

2013

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Baeten, Laurie A.; Pappert, Ryan; Young, John; Schriefer, Martin E.; Gidlewski, Thomas; Kohler, Dennis; and Bowen, Richard A., "IMMUNOLOGICAL AND CLINICAL RESPONSE OF COYOTES (CANIS LATRANS) TO EXPERIMENTAL INOCULATION WITH YERSINIA PESTIS" (2013). *USDA National Wildlife Research Center - Staff Publications*. 1443. https://digitalcommons.unl.edu/icwdm_usdanwrc/1443

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IMMUNOLOGICAL AND CLINICAL RESPONSE OF COYOTES (*CANIS LATRANS*) TO EXPERIMENTAL INOCULATION WITH *YERSINIA PESTIS*

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ABSTRACT: Multiple publications have reported the use of coyotes (*Canis latrans*) in animal-based surveillance efforts for the detection of *Yersinia pestis*. Coyotes are likely exposed via flea bite or oral routes and are presumed to be resistant to the development of clinical disease. These historic data have only been useful for the evaluation of the geographic distribution of *Y. pestis* in the landscape. Because the canid immunologic response to *Y. pestis* has not been thoroughly characterized, we conducted experimental inoculation of captive-reared, juvenile coyotes ($n=8$) with *Y. pestis* CO92 via oral or intradermal routes. We measured the humoral response to *Y. pestis* fraction 1 capsular protein (anti-F1) and found a significant difference between inoculation groups in magnitude and duration of antibody production. The anti-F1 titers in animals exposed intradermally peaked at day 10 postinoculation (PI; range=1:32 to 1:128) with titers remaining stable at 1:32 through week 12. In contrast, orally inoculated animals developed higher titers (range=1:256 to 1:1,024) that remained stable at 1:256 to 1:512 through week 6. No clinical signs of disease were observed, and minimal changes were noted in body temperature, white blood cell counts, and acute phase proteins during the 7 days PI. Gross pathology was unremarkable, and minimal changes were noted in histopathology at days 3 and 7 PI. Rechallenge at 14 wk PI via similar dosage and routes resulted in marked differences in antibody response between groups. Animals in the orally inoculated group produced a striking increase in anti-F1 titers (up to 1:4,096) within 3 days, whereas there was minimal to no increase in antibody response in the intradermal group. Information gathered from this experimental trial may provide additional insight into the spatial and temporal evaluation of coyote plague serology.

Key words: *Canis latrans*, coyote, plague, serology, *Yersinia pestis*.

INTRODUCTION

Plague (*Yersinia pestis*) is a flea-borne disease that continues to have endemic foci throughout the world. Human infection with this zoonotic disease is highly dependent on peridomestic exposure to infected animals or the fleas that infest them (e.g., Christie et al., 1980; Gage et al., 2000; Gould et al., 2008). Although plague is generally maintained in a sylvatic cycle between rodent species and their associated fleas, it can spill over and infect a variety of wild and domestic animals. There are substantial differences among species in their susceptibility to the

disease manifestations of *Y. pestis* infection. For example, infection is highly lethal in prairie dogs (*Cynomys* spp.) and black-footed ferrets (*Mustela nigripes*) and moderately virulent in cats (*Felis catus*; Rust et al., 1971), whereas canids (*Canis* spp.) are considered refractory (Rust et al., 1971) but can act as a source for disease transmission (Poland et al., 1973; Wang et al., 2011).

In recent years, coyotes (*Canis latrans*) and red foxes (*Vulpes vulpes*) have increased in number in urban environments of North America. These rodent hunters can acquire *Y. pestis* infection through the bite of infected rodent fleas or through

ingestion of infected rodents. Although there is no evidence of antemortem transmission from these canids, there are historic reports in which coyote carcasses have been linked to human plague cases (Poland et al., 1973; von Reyn et al., 1976). There is a clear potential for nondomestic canids to transfer infected fleas over significant distances (Mcgee et al., 2006; Boone et al., 2009) as compared with peridomestic rodents. Additionally, the increase in urbanization of wildlife enhances the risk of disease transmission to domestic dogs and cats, which, in turn, could result in increased exposure and disease in human populations (Gage et al., 2000; Gould et al., 2008).

