

High prevalence of *Yersinia pestis* in black-tailed prairie dog colonies during an apparent enzootic phase of sylvatic plague

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Abstract Sylvatic plague (*Yersinia pestis*) was introduced into North America over 100 years ago. The disease causes high mortality and extirpations in black-tailed prairie dogs (*Cynomys ludovicianus*), which is of conservation concern because prairie dogs provide habitat for the critically endangered black-footed ferret (*Mustela nigripes*). Our goal was to help elucidate the mechanism *Y. pestis* uses to persist in prairie ecosystems during enzootic and epizootic phases. We used a nested PCR protocol to assay for plague genomes in fleas collected from prairie dog burrows potentially exposed to plague in 1999 and 2000. No active plague epizootic was apparent in the 55 prairie dog colonies sampled in 2002 and 2003. However, 63% of the colonies contained plague-positive burrows in 2002, and 57% contained plague-positive burrows in 2003. Within plague-positive colonies, 23% of sampled burrows contained plague-positive fleas in 2002, and 26% contained plague-positive fleas in 2003. Of 15 intensively sampled colonies, there was no relationship between change in colony area and percentage of plague-positive burrows over the two years of the study. Some seasonality in plague prevalence was apparent because the highest percentages of

plague-positive colonies were recorded in May and June. The surprisingly high prevalence of plague on study area colonies without any obvious epizootic suggested that the pathogen existed in an enzootic state in black-tailed prairie dogs. These findings have important implications for the management of prairie dogs and other species that are purported to be enzootic reservoir species.

Keywords *Cynomys ludovicianus* · Disease · Montana · Nested PCR · *Pla* gene

Introduction

Sylvatic plague is caused by the bacterium *Yersinia pestis*, which was introduced into North America from Asia about 100 years ago (Kartman 1970; Perry and Fetherston 1997; Gage and Kosoy 2005). Like other introduced diseases, plague poses a significant threat to native species, particularly those that are rare and endangered (McCallum and Dobson 1995; Thorne and Williams 1988; Woodroffe 1999; Daszak et al. 2000). Plague causes high mortality in black-tailed prairie dogs (*Cynomys ludovicianus*), and is a major factor in the range-wide decline of the species (Biggins and Kosoy 2001; Cully and Williams 2001). Fleas are the primary vectors of sylvatic plague and sociality of prairie dogs facilitates rapid spread of the disease between individuals and among colonies.

Plague has acted in concert with habitat conversion, poisoning, and recreational shooting over the last century to decrease the area occupied by black-tailed prairie dogs by 90–99%, which corresponds to a loss of approximately 40 million hectares of habitat (Anderson et al. 1986; Miller et al. 1994; Van Putten and Miller

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1999; Forrest 2005). Status of the black-tailed prairie dog is of considerable conservation concern for biologists working to maintain biodiversity because the species creates habitat or is prey for several rare and declining species (Miller et al. 1994; Kotliar et al. 1999; Miller et al. 2000). For example, the highly endangered black-footed ferret (*Mustela nigripes*) depends on black-tailed prairie dog colonies for its primary habitat, as do several non-endangered species, including mountain plovers (*Charadrius montanus*), burrowing owls (*Athene cunicularia*), and hawks (*Buteo* spp.) (Knowles et al. 1982; Thorne and Williams 1988; Seery and Matiatos 2000; Restani et al. 2001). Negative effects of plague on prairie dogs indirectly hamper conservation efforts of these species. Black-footed ferrets also suffer a direct effect of plague epizootics because they are highly susceptible to the disease (Williams et al. 1994).

Host species of sylvatic plague are classified as either epizootic (amplifying/susceptible) or enzootic (resistant/reservoir) (Lechleitner et al. 1968; Perry and Fetherston 1997; Gage and Kosoy 2005). In general, resistant hosts transmit the pathogen via fleas to susceptible hosts, thereby triggering an epizootic. It is during epizootic phases that declines in abundance of susceptible species like prairie dogs are observed. Colonies hundreds of hectares in size can be decimated by plague within only a few weeks (Lechleitner et al. 1968; Rayor 1985; Menkens and Anderson 1991; Cully and Williams 2001). In addition to the immediate demographic consequences, these population bottlenecks reduce long-term genetic diversity of prairie dog populations (Trudeau et al. 2004). During the enzootic or maintenance phase, however, there is no widespread mortality in susceptible species and spread of the disease appears highly restricted (Gage and Kosoy 2005).

