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Use of Rhodamine B as a Biomarker for Oral Plague Vaccination of Prairie Dogs

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ABSTRACT: Oral vaccination against *Yersinia pestis* could provide a feasible approach for controlling plague in prairie dogs (*Cynomys* spp.) for conservation and public health purposes. Biomarkers are useful in wildlife vaccination programs to demonstrate exposure to vaccine baits. Rhodamine B (RB) was tested as a potential biomarker for oral plague vaccination because it allows nonlethal sampling of animals through hair, blood, and feces. We found that RB is an appropriate marker for bait uptake studies of <60 days in black-tailed prairie dogs (*C. ludovicianus*) when used at concentrations <0.5% of bait mass dosed to deliver >10 mg RB per kg target animal mass. Whiskers with follicles provided the best sample for RB detection.

Key words: Plague, prairie dog, rhodamine B, vaccination.

Plague, a zoonotic disease caused by *Yersinia pestis*, is well established in wild rodents in western USA (Gage and Kosoy, 2005), causing high rates of mortality in prairie dogs (*Cynomys* spp.) and other species and a variety of collateral ecologic effects. Recently an oral plague vaccine developed for prairie dogs was shown to be highly effective in laboratory trials (Rocke et al., 2010). Baits for field use have been identified (Rocke, unpubl. data), and efforts to develop a vaccination program for select prairie dog populations are underway.

To evaluate the efficacy of oral plague vaccination, a biomarker would be useful to provide evidence of bait consumption in target animals, identify nontarget species that may also ingest baits, and determine optimal methods of bait distribution. Rhodamine B (RB) is an industrial and analytical xanthene dye previously used as a marker for ecologic and behavioral research (Lindsey, 1983; Fisher, 1999). Wildlife uses of RB include short-term (days to weeks) and long-term studies (months). Short-term effects (e.g., marking of the gut, feces, and urine) last for up to 1 wk (Lindsey, 1983; Fry et al., 2009). Longer-term effects include marking of claws, hairs, and whiskers (Fisher, 1999), manifested as fluorescent bands visible under a fluorescence microscope. Studies in mice indicate that RB deposits into the hair from systemic circulation within 5 min of dosage due to the dye's association with fibers forming in the hair follicle (Strout and Ruth, 1998). Fluorescent marking has been described in rodent species including mountain beavers (*Aplodontia rufa*) and pocker gophers (*Thomomys mazama*; Lindsey, 1983), coypus (*Myocastor coypus;* Fichet-Calvet, 1999), and house mice (*Mus musculus*; Jacob et al., 2002). The effect of RB on bait acceptance differs among species, but concentrations as high as 0.5% in bait have been successfully used in rodents (Jacob et al., 2002; Papillon et al., 2002). Our objective was to determine the effectiveness of RB as a marker for prairie dogs that consume baits intended for vaccine delivery.

The black-tailed prairie dogs (*Cynomys ludovicianus*, both sexes) used for this study were originally trapped in Colorado and South Dakota in 2007 ($n=15$) and 2009 ($n=10$). They were housed in captivity at the United States Geological Survey (USGS), National Wildlife Health Center (NWHC), Madison, Wisconsin, USA, on the floor in a 16.7-m^2 space; the project was approved by NWHC's Institutional Animal Care and Use Committee (IACUC; Protocol EP090803). Prairie dog diet (Exotic Nutrition, Newport News, Virginia, USA) was provided daily, and fresh vegetables (carrot and sweet potato)

were provided every other day. Water and timothy hay were available ad libitum. Bedding covered the floor, and custom-made, stainless steel nest boxes were used for shelter. A powdered bait formulation, previously demonstrated to be palatable to prairie dogs, was supplied by a commercial source (Animal Health Technologies, Abingdon, Virginia, USA). Baits made at the NWHC with this formulation were readily accepted by prairie dogs (Rocke, unpubl. data) and often given as treats. Concentrations of RB powder (Sigma-Aldrich, St. Louis, Missouri, USA) ranging from 0.05% to 1% (by weight of total bait) were mixed with this bait formulation at the NWHC. In a series of three trials, these RB baits were fed to prairie dogs to optimize concentration and dosage to ensure both palatability and marking.