Canids often survive infection with *Y. pestis* and produce detectable and specific antibody responses. They are, thus, considered useful sentinels of disease, and many animal-based surveillance programs conducted during the past century have been based on serologic data obtained from wild coyotes (e.g., Salkeld et al., 2006; Brinkerhoff et al., 2009). However, the mechanism of their infection, its frequency and timing, and the duration of an antibody response in these natural settings are difficult to measure. We seek to better describe the serologic and clinical responses of coyotes following controlled routes of oral and intradermal (ID) infection with *Y. pestis*. These data provide a baseline characterization for better understanding surveillance data in coyotes.

MATERIAL AND METHODS

Experimental animals

Juvenile, captive-reared coyotes ($n=12$; US Department of Agriculture—Animal and Plant Health Inspection Services [USDA-APHIS], Wildlife Services, Predator Field Station, Millville, Utah, USA) obtained at approximately 4 mo of age were housed in animal biosafety level 3 isolation at the Animal Disease Laboratory (Colorado State University, Fort Collins, Colorado, USA). Before transfer, coyotes were vaccinated with standard canine vaccines (Vanguard® Plus5/CV and Defensor®3; Pfizer Animal Health, New York, New York,

USA). Animals were housed individually in standard, elevated dog runs (1.2 m×2.1 m; 2.6 m²), four runs per room, each with a manufactured polyvinyl chloride (PVC) den box (53 cm diameter×56 cm height). Animals were provided water and commercial dry dog food ad libitum, supplemented with dead mice, peanut butter, eggs, fruit, rawhide, long bones, or pig ears as daily environmental enrichment. Clinical evaluations were made twice daily on each animal throughout the study to detect changes in behavior, food consumption, fecal output, and activity level. At approximately 5 mo of age, temperature recorders (Thermochron iButton®, Maxim Integrated Products, Inc., Sunnyvale, California, USA) were surgically implanted into the peritoneal cavity of all coyotes via a caudal left flank incision. All coyotes were treated with topical selamectin (Revolution®, Pfizer Animal Health, Madison, New Jersey, USA) at 6 mg/kg approximately 4 wk before inoculation. Coyotes used in the inoculation trials were stratified by litter (three litters) and then randomized into two inoculation groups. One animal from each inoculation group was humanely euthanized on day 3 and day 7 postinoculation (PI) to assess gross and microscopic lesions during early infection; all others were euthanized at the termination of the study. Control animals ($n=2$) were age-matched but maintained at the captive breeding facility in outdoor pens and euthanized at the termination of the study. The tissues collected during postmortem examination included all major internal organs (liver, lung, spleen, heart, kidney, and adrenal glands) and lymphoid tissues, including palatine tonsil, retropharyngeal lymph nodes (RPLN), submandibular lymph nodes (SMLN), bronchiolar or tracheal lymph nodes, and mediastinal, prescapular, axillary, popliteal, and mesenteric lymph nodes. Tissues were fixed in 10% neutral-buffered formalin for histologic examination or stored frozen at -80 C until further analysis. Histopathology descriptions are based on routine H&E staining and *Y. pestis*-specific immunohistochemistry (IHC; Guarner et al., 2002).

