Protection of Black-Tailed Prairie Dogs (*Cynomys ludovicianus*) against Plague after Voluntary Consumption of Baits Containing Recombinant Raccoon Poxvirus Vaccine

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Prairie dogs (*Cynomys spp.*) are highly susceptible to *Yersinia pestis* and significant reservoirs of plague for humans in the western United States. A recombinant raccoon poxvirus, expressing the F1 antigen of *Y. pestis*, was incorporated into a palatable bait and offered to 18 black-tailed prairie dogs (*Cynomys ludovicianus*) for voluntary consumption; 18 negative control animals received placebo baits. Antibody titers against *Y. pestis* F1 antigen increased significantly (*P* < 0.01) in vaccinees, and their survival was significantly higher upon challenge with *Y. pestis* than that of negative controls (*P* < 0.01).

Sylvatic plague, caused by the bacterium *Yersinia pestis*, is a disease of rodents that can afflict humans, as well as other mammals, and is transmitted primarily via fleas. Currently, the most significant reservoir of plague for humans in North America is wild rodents, particularly prairie dogs and several squirrel species (11). Important trends in plague epidemiology are the increased transmission of plague from wild rodents to domestic cats as residential areas encroach on enzootic plague foci and the increased transmission of the disease from cats to their owners and veterinarians (17).

Plague epizootics in prairie dog populations with mortality rates as high as 95 to 99% have been well documented for many decades (1, 5, 6, 18, 21). Not only do these outbreaks decimate local populations of prairie dogs, they also impact other species that depend on prairie dogs for food and shelter (2), such as the endangered black-footed ferret (*Mustela nigripes*). Although flea infestations in prairie dogs have been reduced in the past by application of insecticides to burrows (10), this method is labor intensive and generally applied after an outbreak has begun. Prophylactic control of the disease through immunization could be more effective. Recently, Osorio et al. (16) described a recombinant raccoon poxvirus (RCN) that expresses the F1 antigen of *Y. pestis* (herein designated RCN-F1) and protects mice from virulent plague challenge. In preliminary experiments with black-tailed prairie dogs (*Cynomys ludovicianus*), all animals vaccinated with RCN-F1 via intramuscular (i.m.) injection survived subcutaneous challenge with virulent *Y. pestis* (T. E. Rocke, unpublished data). However, efficient large-scale protection of free-ranging wildlife populations necessitates voluntary consumption of vaccine (19, 20). The purpose of this study was to investigate the ability of RCN-F1 to elicit a protective immune response against *Y. pestis* infection in black-tailed prairie dogs after voluntary ingestion of palatable vaccine-laden baits.

**Experimental animals.** Adult black-tailed prairie dogs captured from wild colonies in South Dakota were purchased from a commercial supplier and transported to the U.S. Geological Survey National Wildlife Health Center (Madison, Wis.). Upon arrival at the National Wildlife Health Center, animals were inspected for external parasites, treated with an anthelmintic injection (Ivomec; Merck & Co., Inc, West Point, Pa.), and marked with uniquely numbered ear tags. Prairie dogs were group housed in isolation rooms with approximately 180 square ft of floor space. Beta chips covered the floor, and Rubbermaid nest boxes connected by lengths of polyvinyl chloride pipe were used to mimic a burrow system. An alfalfa-based pelleted food was fed free choice (approximately 50 g per animal per day), and fresh vegetables (broccoli, carrot, green beans, and sweet potato chunks) were given once daily. Water was available ad libitum.

**Vaccine and bait preparation.** The raccoon poxvirus-vectored recombinant plague vaccine RCN-IRE5-IPA-YpF1 (designated RCN-F1 in this paper) was produced as previously described (16) and stored at −70°C in 2-ml aliquots until bait production. Virus stocks were thawed and diluted to 5 × 10^7 50% tissue culture infective doses (TCID<sub>50</sub>)/ml in Hanks’ medium (Gibco BRL, Carlsbad, Calif.) supplemented with 5% glycerin (Sigma, St. Louis, Mo.) immediately before use.