The objective of the first trial was to determine if RB consumption (at concentrations and dosages established for other species) would mark prairie dogs. For bait consumption, prairie dogs were kenneled individually for no longer than 2 hr using small dog kennels to which they had been acclimated. Animals were weighed and one bait (mean weight 5.78 g; range 3.7–10.0 g) with RB concentration ranging from 0.45% to 1% (representing dosages of 10–34 mg RB/kg body weight) was offered to each animal. Two control animals received baits with no RB. After 2 hr, the animals were released, and any bait remaining was weighed. The dosage of RB consumed by each animal was calculated based on RB concentration of the bait, amount eaten, and the animal's weight. Two animals offered baits with 0.8% RB only ate a small portion (<1.5 g), and thus were offered a second bait with 0.5% RB. Animals were examined daily for signs of illness and weighed again approximately 1 wk later. No signs of illness were observed, and matched pairs analysis indicated no statistically significant differences in weights before and after consumption (mean difference: -7.0 g$\pm$8.0; t-ratio$=-0.88$; $P=0.21$).

To detect external marking, animals were examined under both natural light and a handheld long-wave (365 nm), ultraviolet (UV) light (Spectronics Corporation, Westbury, New York, USA). Fresh feces were collected from day 1 to day 12 postingestion (PI); one to three whiskers from each animal were cut from the base in this trial (or extracted in subsequent trials) and hair (both down and ward hair) from the back was extracted periodically until day 58 PI. Feces, hairs, and whiskers were examined under natural light and under the handheld UV light for RB staining and fluorescence. Hairs and whiskers were also observed under a fluorescence microscope (excitation wavelength: 540 nm, emission wavelength: 625 nm; Olympus CK40, Melville, New York, USA). Blood was obtained from four animals (0.5 ml, saphenous/femoral vein) on days 1, 3, 5, 7, 9, 12, and 16 PI. Blood was centrifuged and serum stored at -20 C. Serum samples were analyzed for RB fluorescence on a FlexStation 3 Microplate Reader (Molecular Devices, Sunnyvale, California, USA). Subsets of animals were euthanized from day 1 to day 58 PI following IACUC protocols.

During the first 24 hr PI, fluorescence of the front nails, maxillary incisor teeth, or fur around the mouth was detected under UV light in all 10 animals (Table 1). External marking after day 5 PI was seen in seven of 10 animals. Rhodamine B staining was also detected in feces under natural light on day 1 (10 of 10 animals) but not on day 3 (0 of 10 animals). The presence of RB was observed under UV light in feces from day 1 (10 of 10 animals) to day 3 PI (8 of 10 animals); RB was not detected in feces after day 5. The intensity of fluorescence in feces under the UV light visually decreased over time. Marking of whiskers was detected in all 10 animals, but the dye band was not observed until day 15 PI in some cases, with variations related to cut placement. In whiskers collected later the fluorescent band was farther from the root. By 58 days

TABLE 1. Number of black-tailed prairie dogs (*Cynomys ludovicianus*) fed rhodamine B baits (0.45–1%) that had marked feces as observed under natural and ultraviolet (UV) light, or external marking under UV light and marking of whiskers under fluorescence microscope, at various dosages and days postingestion (dpi).

		No. with marked feces					No. with external marking		No. with marked whiskers
		Under natural light		Under UV light					
n	Dosage (mg RB/kg body weight)	1 dpi	3 dpi	1 dpi	3 dpi	5 dpi	1 dpi	5 dpi	17–58 dpi
2	0	0	0	0	0	0	0	0	0
2	10–14	2	0	2	2	0	2	2	2
4	20–24	4	0	4	3	0[a]	4	2[a]	4
4	26–34	4	0	4	3	0	4	3	4

[a] One of the animals was not sampled on this day.