Epizootic outbreaks of sylvatic plague are typically explained by the presence of resistant reservoir species, which maintain the disease in the environment and are the source of subsequent epizootics in susceptible hosts (Perry and Fetherston 1997; Cully and Williams 2001; Gage and Kosoy 2005). Plague can theoretically be retained in the environment without causing devastating epizootics in susceptible hosts (Keeling and Gilligan 2000; Davis et al. 2004), possibly by cycling within and between reservoir species that are not susceptible to the disease (Barnes 1982). Many common and widespread species are believed to be resistant to the disease, including most carnivores and some rodents (Lechleitner et al. 1968; Barnes 1982; Perry and Fetherston 1997; Biggins and Kosoy 2005). Diseases with purported reservoir hosts are not constrained by the density of their epizootic hosts, which extirpates

these populations, yet allows the disease to persist in the environment in enzootic hosts (Barnes 1982). Mitigating plague's effects on the prairie dog ecosystem is proving difficult due its unpredictable and recurrent nature and the distribution and abundance of both susceptible and resistant hosts (Davis et al. 2004).

Montana contains large areas important for conservation of black-tailed prairie dogs and their habitat associates. Colonies occupy an estimated 36,000 hectares, most of which are located in the north-central part of the state (Faunawest Wildlife Consultants 1999). Here colonies are separated by short distances and vary widely in overall size and population density. Our study area, the Fort Belknap Indian Reservation (FBIR), resides within this region and contains about 200 prairie dog colonies as well as reintroduced black-footed ferrets, mountain plovers, and burrowing owls. Fleas from prairie dog burrows on two colonies tested positive for sylvatic plague in 1999, a discovery which coincided with disappearances or significant declines of colonies in 1999 and 2000. Of the 8 flea species common to FBIR, about 70% of the flea fauna is composed of *Oropsylla hirsuta* and *O. tuberculata cynomuris* (Young unpublished data), which feed exclusively on prairie dogs (Cully and Williams 2001). *Pulex irritans* has a wide host preference and constitutes about 15% of the flea assemblage (Young unpublished data).

Collecting and testing fleas for the presence of *Y. pestis* is a potential tool in the surveillance of sylvatic plague. *Y. pestis* accumulates in the foregut of infected fleas (Perry and Fetherston 1997), and PCR-based methods provide a useful and sensitive means to detect the pathogen (Englethaler et al. 1999). We used a very sensitive PCR procedure to detect the presence of *Y. pestis* genomes within fleas collected from black-tailed prairie dog burrows on FBIR in 2002 and 2003. Our goal was to help elucidate the mechanism *Y. pestis* uses to persist in the prairie ecosystem during enzootic and epizootic phases by testing fleas collected from prairie dog colonies that had potentially been exposed to plague in 1999 and 2000.

Methods

Sample collection and DNA extraction

Between May and August 2002 and 2003, we collected fleas from burrows of 55 black-tailed prairie dog colonies on FBIR. We collected fleas by pushing a white flannel cloth into active prairie dog burrows (Ubico et al. 1988). Burrows were determined to be active by the presence of fresh droppings and diggings. Fleas

were removed from the cloth and stored in 3.6 ml cryotubes filled with 95% ethanol. We did not identify fleas to species and pooled all fleas sampled from a burrow on each sampling occasion for DNA extraction and plague assays. Sampled burrows were distributed throughout the extent of each colony, and we took a GPS location at each burrow. We sampled 36 colonies at least once during both years of study.

The perimeters of 15 colonies were mapped with GPS technology from 2001 to 2003 (Geoscience Associates 2003), and we extensively sampled these 15 colonies for fleas throughout 2002 and 2003: late May/early June, late June/early July, and late July/early August. We sought to associate any change in area of the prairie dog colony with seasonal prevalence of plague. Following convention, we used area of prairie dog colonies as an index to abundance (i.e., population size) (Miller et al. 1994; Van Putten and Miller 1999; Cully and Williams 2001; Forrest 2005). Study colonies were selected based on previous declines in size suggestive of past plague epizootics or their proximity to infected colonies.

