Detection of *Yersinia pestis* DNA in Prairie Dog–Associated Fleas by Polymerase Chain Reaction Assay of Purified DNA

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**ABSTRACT:** We evaluated, refined, and applied well-established polymerase chain reaction (PCR) techniques for detecting *Yersinia pestis* DNA in fleas (mainly *Oropsylla* spp.) collected from prairie dog (*Cynomys* spp.) burrows. Based on results from PCR of avirulent *Y. pestis* strain A1122 DNA, we used DNA purification and primers for the plasminogen activator gene to screen field-collected fleas. We detected *Y. pestis* DNA in flea pools from two black-tailed prairie dog (*Cynomys ludovicianus*) colonies with evidence of recent plague epizootics, and from one of four white-tailed prairie dog (*Cynomys leucurus*) colony complexes (Wolf Creek) where evidence of epizootic plague was lacking. Relative flea abundance and occurrence of *Y. pestis* DNA among flea pools appeared to vary over time at Wolf Creek. Both DNA purification and primer sequences appeared to influence the likelihood of detecting *Y. pestis* DNA by PCR in fleas collected from prairie dog burrows in the absence of observed epizootic plague. Presence of *Y. pestis* plasmid DNA in fleas collected from prairie dog burrows at Wolf Creek may represent evidence that infected fleas were somehow being maintained in that system between epizootics, consistent with the hypothesized enzootic maintenance of plague in prairie dog colony complexes elsewhere.

**Key words:** *Cynomys* spp., flea, *Oropsylla* spp., PCR, plague, polymerase chain reaction, prairie dog, *Yersinia pestis*.

*Yersinia pestis*, the etiologic agent of plague, infects a variety of rodent and lagomorph species virtually worldwide (Biggins and Kosoy, 2001). Since plague’s introduction into western North America, epizootics involving prairie dogs (*Cynomys* spp.) and other small mammal species have occurred regularly (Biggins and Kosoy, 2001; Cully and Williams, 2001; Gage and Kosoy, 2005). Plague epizootics cause dramatic reductions in prairie dog populations and have contributed to declines of these species and others that depend on prairie dogs as prey or landscape modifiers (Biggins and Kosoy, 2001; Cully and Williams, 2001; Augustine et al., 2008; Biggins et al., 2010).

The ecology of plague in North American ecosystems has been described as encompassing both epizootic and enzootic cycles of *Y. pestis* transmission (Biggins and Kosoy, 2001; Cully and Williams, 2001; Gage and Kosoy, 2005; Hanson et al., 2007; Biggins et al., 2010), with the former more extensively studied and thus more clearly understood. Fleas of several species appear to be the main plague vectors for prairie dogs (reviewed by Cully and Williams, 2001). During epizootics, bacterial loads in prairie dog fleas (*Oropsylla* spp.) reach $10^3$ to $10^7$ colony forming units (CFU) and *Y. pestis* can be detected readily by laboratory rodent inoculation and polymerase chain reaction (PCR; Pollitzer, 1954; Hinnebusch and Schwan, 1993; Anderson and Williams, 1997; Engelthaler et al., 1999; Engelthaler and Gage, 2000). Because of this relationship, fleas are often collected and tested to confirm suspected plague epizootics (Pollitzer, 1954; Hinnebusch and Schwan, 1993; Engelthaler et al., 1999).

Approaches for detecting and studying enzootic plague and the earliest phases of epizootics in prairie dog colonies also would be valuable to wildlife managers (Biggins et al., 2010). Here, we describe evaluation, refinement, and field application of established PCR techniques for detecting *Y. pestis* DNA in fleas as a potential tool for enzootic plague surveillance and control.

To establish *Y. pestis* PCR capability and optimize assay performance in our laboratory, we used a stock solution
containing DNA from a well-characterized, avirulent *Y. pestis* strain (A1122; Orientalis biovar, pigmentation locus negative; Jawetz and Meyer, 1943) and laboratory-raised fleas (*Xenopsylla cheopsis*). The stock DNA solution contained about 0.5 ng DNA/μl (1 ng DNA equivalent to about $2 \times 10^5$ CFU of *Y. pestis* strain A1122). Both the stock DNA solution and fleas were provided by the US Centers for Disease Control and Prevention (CDC; Fort Collins, Colorado, USA). We first assessed how DNA purification and primer selection affected PCR sensitivity.