Yersinia pestis challenges

At approximately 6 mo of age, six animals were inoculated by ID injection (50 μ L) bilaterally in the prescapular region with a target dose of 10^3 colony-forming units (cfu) *Y. pestis* CO92. Bacteria for the inoculum were cultivated from frozen stock, grown in brain heart infusion (BHI) broth at 28 C for 48 hr, and diluted in phosphate-buffered saline to the desired concentration. The dose inoculated was determined by back titration of the

inoculum on BHI agar plates. The other six coyotes were offered one to three *Y. pestis*-infected mice for consumption. These 6-wk-old imprinting control region (ICR) mice were inoculated subcutaneously (SC) with 10^2 cfu of *Y. pestis* CO92 (50% lethal dose [LD₅₀] of approximately 20) in a 0.1-mL volume, and mice were euthanized by cervical dislocation once moribund (approximately 48 hr PI). At 14 wk PI, four coyotes from each inoculation group were rechallenged with *Y. pestis* using the same route and dosage as the original challenge. The coyotes were manually or chemically restrained for serial venous blood sample collection in gel serum separation and ethylenediaminetetraacetic acid tubes (Vacutainer®, Becton Dickson, Franklin Lakes, New Jersey, USA). Complete blood cell counts were performed with an automated hematology analyzer (HemaTrue Hematology Analyzer, Heska, Des Moines, Iowa, USA). Whole blood and selected tissues were cultured for *Y. pestis* using standard techniques (Chu, 2000). Briefly, whole blood was plated directly or diluted 1:5, and tissues (organs=1 g; lymph nodes=0.5 g) were manually homogenized in 1 mL of BHI broth followed by plating. Feces were cultured after 24 hr enrichment in buffered-peptone water. All samples were plated to the selective and differential media; cefsulodin-irgasan-novobiocin agar (Becton Dickinson) and incubated at 37 C with 5% CO₂ for ≥72 hr. Isolates were confirmed as *Y. pestis* based on characteristic colony morphology and Gram stain; biochemical confirmation was performed using Micro-ID® (Thermo Fisher Scientific, Lenexa, Kansas, USA).

Serology

Serum was harvested, heat inactivated at 56 C for 30 min, and stored at -80 C before analysis. Titers of antibody reactivity to *Y. pestis* fraction 1 capsular protein (anti-F1) were determined by passive hemagglutination as previously described (Chu, 2000). C-reactive protein (CRP) and serum amyloid A (SAA) values were determined by enzyme-linked immunoassay (ELISA) (PHASE™ Canine CRP assay and PHASE™ Serum Amyloid A multispecies assay, Tridelata Development Limited, Maynooth, Kildare, Ireland) according to manufacturer specifications.

Statistical analysis was performed using SAS 9.2 (SAS Institute, Cary, North Carolina, USA). Repeated-measures analysis of variance was used to determine differences among and between groups by means of Proc Mixed procedures. Unless otherwise noted, *P*-values of <0.05 were considered statistically significant.

RESULTS

All animals used in this study were antibody negative (anti-F1 titers <1:4) before inoculation with *Y. pestis*. No mortality occurred, and no appreciable signs of morbidity were noted during daily observations of the 12 coyotes PI. Food consumption and activity levels of the coyotes did not change from baseline observations throughout the study. *Yersinia pestis* was not isolated from blood cultures or feces collected between day 0 and day 7 PI. Complete blood cell counts were normal (Gates and Goering, 1976), except white blood cell counts (WBCs), which peaked at a mean (range) of $21.0 \times 10^3/L$ (20.5 – $21.5 \times 10^3/\mu L$) on day 2 PI in the group inoculated orally. Increases in WBCs were attributed to increases in absolute and percentage of granulocyte counts only.

Intra-abdominal body temperatures, as measured by iButtons, were recorded for each animal at 1- or 3-hr intervals throughout the study. Broad circadian rhythm variations were noted with preinoculation temperatures recorded between 36.1–39.9 C. High body temperatures were noted during high-stress conditions, such as manual restraint and husbandry manipulations. All these factors made assessment of febrile conditions in the coyotes difficult. However, comparison of PI average daily body temperatures compared with mean preinoculation values demonstrated a 0.6 C (0.3–0.9 C) increase on day 1 and 0.3 C (0.1–0.6 C) on day 2 PI for animals in the oral group. Although statistically significant ($P < 0.0001$ and $P = 0.02$, respectively), the clinical relevance is arguable. No statistical difference was noted in body temperatures taken from animals in the ID group between 1 and 7 days PI.