Observation of the prairie dogs’ food preference suggested that sweet potato was the most palatable vegetable in their laboratory diet. Finely shredded sweet potato was lightly packed in 10-g lots into wells of plastic ice cube trays, and 8 ml of liquid gelatin (9.3 g of powdered gelatin [Difco, Irvine, Calif.] in 150 ml of warmed Hanks’ medium) was added, followed by 1 × 10^7 TCID<sub>50</sub> of RCN-F1 vaccine/ml in 200 μl of Hanks’ medium with glycerin. The vaccine was gently mixed through the liquid gelatin and sweet potato. For the negative control baits, 200 μl of Hanks’ medium with glycerin alone was inserted into the bait. The ice cube trays were then refrigerated for 30 to 90 min until the gelatin baits were solidified.

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To ensure that bait production did not reduce vaccine vector viability, virus was extracted from two vaccine-laden baits within 24 h after preparation by homogenization and low-speed centrifugation. Identical processing was performed on two negative control baits containing no RCN-F1. Extracted supernatants were serially diluted (10^x), Vero cells were added and, after 3 days at 37°C and 5% CO₂, wells were stained with trypan blue and observed for disruption of the Vero cell monolayer, consistent with viral cytopathic effect (CPE). The supernatant from the vaccine-bait preparation had a titer of 2 × 10^6 TCID₅₀/ml for the positive control sample. The difference between these two titers was probably due to incomplete extraction of virus from the bait. Even if formulation led to some reduction in viral titer, we assume at least one priming bait but then ate at least one boosting bait. One animal in the negative control group failed to eat any baits and was eliminated from further analyses.

**Y. pestis challenge.** Six weeks post-priming vaccination, all animals were challenged with the CO92 wild-type isolate of *Y. pestis* (provided by the U.S. Army Medical Research Institute of Infectious Diseases). Stock aliquots of the bacteria, prepared and quantified as previously described (16), were diluted 1,000-fold in sterile saline. A volume of 0.2 ml of this solution was administered to each prairie dog by s.c. injection in the right hip region. Plate counts of the challenge inoculum indicated a dose of 132,000 CFU (6,600 mouse 50% lethal doses), and concurrent mouse tests confirmed its virulence. Because as many as 11,000 to 24,000 bacteria per bite may be regurgitated by flea vectors (3), our challenge dose represents approximately that delivered by 6 to 12 infectious flea bites. Several attempts were made prior to this experiment to determine a 50% lethal dose for our *Y. pestis* challenge inoculum in black-tailed prairie dogs (Rocke, unpublished). However, unlike inbred mice, reproducible results could not be achieved with prairie dogs. Prairie dogs were monitored for 21 days for signs of illness or death, after which all survivors were humanely euthanized. All carcasses were frozen for future necropsy.

Survival rates of orally vaccinated prairie dogs differed significantly (*P < 0.01*) from those of negative controls via the Fisher exact test (22). Ten of the 18 (55.6%) vaccinees survived challenge (Table 1) compared to only 2 of 17 (11.8%) controls that ingested one placebo bait. In contrast, time to death for those animals that did not survive challenge was not significantly different (*P > 0.2*) between the vaccinated and control groups via the Mann-Whitney ranks test (22). Although not included in our analyses, two of four animals vaccinated via the i.m. route also survived challenge.

Plague-induced mortality in challenged animals was verified by isolation of *Y. pestis*-specific DNA sequences from tissue culture by PCR. Selected frozen carcasses were thawed and necropsied, and tissue samples from lung, liver, and spleen were cultured in brain heart infusion broth (Difco) and on

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* ND, not determined.
blood agar plates (Becton-Dickinson, Franklin Lakes, N.J.) at 28°C for up to 72 h. The DNA was subsequently extracted from the culture broth and stored at -20°C. For PCR, primers specific for the Y. pestis F1 gene (12) were used to amplify DNA fragments that were fractionated and directly visualized using standard techniques. Y. pestis DNA fragments were recovered by PCR from the lungs of 10 of 12 necropsied animals that had succumbed to plague challenge and from the liver or spleen of 4 of the 12. Y. pestis DNA was not recovered from any sampled tissue of the three necropsied animals that survived challenge.