To evaluate DNA purification, we prepared two sets of flea homogenates by placing individual fleas in four separate 2-ml microcentrifuge tubes containing 180 μl phosphate-buffered saline (PBS) and three sterile glass beads. Each tube in a set was spiked with 5 μl *Y. pestis* strain A1122 DNA solution undiluted or diluted $10^{-3}$, $10^{-4}$, or $10^{-5}$ in sterile water. Each tube was homogenized using the Qbiogene Fast Prep (Carlsbad, California, USA) at 5.0 m/sec for 20 sec. Flea homogenate samples were heated to 95 C for 10 min, then immediately centrifuged for 30 sec at maximum speed (13,000 rpm) to pellet flea tissue. We purified DNA from flea material from one dilution series of spiked flea homogenates using the Qiagen supplementary protocol for purification of total DNA from insects using the DNeasy blood and tissue kit (Qiagen, Valencia, California, USA).

To evaluate primer selection, we compared primers designed from *Y. pestis* genes encoding either plasminogen activator (*pla*; Sodeinde and Goguen, 1989; Hinnebusch and Schwan, 1993; Engelthaler et al., 1999) or capsular antigen fraction 1 (*caf1*; Galyov et al., 1990; Begier et al., 2006). Both primers have been used extensively by the CDC and others for plague detection (Begier et al., 2006). For *pla* PCR, we initially used primer sequences described by Engelthaler et al. (1999) and Begier et al. (2006) but found them insufficiently specific for use on purified DNA from field samples. We subsequently noticed differences in published sequences for the forward (*pla-f*) primer; the reason for deleting the guanine nucleotide from the 3’ terminus was unclear, but this deletion appeared to explain the compromised specificity. Thereafter, we used the original primer sequences for *pla* PCR (Hinnebusch and Schwann, 1993): forward (*pla-f*) ATCTTTCGCTGAGAAC, reverse (*pla-r*) CTTGGATGTTGAGCTTCCTA; product 478 base pairs. We used sequences reported by Begier et al. (2006) for *caf1* PCR: forward (*caf1-f*) ATACTGCAGATGAAAAATCAGTTCC, reverse (*caf1-r*) ATAAAAGCTTTATTTGATGAGGT; product 531 base pairs.

Our PCR protocol was modified from procedures originally described by Hinnebusch and Schwan (1993). For each PCR assay, 1 μl of each primer (*pla-f* and *pla-r* or *caf1-f* and *caf1-r*; 100 μM primer/μl), 18 μl deionized, distilled water, and 5 μl flea homogenate or DNA purified from flea material was combined in a 0.2-ml PCR tube containing a puReTaq Ready-To-Go PCR bead (illustra™, GE Healthcare Bio-Sciences Corp, Piscataway, New Jersey, USA) for a final volume of 25 μl. Each PCR bead contained 2.5 units puReTaq DNA polymerase, 10 mM Tris HCl, 50 mM KCl, 1.5 mM MgCl₂, 200 μM of each deoxynucleoside triphosphate, and stabilizers, including bovine serum albumin. In each assay, we also used positive control tubes with 5 μl *Y. pestis* A1122 DNA (equivalent to about $5 \times 10^5$ CFU) and negative control tubes with 5 μl sterile water with the reagents listed above; control tubes included no flea material. We amplified DNA in a PTC-100 thermal cycler (MJ Research, Waltham, Massachusetts, USA) with the following thermocycling program: initial denaturation at 94 C for 10 min, followed by 35 cycles of denaturing at 94 C for 1 min, annealing at 55 C for 1 min, and primer extension at 72 C for 30 sec. After the last cycle, primer
extension was continued at 72°C for 10 min. Samples were analyzed by electrophoresis on 2% agarose gels with ethidium bromide.

