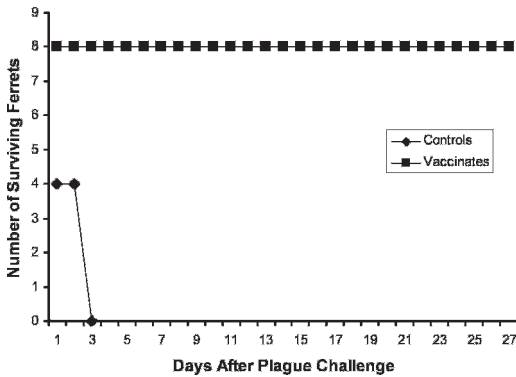


FIGURE 2. Survival of black-footed ferrets vaccinated with F1-V protein and control animals that received a placebo after challenge via consumption of a *Y. pestis*-infected mouse.

pestis-infected mouse (Fig. 2). Gross and histologic lesions consistent with plague were observed in all dead control animals, including marked congestion and hemorrhage in the liver, spleen, lymph nodes, and intestines. *Yersinia pestis* was isolated from nasal swabs and liver in every case.

Antigen-specific titers of all survivors rose significantly after challenge (Fig. 1). Mean anti-F1 and anti-V antibody titers of surviving vaccinates were significantly higher ($P=0.0187$ and 0.0368 , respectively) than their mean prechallenge titers. Upon euthanasia and necropsy of surviving vaccinates at the end of the experiment, no obvious lesions characteristic of plague were observed at the organ or tissue level, and no *Y. pestis* was isolated from tested tissue samples.

Duration of antibody

All 18 vaccinates developed a significant increase in antibody titers to F1 and V antigens (Fig. 3) after the initial vaccine dose ($P<0.0001$) at 60 days-of-age and an even higher increase in titer after the booster injection ($P<0.0001$) at 120 days-of-age. Antibody titers remained high for at least 2 yr PV (Fig. 2), and there was no difference in combined log titers (anti-F1 and anti-V) between males and females ($P=0.3584$). Controls remained antibody

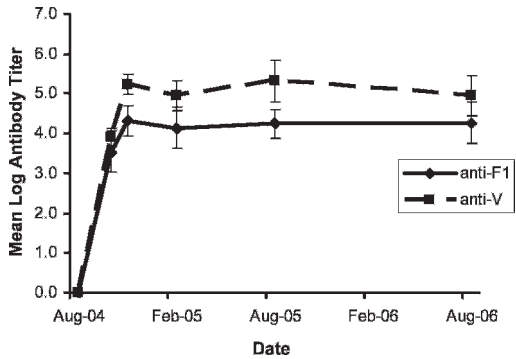


FIGURE 3. Geometric mean titers of anti-F1 and anti-V antibodies in black-footed ferret kits vaccinated with F1-V protein at 0, 3 mo, 6 mo, and 1 yr ($n=18$) and 2 yr ($n=14$) postvaccination. Animals received the first vaccine dose in August 2004 and received a similar boost in October 2004.

negative throughout the 2-yr duration of the study.

DISCUSSION

In this study, we found that black-footed ferret kits vaccinated and boosted SC with F1-V protein were protected against plague contracted via ingestion of a *Y. pestis*-infected mouse. All vaccinated animals developed high antibody titers against F1 and V antigens, survived oral *Y. pestis* challenge, and showed a significant increase in antigen-specific titers after challenge. Together with our earlier studies (Rocke et al., 2004, 2006), these results demonstrated that the F1-V protein is an effective vaccine against plague for black-footed ferrets, eliciting protective immunity against both an oral challenge (consumption of infected prey) and an SC injection of the bacteria (simulated flea-bite exposure). Our results are consistent with numerous other studies that have demonstrated the protective efficacy of F1-V fusion protein against *Y. pestis* infection in mice (Anderson et al., 1998; Williamson et al., 1999; Glynn et al., 2005; Powell et al., 2005) and reaffirm the utility of F1-V protein as a plague vaccine.

The antibodies elicited against *Y. pestis* antigens after captive ferret kits were