We extracted DNA from whole fleas with the Easy DNA kit (Invitrogen Life Technologies, Carlsbad, CA). Manufacturer's protocols were followed in modified form for mouse tail extraction (protocol 8, Invitrogen Life Technologies) using 0.1 of the recommended volume. Fleas were washed in 0.15% saline solution, added to 1.5 ml micro-centrifuge tubes, and crushed with a closed 1000 μ l pipette tip. A chloroform extraction followed as described by the manufacturer. Supernatant was washed with 100 μ l of 100% ethanol (-20°C) and set on ice for 30 min. Centrifugation followed at maximum speed for 10 min at 4°C . We decanted ethanol and dried the resulting pellet in a vacuum centrifuge. The pellet was resuspended in 30 μ l of TE and stored at -70°C .

Bacterial strains and cultivation

We obtained *Y. pestis* strain A1122 var. *orientalis* from the Centers for Disease Control, Ft. Collins, CO. This strain lacks the 70-kb pCD1 virulence plasmid and is not pathogenic. *Y. pseudotuberculosis* (ATCC #29833) and *Y. enterocolitica* (ATCC #23715) were obtained from the American Type Culture Collection. Cultures were grown in Brain-Heart Infusion (BHI) broth with agitation at 37°C . We stored 1-ml aliquots from overnight broth cultures at -70°C . Counts were performed to determine total numbers of colony-forming units (CFU) in each stock culture. Total genomic DNA was extracted using Easy-DNA (Invitrogen Life Technologies) from $\sim 1.3 \times 10^7$ CFU *Y. pestis*, 4×10^9 CFU

Y. pseudotuberculosis, and 3.2×10^9 CFU *Y. enterocolitica*; CFU:bacteria ratio for *Y. pestis* is probably <1 due to the tendency of this organism to clump in broth medium. DNA was dissolved in 50 μ l tris-EDTA (TE, 10 mM tris-HCL pH 8.5/1 mM EDTA).

Nested PCR

A nested PCR, based on an assay described by Hinnebusch and Schwan (1993), was designed to amplify a 110-bp fragment of the plasmid-encoded *pla* (plasminogen activator) gene unique to *Y. pestis* (Sodeinde et al. 1988). A nested PCR protocol was used because the desired sensitivity could not be achieved with a single primer set. All PCRs were carried out using either a MJ Research PTC 200 Thermocycler or an Eppendorf Mastercycler Personal Thermocycler. The initial step was carried out using primers ypl3 and ypl4 (5'-3' catccggetcagcttattatgtacc, 5'-3' ctgtaggaagctcaacatccaag) modified from primers yp1 and yp2 described by Hinnebusch and Schwan (1993), in a 50 μ l reaction containing 1 Hotstart *Taq* Polymerase bead (Promega Taqbead 1.25 u/bead), $1 \times$ MgCl₂ free buffer, 0.2 mM dNTP, 1.5 mM MgCl₂, 1.0 μ M primers. Settings were 1 cycle at 95°C for 5 min, 30 cycles at 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min, 1 cycle at 72°C for 8 min. This initial PCR product was 460 bp in length. Nested PCR used primers ypl5 and ypl6 (5'-3' cacacctaagcceaagctcttggcg, 5'-3' gtggagattctgtctctattggcg). Nested PCR was carried out by using 5 μ l of product from the initial PCR step in a 50 μ l reaction containing the same concentration of reagents and subjected to the same conditions as the initial PCR step. Sensitivity and specificity of the assay were determined by amplifying 10- μ l templates of 10-fold *Y. pestis* DNA dilutions and 10 μ l 1:10 dilutions of *Y. enterocolitica* and *Y. pseudotuberculosis* DNA. PCR products were examined by electrophoresis on 1.5% or 2% TBE agarose gels stained with ethidium bromide. Gels were photographed with an electrophoresis documentation system (Kodak Electrophoresis Documentation and Analysis System 120). Amplification of the *pla* fragment from *Y. pestis* DNA extracted from fleas was carried out with 5 μ l of undiluted template. DNA extracted from *Y. enterocolitica* and *Y. pseudotuberculosis* and sterile water (5 μ l each) were used as negative controls, and DNA extracted from *Y. pestis* strain A1122 and diluted 10^{-6} (5 μ l) was used as a positive control in all PCRs.

Later, one negative and several positive flea DNA samples and the above-described positive and negative controls were reamplified to provide an example of assay results (Fig. 1); for this purpose we used 1 μ l of

template and electrophoresed the resulting amplicons on a 2% agarose gel.