PI, the band was at or near the whisker tip. Hair analysis under the fluorescence microscope appeared reliable on days 1–2 PI. However, due to natural fluorescence seen in the distal one third of some hair samples, we elected not to use hairs for longer-term evaluation. Neither marking of feces or whiskers nor external marking was observed in controls.

Serum was collected from four animals to assess RB detection in blood. In all samples tested, RB fluorescence was detected only on days 1 and 3 PI. No fluorescence was detected in control sera. Data were insufficient to show a correlation between RB dose and detectable serum fluorescence.

In the second trial, the effect of RB concentration on bait palatability was tested using a cafeteria-style design. Prior to the trial, all five subject animals readily consumed baits without RB. Several days later, the same group-housed animals were offered baits (3–4 g) with 0.3%, 0.5%, and 0.8% RB on three trays (10 baits per concentration, 30 baits in total). Trays were monitored after 3 hr and after 24 hr. Baits with 0.3% RB were consumed readily, but consumption decreased with higher concentrations (Table 2). Baits with 0.8% RB were not consumed, as was observed during the first trial. Animals were euthanized at 36 hr PI. The front nails, maxillary incisor teeth, or fur around the mouth fluoresced under handheld UV light in all five animals. Rhodamine B staining was also detected in feces under natural and UV light in all animals. In this trial, whiskers were extracted, and fluorescence was detected in the follicles of both whiskers and hairs from all animals.

In the third trial, we aimed to establish the lowest dosage that would ensure marking using RB concentrations (0.05–0.3%) shown to be readily accepted by prairie dogs. Ten animals received 3-g to 4-g baits with dosages of 1.5–15.0 mg RB/kg body weight. Baits were eaten readily, and animals were euthanized at 36 hr PI. The front nails, maxillary incisor teeth, or fur around the mouth fluoresced under handheld UV light in all animals consuming >3 mg of RB/kg of body weight and in two of four animals that consumed dosages <3 mg RB/kg (Table 3). Staining was also detected in feces under natural light after dosages ≥3 mg/kg (six of six animals) and under UV light with a dosage as low as 1.5 mg RB/kg (three of four animals). In

TABLE 2. Palatability of 4-g baits with rhodamine B (RB) concentrations ranging from 0.3% to 0.8% in black-tailed prairie dogs. The experiment was conducted cafeteria-style, with all RB baits offered at the same time to five animals. Prior to the trial with RB baits, these five animals readily consumed baits without RB (10 baits within 3 hr).

		Baits consumed (*n*)	
RB concentration (%)	Baits offered (*n*)	Within 3 hr	Within 24 hr
0.3	10	6	10
0.5	10	1	4
0.8	10	0	0

TABLE 3. Effect of rhodamine B (RB) concentrations palatable to prairie dogs on marking of feces under natural and ultraviolet (UV) light, external marking under UV light, and marking of whiskers examined with a fluorescence microscope.

n	Dosage (mg RB/kg body weight)	% RB	Marked feces (*n*)		Animals with external marking (*n*)	Animals with marked whiskers (*n*)
			Under natural light	Under UV light		
2	0	0	0	0	0	0
4	1.5–2	0.05	0	3	2	0
2	3–4	0.1	2	2	2	0
2	8	0.2	2	2	2	2 (low)
2	12–15	0.3	2	2	2	2

animals consuming 8–15 mg RB/kg, dye was evident in whisker follicles within 36 hr PI, but not in whiskers from animals consuming <8 mg RB/kg.

We found that RB is an effective biomarker for prairie dogs for bait uptake studies of <58 days using concentrations <0.5% and dosages >10 mg RB/kg body weight. In evaluating bait uptake for plague vaccine trials, we recommend using 4-g to 5-g baits with about 0.35% RB to mark prairie dogs. Although external staining was visible in feces and on different parts of the body under UV light for up to 3 days PI, examining plucked whiskers (with follicles) using a fluorescence microscope provided the best means of detecting bait consumption in prairie dogs from 1 day to 2 days PI until at least 58 days PI.

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