Anti-RCN antibody. Blood samples (300 µl) were collected from the medial saphenous vein of each prairie dog before the priming and booster vaccinations and before challenge; blood samples were also obtained from survivors postchallenge. Serum was collected and stored at -20°C until analyses.

A modification of a low antibody titer microneutralization assay was used to determine serum anti-RCN antibody titers. Prairie dog serum samples were serially diluted and tested for the ability to neutralize 1 × 10^3 PFU/ml in microtiter plates. After incubation for 2 h at 37°C, incubated samples were added to duplicate wells of previously seeded 96-well plates containing Vero cells. After additional incubation, fixation, and staining, CPE was scored in relation to negative control wells and the highest dilution of serum with reduced infection (25 to 50% reduction in CPE) was recorded.

Of 17 vaccinees tested, all developed anti-RCN antibody titers of 1:15 or higher (maximum, 1:1,875) postboost (Table 1); the serum from one individual was not tested because it was hemolytic. The geometric mean titer (GMT) of the oral vaccinees was 1:176, while all the negative controls had anti-RCN antibody titers of <1:15. All four animals immunized with RCN-F1 by i.m. injection developed anti-RCN antibodies, confirming infectivity of the virus; their GMT was 1:839.

Anti-F1 antibody titers. Antibody titers to Y. pestis F1 antigen were determined by using a modified enzyme-linked immunosorbent assay ELISA protocol (4) with F1 antigen supplied by the Centers for Disease Control and Prevention. Briefly, serum samples were serially diluted fourfold from 1:50 to 1:12,800; test samples were run in duplicate. Each plate also contained four replicates of a negative control serum sample and two replicates of a positive control serum sample. A horse-radish peroxidase-labeled anti-prairie dog immunoglobulin G custom prepared by Bethyl Laboratories (Montgomery, Tex.) was diluted 1:100 and used as the secondary antibody. Titers <1:50 were treated as equal to 1:50, and those >1:12,800 were treated as equal to 1:12,800.

Baseline antibody titers against F1 antigen were all <1:50. Fourteen of the 18 vaccinees developed anti-F1 antibody titers of ≥1:100 after vaccination (Table 1). The postprime GMT of oral vaccinees was 1:177, and their postboost GMT was 1:416. Reciprocal titers were log10 transformed, and the difference in each animal’s transformed titer from baseline to postprime and from postprime to postboost was calculated. Anti-F1 antibody titers increased significantly in vaccinated animals after both the initial dose (P < 0.01, Wilcoxon signed rank test) and the second booster dose (P < 0.01; Fig. 1). These data demonstrate that voluntary ingestion of RCN-F1-laden baits by prairie dogs elicited a humoral immune response to F1 protein.

Anti-V antibody. Antibody titers to another Y. pestis protein, V antigen, was measured similarly by enzyme-linked immuno-
RCN is highly attenuated and shown to be safe in numerous animal species, including black-footed ferrets (Rocke, unpublished), raccoons, skunks, bobcats, cats, dogs, and sheep (7, 8, 9, 13). Following immunization of mice and cats with RCN-vectored vaccines in other studies, RCN infection was limited to the site of exposure and no viremia or viral shedding was detected (15).

Oral vaccination through consumption of vaccine-laden baits may have application in managing plague epizootics in free-ranging prairie dog populations. Immunization of natural prairie dog colonies via oral, vaccine-laden baits could directly reduce plague mortality in exposed individuals and could also reduce the source of bacteria for flea transmission.

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REFERENCES