Both DNA purification and primer selection influenced apparent PCR sensitivity (Fig. 1). The pla PCR was about 10² times more sensitive than caf1 PCR when either flea homogenate or purified DNA was used and purifying DNA increased sensitivity an additional 10-fold (Fig. 1). We attributed the difference in apparent sensitivity between primers to the ~100-fold greater number of pla gene copies carried in the Y. pestis genome (Parkhill et al., 2001) and perhaps to a reduced number of caf1 target sequence copies from chromosomal integration of the pFra plasmid (Protsenko et al., 1991), but did not explore this further. Repeating these comparisons using 1 μl of flea homogenate, purified DNA from flea material, or Y. pestis A1122 DNA stock solution produced the same outcome.

Based on these findings, we used DNA purification and pla PCR to screen fleas for evidence of Y. pestis DNA. Assay methods for field samples were as above, except that we used brain-heart infusion broth instead of PBS and homogenized fleas with a TissueLyser II (Qiagen) set at 20 oscillations/sec for 1 min.

We collected fleas from six field sites for plague surveillance. In north-central Colorado, USA, we sampled black-tailed prairie dog (Cynomys ludovicianus) burrows at two sites (Pineridge, Soapstone Prairie) shortly after or during plague epizootics; prairie dogs were completely absent at Pineridge and plague had been confirmed there by the CDC, whereas the few live prairie dogs and carcasses at Soapstone Prairie suggested an epizootic was ongoing. We also sampled white-tailed prairie dog (Cynomys leucurus) burrows at four sites in northwestern Colorado (Coyote Basin, Little Snake, Snake John Reef, Wolf Creek) where recent epizootic plague had not been observed (Holmes, 2008) (Table 1). Sampling in north-central Colorado was opportunistic and targeted prairie dog colonies where epizootic plague had recently occurred; in contrast, sites in northwestern Colorado were stratified based on established prairie dog activity transects (Holmes, 2008) and randomly sampled using site-specific transects (D. Walsh and D. Martin, unpubl. data). At all sites, burrows were swabbed by placing a ~25×25-cm piece of white flannel cloth...
("flag") down the entrance using a 5-m pipe snake (modified from Ecke and Johnson, 1952). We swabbed each burrow twice using a different flag, placed each flag into a labeled, sealable plastic bag, and stored them at $-20\,^\circ\text{C}$ until processed. We also assessed prairie dog activity at sampled burrows using established methods (Biggins et al., 1993).

In the laboratory, we used sterile forceps to place fleas from each flag into a labeled, microcentrifuge tube containing 2% saline and stored them at $-70\,^\circ\text{C}$ until assayed. Except for some samples collected in 2007, we identified fleas to species using published keys (Hubbard, 1947; Furman and Catts, 1982; Lewis, 2002). Where fleas were not identified to species, we pooled by transect and burrow; fleas identified to species were separated by species, then pooled by transect and burrow. When $>10$ individual fleas occurred in a transect-burrow or species-transect-burrow collection, we divided them into multiple pools with $\leq10$ fleas each, except where noted. We homogenized fleas and screened purified DNA using $plaq\text{PCR}$ as above.

From the two sites where epizootic plague was confirmed or suspected, we detected $Y.\,pestis$ DNA in seven of 12 flea pools (1–19 fleas/positive pool) from Pineridge and 34 of 38 pools (1–10 fleas/positive pool) from Soapstone Prairie (Table 1). In addition, we detected $Y.\,pestis$ DNA in six fleas (all $Oropsylla\,hirsuta$) from two prairie dog carcasses found at Soapstone Prairie next to burrows that also yielded PCR-positive fleas; the CDC later cultured $Y.\,pestis$ from these carcasses.

The intensity of amplicon bands varied among samples (Fig. 2). Sequencing of DNA (Amplicon Express, Pullman, Washington, USA) recovered from strong $plaq\text{PCR}$ bands (Pineridge flea pool FTC-13 and Soapstone Prairie pools SB and SE) confirmed the presence of $Y.\,pestis$ plasmid DNA (GenBank M27820); however, only two of these three pools (SB,

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**Table 1.** Numbers of burrows or carcasses sampled and yielding fleas, and numbers of fleas and pooled flea samples tested for presence of $Yersinia\,pestis$ DNA using polymerase chain reaction (PCR). Samples were collected from prairie dog ($Cynomys$ spp.) burrows or carcasses in Larimer or Moffat counties, Colorado, USA, during 2007–08.