Results

Y. pestis DNA diluted 10^{-1} through 10^{-6} yielded 110-bp products. In a later experiment, similar results were obtained with 1 μ l of the 10^{-6} dilution (Fig. 1), for an apparent sensitivity of ~ 0.3 CFU per sample (actual sensitivity is less than this because of bacterial clumping and the likely presence of multiple copies of the *pla*-encoding plasmid per *Y. pestis* genome). No products were amplified from *Y. enterocolitica* or *Y. pseudotuberculosis* at any concentration tested. These results confirm that the nested PCR used in this study is both specific and highly sensitive.

We sampled fleas from 55 colonies in 2002 and 2003, with 36 colonies sampled at least once both years (Table 1). Fig. 1 shows a sampling of *pla* amplicons from fleas collected in this study. Overall, 63% ($n = 30$ colonies) of colonies had at least one plague-positive flea sample in 2002, and 57% ($n = 24$ colonies) of colonies had at least one plague-positive flea sample in 2003. Plague-positive colonies were distributed throughout FBIR. For plague-positive colonies, 23% (mean \pm SE, 3.9 ± 0.5 plague-positive burrows/colony) of sampled burrows had plague-positive fleas in 2002, and 26% (2.3 ± 0.3 plague-positive burrows/colony) had plague-positive burrows in 2003.

We collected fleas from 15 colonies over 3 time periods in 2002 and 2003 (Table 2). Although percentages of plague-positive colonies varied among the 3 sample periods, plague was most detectable between late May and early July (Fig. 2). These results were also consistent with the entire dataset: for all

plague-positive burrows sampled in 2002, 72% were from late June/early July, and 72% of all plague-positive burrows sampled in 2003 were from late May/early June.

Of the 15 intensively sampled colonies, 4 colonies increased in area (ha) between 2001 and 2002, and between 2002 and 2003 (Table 3). Six colonies decreased in area between 2001 and 2002, and a different group of five colonies decreased in area between 2002 and 2003. No colony decreased in area both years. Of the 15 intensively sampled colonies, a regression of percentage change in colony area between 2001 and 2002 against percentage of plague-positive burrows in 2002 was nonsignificant ($r^2 = 0.207$, $F = 3.38$, $df = 14$, $P = 0.09$). A similar nonsignificant result was obtained for colony area changes between 2002 and 2003 ($r^2 = 0.028$, $F = 0.371$, $df = 14$, $P = 0.55$).

Discussion

Results of our study indicate that, under some conditions, reservoir species may be unimportant in maintaining sylvatic plague in an enzootic state in the prairie dog ecosystem and that some black-tailed prairie dogs may be enzootic hosts of the pathogen. First, an unexpected high percentage (57–63%) of prairie dog colonies tested positive for *Y. pestis*. Wide distribution and high prevalence of plague-positive fleas on FBIR was not an artifact of sampling because we collected fleas from dozens of colonies across a large geographic area (ca 50 km \times 80 km). Second, despite high prevalence of plague-positive fleas, there was no evidence of an ongoing epizootic, which would have been manifest in significant and consistent declines in the area of prairie dog colonies (Van Putten and Miller 1999; Cully and Williams 2001; Trudeau

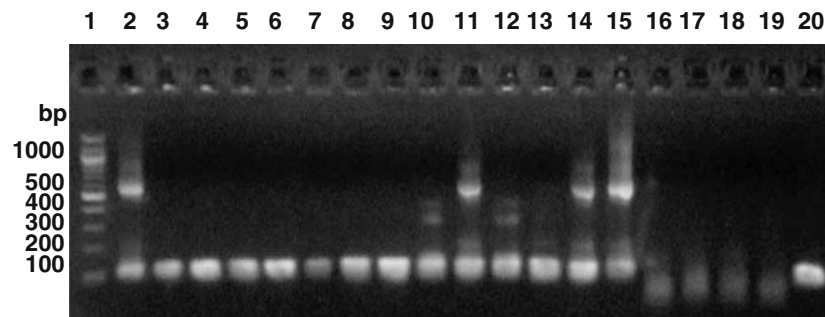


Fig. 1 Nested PCR results from selected flea samples. 1- μ l samples were amplified as described in Materials and Methods; 5 μ l each were electrophoresed on a 2% agarose gel. Lane 1: 100-bp ladder; Lanes 2–15: PCR-positive *Y. pestis* DNA; Lane 16: PCR-negative *Y. pestis* DNA; Lane 17: sterile water; Lane 18:

Y. pseudotuberculosis DNA (10^{-3} diluted, representing $\sim 8 \times 10^4$ CFU); Lane 19: *Y. enterocolitica* DNA (10^{-3} diluted, representing $\sim 6.4 \times 10^4$ CFU); Lane 20: *Y. pestis* strain A1122 DNA (10^{-6} diluted, representing ~ 0.3 CFU)

Table 1 Sampling effort of flea collection and results of PCR-based sylvatic plague assays from black-tailed prairie dog colonies on the Fort Belknap Indian Reservation, Montana, 2002 and 2003. Results are mean ± SE

Year	Number of colonies sampled	Number of burrows sampled/colony	Number of fleas collected/colony	Number of fleas collected/burrow	Number of plague-positive colonies	Number of plague-positive burrows/colony
2002	48	10.9 ± 0.9	17.6 ± 1.9	1.5 ± 0.1	30	3.9 ± 0.5
2003	42	6.6 ± 0.6	8.5 ± 0.8	1.5 ± 0.1	24	2.3 ± 0.3

et al. 2004; Pauli et al. 2006). Prairie dog colonies fluctuated in size from 2001 to 2003, with some individual colonies both increasing and decreasing across years. Third, we collected fewer fleas from individual burrows than reported by previous studies which sampled fleas during ongoing epizootics (Lechleitner et al. 1968; Ubico et al. 1988; Cully et al. 2000). Low flea activity on FBIR provided further support that no epizootic occurred in 2002 and 2003. In sum, we failed to observe the widespread declines of colonies that would have been expected if reservoir species acted solely to maintain plague and if black-tailed prairie dogs were strictly an epizootic host.

Most studies implicate reservoir species as maintaining sylvatic plague in the prairie dog ecosystem between epizootics (Rayor 1985; Ubico et al. 1988; Menkens and Anderson 1991; Anderson and Williams 1997; Cully and Williams 2001). However, alternative mechanisms have been proposed for plague maintenance in other rodents and these mechanisms may pertain to black-tailed prairie dogs as well. For example, Keeling and Gilligan (2000) modeled plague persistence in rat metapopulations and reported that plague could exist enzootically even when 50% of the rat population was susceptible. Epizootics occurred when >80% of the rat population was susceptible. Enzootic maintenance of plague also occurs in Asian rodent populations such as great gerbils (*Rhombomys opimus*) where plague is native (Davis et al. 2004). These theoretical and empirical results suggest some parameters by which the disease can be maintained in

an enzootic phase within a susceptible rodent host such as the black-tailed prairie dog (see also Webb et al. 2006). Black-tailed prairie dogs might also exhibit greater resistance to the disease than is currently believed (Pauli et al. 2006). Although sylvatic plague has been in North America for only 100 years, some degree of resistance could evolve within decades (Altizer et al. 2003). This possibility exists for black-tailed prairie dogs because populations typically harbor high levels of genetic diversity (Chesser 1983; Daley 1992; Altizer et al. 2003).

Poor vector to host transfer of the disease is also a possible explanation for the high level of plague found in fleas at our site. Different species of fleas have been shown to vary in their ability to transmit the disease, with the highest level of transmission seen with the rat flea *Xenopsylla cheopis* (Burroughs 1947; Holdenried 1952). *O. hirsuta* and *O. tuberculata cynomuris* are the most common fleas inhabiting black-tailed prairie dog burrows at our site and may be important vectors. Although their ability to transfer plague has not been extensively studied, recent evidence from Hinnebusch et al. (1998) indicates that *Y. pestis* may not replicate to high enough numbers in *O. hirsuta* to readily create the “blocked” condition required for transmission. These investigators observed that while 48% of *O. hirsuta* fleas collected from a prairie dog colony after a plague outbreak were positive for *Y. pestis*, <2% carried sufficient numbers of bacteria to be considered “blocked.” This is consistent with our observation that larger numbers of fleas were PCR positive in this study

Table 2 Sampling effort of flea collection and results of PCR-based sylvatic plague assays from 15 intensively sampled black-tailed prairie dog colonies on the Fort Belknap Indian Reservation, Montana, 2002 and 2003. Results are mean ± SE