vaccinated with F1-V protein appear to last at least 2 yr (the last time point tested) and may possibly last longer, since they showed little drop in titer. Although we did not challenge these animals, we believe their titers continued to be protective against plague. Anderson et al. (1998) demonstrated that F1-V-vaccinated mice retained antibodies protective against pneumonic plague for as long as 1 yr, the last time point tested in their study. Furthermore, other investigators have shown that the combined anti-F1 and anti-V log titer correlates better with protection against plague in mice than do either of the individual log-transformed anti-F1 or anti-V titers (Williamson et al., 1999; J. Adamovicz, pers. comm.). By analyzing titer data (Fig. 4) generated from vaccinated black-footed ferrets in this study, a previous study (Rocke et al., 2006), and unpublished data (Rocke), we determined that the mean combined anti-F1 and anti-V log titer of vaccinates that survived *Y. pestis* challenge in our studies (9.14 ± 1.05 , $n=21$) was significantly higher ($P < 0.0009$) than the combined log titer of vaccinated ferrets that died upon challenge (7.69 ± 0.68 , $n=11$). We used probit analysis (SAS) to examine this relationship further and found that an animal with a combined log titer of 9.05 (confidence interval of 8.50 to 13.26) had a 90% chance of surviving *Y. pestis* exposure. The combined log titers of vaccinated ferrets 1 yr PV ranged from 8.62 to 10.43, with a mean of 9.56 ± 0.64 . These values suggest that the majority of these animals were probably protected against plague 1 yr PV. At 2 yr PV, combined log titers ranged from 8.02 to 11.03, with a mean of 9.22. Probit analysis suggests that all the vaccinated animals had a 50% chance of surviving *Y. pestis* exposure (Fig. 4) and most (8/14) had a greater than 90% chance of survival.

At NBFFCC, zoos, and other facilities where prairie dog meat is fed to captive ferrets, there is a minimal but continual risk of *Y. pestis* exposure. To mitigate this

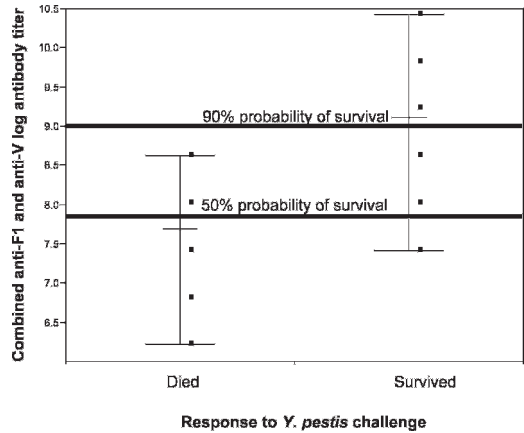


FIGURE 4. Combined anti-F1 and anti-V antibody titers of black-footed ferrets immunized with F1-V fusion protein that either died or survived upon *Y. pestis* challenge. Data are combined from this study and previous studies (Rocke, et al., 2006, unpubl. data) and include animals that received either 1 or 2 subcutaneous (SC) injections of vaccine at a dose of 100 μ g F1-V protein per animal and animals challenged either by SC injection of *Yersinia pestis* or via consumption of a *Y. pestis*-infected mouse. The horizontal lines indicate the combined anti-F1 and anti-V log titers at which 50% (7.97) and 90% (9.05) of the animals would survive *Y. pestis* challenge as determined by probit analysis (with 95% confidence intervals of 6.91 to 8.57 and 8.50 to 13.26, respectively).

risk, prairie dogs used for ferret feeding are quarantined for at least 21 days. Vaccination of captive ferrets with F1-V protein should reduce the risk of *Y. pestis* exposure even further. This vaccine could also be used to immunize ferret kits that are released as part of reintroduction programs and whenever wild ferrets are trapped for monitoring programs. In 2004, a study was begun to measure survival rates of both field-born and field-released ferret kits vaccinated with F1-V in comparison to animals not vaccinated. Although survival data are not available yet, six field-released vaccinates trapped approximately 1 yr later had significant antibody titers to F1 and V (combined log titer range of 7.42 to 9.83).

Finally, although trapping and vaccination is a labor-intensive prospect, the vaccine might also be useful when plague

threatens important ferret populations that have been reestablished in their native range. In 2005, plague killed numerous prairie dogs on the Pine Ridge Indian Reservation in South Dakota. This outbreak occurred just 48 km south of the Conata Basin, where the largest number of black-footed ferrets resides (about 250 animals), representing half of the free-ranging population. In an effort to protect at least some of these animals should the disease move northward, 100 ferrets were captured and vaccinated with F1-V fusion protein antigen. That effort is still ongoing, and the findings will be reported separately. Currently, the F1-V vaccine used in this study is strictly available for animal testing on a limited basis. However, commercial production of this or a similar vaccine is planned upon further definition of its efficacy in preventing plague in animals.

Even though we demonstrated that administration of F1-V elicited a robust protective response, we view vaccination of ferrets as an emergency, stop-gap measure to prevent plague in free-ranging populations of ferrets. A more effective strategy may be to devise a practical method to directly control the disease in prairie dogs, the primary source of infection for ferrets. This wildlife disease management goal appears to be approachable through targeted immunization of prairie dogs using oral baits laden with a different plague vaccine (Mencher et al., 2004).

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