<table>
<thead>
<tr>
<th>Site</th>
<th>Prairie dog species</th>
<th>Epizootic (date)</th>
<th>Sampling date</th>
<th>Sample source</th>
<th>Number sampled (with fleas)</th>
<th>Number of fleas</th>
<th>Number of pools</th>
<th>Total PCR-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pineridge</td>
<td>black-tailed</td>
<td>yes (May 2007)</td>
<td>August 2007</td>
<td>burrow</td>
<td>133 (14)</td>
<td>39</td>
<td>12</td>
<td>7</td>
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<tr>
<td>Soapstone Prairie</td>
<td>black-tailed</td>
<td>yes (June 2008)</td>
<td>June 2008</td>
<td>carcass</td>
<td>2 (2)</td>
<td>6</td>
<td>6</td>
<td>6</td>
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<tr>
<td>Coyote Basin</td>
<td>white-tailed</td>
<td>no</td>
<td>May 2008</td>
<td>burrow</td>
<td>90 (16)</td>
<td>36</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>no</td>
<td>July 2008</td>
<td>burrow</td>
<td>90 (14)</td>
<td>26</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>no</td>
<td>September 2008</td>
<td>burrow</td>
<td>70 (11)</td>
<td>43</td>
<td>12</td>
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<tr>
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<td>burrow</td>
<td>70 (7)</td>
<td>45</td>
<td>12</td>
<td>0</td>
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<tr>
<td></td>
<td></td>
<td>no</td>
<td>September 2008</td>
<td>burrow</td>
<td>60 (6)</td>
<td>9</td>
<td>6</td>
<td>0</td>
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<tr>
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<td>no</td>
<td>August 2007</td>
<td>burrow</td>
<td>121 (44)</td>
<td>136</td>
<td>49</td>
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<tr>
<td></td>
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<td>burrow</td>
<td>60 (10)</td>
<td>24</td>
<td>11</td>
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<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>no</td>
<td>September 2008</td>
<td>burrow</td>
<td>61 (7)</td>
<td>14</td>
<td>7</td>
<td>0</td>
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<tr>
<td>Wolf Creek</td>
<td>white-tailed</td>
<td>no</td>
<td>August 2007</td>
<td>burrow</td>
<td>246 (132)</td>
<td>798</td>
<td>232</td>
<td>32</td>
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<tr>
<td></td>
<td></td>
<td>no</td>
<td>May 2008</td>
<td>burrow</td>
<td>529 (184)</td>
<td>640</td>
<td>289</td>
<td>5</td>
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<tr>
<td></td>
<td></td>
<td>no</td>
<td>July 2008</td>
<td>burrow</td>
<td>514 (188)</td>
<td>750</td>
<td>245</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>no</td>
<td>September 2008</td>
<td>burrow</td>
<td>527 (139)</td>
<td>544</td>
<td>167</td>
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SE) showed evidence of *Y. pestis* DNA when screened with *cafl* PCR.

Using the same PCR methods, we also detected *Y. pestis* DNA in flea pools from one of four northwestern Colorado sites in the apparent absence of epizootic plague (Table 1). Overall, 50 of 933 (5%; 95% binomial confidence interval [bCI] 4–7%) flea pools from Wolf Creek were PCR-positive. These PCR-positive flea pools represented 43 burrows located in 20 sampling transects scattered among 11 of the 21 Wolf Creek colonies sampled, including colonies where black-footed ferrets (*Mustela nigripes*) resided (Holmes, 2008).

Because our sample storage methods were not regarded as sufficient to maintain viable bacteria until PCR results became available, we did not submit Wolf Creek flea pools for *Y. pestis* culture. However, we confirmed presence of *Y. pestis* plasmid DNA (GenBank M27820) by sequencing DNA (Amplicon Express) recovered from four strong *pla* PCR bands (Wolf Creek flea pools M6, M38, M39, M40, M50) and one weaker *pla* PCR band (pool M38; Fig. 2A). We subsequently screened 46 *pla* PCR-positive purified DNA samples from Wolf Creek using *cafl* PCR; only 10 also were *cafl* PCR-positive, a pattern consistent with dilution series results.