Sample period	Number of burrows sampled/colony	Number of fleas collected/colony	Number of fleas collected/burrow	Number of positive burrows/positive colony	Percentage positive burrows
May/June 2002	4.3 ± 0.5	10.6 ± 2.4	2.6 ± 0.5	1.0 ± 0.0	4.7
May/June 2003	3.3 ± 0.3	4.1 ± 0.5	1.3 ± 0.1	1.4 ± 0.3	28.0
June/July 2002	6.3 ± 0.9	8.8 ± 1.7	1.3 ± 0.1	3.7 ± 0.9	43.6
June/July 2003	3.1 ± 0.3	4.2 ± 0.8	1.5 ± 0.4	1.2 ± 0.2	12.8
July/August 2002	4.5 ± 0.5	5.3 ± 0.7	1.1 ± 0.04	1.7 ± 0.5	14.9
July/August 2003	1.9 ± 0.2	2.1 ± 0.3	1.0 ± 0.03	1.7 ± 0.2	34.5

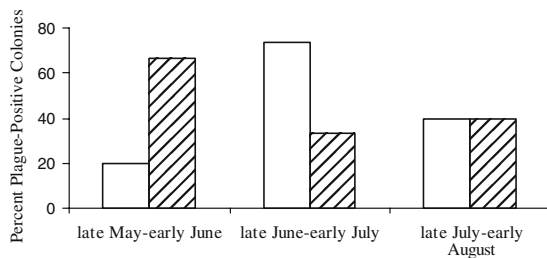


Fig. 2 Percentage of 15 black-tailed prairie dog colonies containing plague-positive fleas on the Fort Belknap Indian Reservation, Montana, 2002 (open bars) and 2003 (shaded bars)

than would have been predicted by the apparent overall health of the prairie dog colonies. Conditions under which transmission of plague begins are not understood but contributing factors likely include enhanced replication of *Y. pestis* in fleas (or higher levels of bacteremia in host animals, leading to uptake of more CFU in a blood meal [Hinnebusch et al. 1998]), greater flea infestation levels, and possible changes in susceptibility or resistance of the resident rodent population.

Although sylvatic plague did not appear to be intensifying or declining from 2001 to 2003 on FBIR, it appeared that some amplification had occurred seasonally. The late June/early July 2002 sample period showed a noticeable increase in the percentage of plague-positive burrows, whereas a similar increase occurred in late May/early June 2003. These effects were no longer apparent by late July/early August of both years. Thus, there may be as yet unknown sea-

Table 3 Area (ha) and percentage change in area of 15 intensively sampled black-tailed prairie dog colonies on the Fort Belknap Indian Reservation, Montana, 2001 to 2003

Colony	Area in 2001	Area in 2002	Percentage Change 2001 to 2002	Area in 2003	Percentage Change 2002 to 2003
85N	8	13	64	109	747
85W	30	16	-49	17	11
85WW	18	31	74	96	209
93	8	16	84	25	61
95	0	7	-	121	573
97S	4	26	515	3	-89
99	20	30	53	28	-9
99N	22	33	53	3	-90
100	52	100	91	17	83
101	14	40	186	8	-79
109	88	9	-89	84	796
111	48	34	-30	54	59
112	68	55	-19	56	2
126	17	4	-77	7	77
127	39	2	-95	3	33

sonal factors that participate in the dynamics of plague at our study site.

Sylvatic plague is the only disease known to threaten black-tailed prairie dog populations with high mortality and local extirpation (Anderson et al. 1986; Miller et al. 1994; Biggins and Kosoy 2001; Cully and Williams 2001; Pauli et al. 2006). *Y. pestis* is retained in the environment and can cause recurrent unpredictable epizootics, adding to the devastating nature of the disease on native biodiversity. The pathogen's reliance on reservoir hosts during enzootic periods is thought to be an important ecological aspect of the disease. Moreover, prairie dogs are thought to lack any immunity to the disease. Our highly sensitive PCR-based technique detected the pathogen in fleas collected from black-tailed prairie dog burrows during an enzootic phase. Thus, our technique could be used to screen currently healthy black-tailed prairie dog colonies that are being considered for black-footed ferret re-introductions and potentially to predict future plague epizootics in such colonies. Clearly, further basic research into the means by which sylvatic plague is maintained in the environment over enzootic periods is needed. Such research would also benefit conservation programs devoted to maintenance of black-tailed prairie dog populations and their habitat associates.

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