Using purified DNA seemed to improve the sensitivity of *pla* PCR on flea pools collected from prairie dog burrows in the apparent absence of epizootic plague (Fig. 2). The number of fleas per pool (relative to the number carrying *Y. pestis* DNA) may have influenced PCR signal strength in direct assays of flea homogenate (Fig. 2B). Assaying 1 μl flea homogenate improved PCR signal strength for one of the five flea pools (M39) shown in Fig. 2B, but two others (M38, M50) remained negative. Hanson et al. (2007) detected PCR-positive fleas in 5–44% of sampled black-tailed prairie dog burrows in the absence of epizootic plague using extracted DNA in a nested assay. These observations suggest the possibility that the occurrence of *Y. pestis* DNA in prairie dog fleas in the absence of observable epizootic plague may be underreported when PCR data are derived from flea homogenates rather than purified DNA.

Occurrence of *Y. pestis* DNA among flea pools and relative flea abundance appeared to vary somewhat over time at Wolf Creek (Fig. 3A). Of 16 PCR-positive flea pools where species was known, 12 were pools of *O. hirsuta* (Fig. 3A). Positive *Oropsylla tuberculata* pools were detected only during May when this species was most abundant (Fig. 3A). The proportion of PCR-positive pools appeared similar for the two species: 12 of 457 *O. hirsuta* pools (2.6%; 95% bCI 1.4–4.5%) and four of 120 *O. tuberculata* pools (3.3%; 95% bCI 0.9–8.3%) were PCR-positive. Two positive pools with identified flea species outside the genus *Oropsylla* were from burrows that also yielded PCR-positive *Oropsylla* spp.
These patterns may suggest a mechanism wherein interspecies differences in *Y. pestis* transmission efficiency between *O. hirsuta* and *O. tuberculata cynomuris* (Hanson et al., 2007; Wilder et al., 2008) and seasonal and annual variation in their abundance (Wilder et al., 2008; Tripp et al., 2009; Fig. 3) influence shifts between enzootic and epizootic plague phases within white-tailed prairie dog colonies.
Individual cases of plague at Wolf Creek were confirmed by the CDC in a desert cottontail rabbit (Sylvilagus audubonii) found there in September 2007 and in a prairie dog carcass found in spring 2008, but we saw no evidence of large-scale prairie dog mortality in 2007 or 2008. During May–September 2008, the proportion of active burrows among those that yielded PCR-positive flea pools (4/14; 95% bCI 8–58%) tended to be lower than among those yielding PCR-negative pools (276/497; 95% bCI 51–60%; Fig. 3B); moreover, the overall proportion of active prairie dog burrows tended to be lower in 2008 than in 2007 (Fig. 3B). Although plague likely contributed to these patterns, the apparent decline in activity among burrows yielding PCR-negative fleas (Fig. 3B) suggested other factors also may have contributed. During April–August 2009, at least eight additional plague-positive prairie dog carcasses were encountered at Wolf Creek and overall prairie dog activity was dramatically lower than in prior years (D. Tripp and B. Holmes, pers. comm.). We conclude from these observations that our burrow flea sampling and PCR screening approach detected evidence of Y. pestis in August 2007 and spring 2008 in the apparent absence of (and perhaps prior to) epizootic plague in the Wolf Creek complex.

Our surveillance approach incorporating structured spatial sampling of prairie dog burrows, flea identification, and pla PCR screening of purified DNA from flea pools provided an effective means of detecting and monitoring plague activity in a white-tailed prairie dog colony complex. This approach also appears to be useful for monitoring plague in Gunnison’s prairie dogs (Cynomys gunnisoni; K. Griffin and A. Seghun, unpubl. data). Primer selection and DNA purification apparently can influence the likelihood of detecting Y. pestis DNA by PCR in fleas collected from natural systems in the absence of observed epizootic plague. The presence of Y. pestis DNA in fleas from scattered loci within the Wolf Creek complex well in advance of noticeable “plague activity” in prairie dogs suggests that infected fleas may somehow be maintained in that system between epizootics, consistent with the hypothesized enzootic maintenance of plague in prairie dog colony complexes elsewhere (Anderson and Williams, 1997; Cully and Williams, 2001; Hanson et al., 2007; Biggins et al., 2010).

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**LITERATURE CITED**


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