To assess the activation of the innate immune response, the acute phase proteins CRP and SAA were measured at 0, 2, 3, 5, and 10 days PI. The CRP and SAA were found to be markedly elevated from baseline in all of the animals in the group

exposed by ingestion. The highest CRP levels (>120 ng/mL) were noted on days 2 and 3 PI, with significant declines on day 5 and approaching baseline by day 10 PI. The SAA levels were >160 ng/mL on days 2 and 3 PI but were undetectable on days 0, 5, and 10. Slight increases in CRP above baseline (10–25 ng/mL) were detected in three of the six animals in the ID exposure group; however, SAA was not detected in this group at any time point.

Gross pathology was unremarkable on animals sacrificed at days 3 and 7 PI from each group. Lesions were not observed at ID inoculation sites. Superficial lymph nodes were not palpable. No gross lesions were noted in internal organs, and *Y. pestis* was not isolated from internal organ (spleen, liver, heart, kidney, and adrenal glands) homogenates. Of all of the lymphoid tissues examined, the palatine tonsils, SMLN and RPLN lymph nodes were slightly edematous on day 3 PI in the coyote that was inoculated by ingestion. However, *Y. pestis* was not isolated from any of the lymphoid tissues cultured, including palatine tonsils, RPLNs, SMLNs, and bronchiolar or tracheal, mediastinal, prescapular, axillary, popliteal, and mesenteric lymph nodes.

Histologic abnormalities were noted in the spleen, liver, lung, and cervical lymph nodes in the animals inoculated by ingestion. Mild lymphoid depletion was noted in the spleen of the day-3 animal inoculated by ingestion, and mild splenitis was noted in the day-7 animal. Mild hydropic degeneration of the central lobular regions of the liver was noted in the day-3 animal inoculated by ingestion. The lungs had small areas of focal, interstitial thickening in both animals. However, *Y. pestis* was not detected by IHC in lung tissues. Mild lymphoid hyperplasia with suppurative tonsillitis and mild suppurative lymphadenitis was noted in the palatine tonsil and SMLN of the day-3 animal inoculated orally. Compression of the subcapsular sinusoids was

noted in the RPLN and SMLN of the day-7, orally inoculated animal. The other lymph nodes were described as reactive or quiescently active (tracheal/bronchial, prescapular, axillary, popliteal, and mesenteric) and did not differ in presentation from that of the control animals.

Histologic changes were less pronounced in the animals inoculated ID. A mild splenitis was noted in the day-3 animal. Focal, alveolar, interstitial thickening was present in both the day-3 and day-7 animals but were negative for *Y. pestis* by IHC. The lymphoid tissue in the ID-inoculated animals were similar to those of the controls, except for the SMLN of the day-3 animal, which had focal areas of compression of the subcapsular sinusoids, but no significant accumulation of neutrophils was noted.

There were major differences in the magnitude of antibody responses between inoculation groups. Anti-F1 titers for animals in the ID exposure group had already reached their maximum levels by day 10 PI, ranging from 1:32 to 1:128 (Fig. 1). These antibody titers remained stable with a group median titer of 1:32 through wk 14 PI. In contrast, the orally inoculated animals had higher anti-F1 titers (1:256 to 1:1,024) that remained stable at 1:256 or 1:512 through wk 6. Between wk 6 and 14, there was a twofold decline in titers (Fig. 2).

Marked differences in antibody production were again noted between groups after rechallenge. Animals in the group exposed by ingestion produced a striking increase in anti-F1 titer of up to 1:4,096 within 7 days after rechallenge, and those antibody titers remained elevated for the next 4 wk (Fig. 2). In contrast, there was minimal to no increase in antibody response noted over 4 wk in the animals reinoculated by the ID route (Fig. 1). To further investigate the lack of an anamnestic response, the animals in the ID group were rechallenged a second time (4 wk after initial rechallenge) and again,

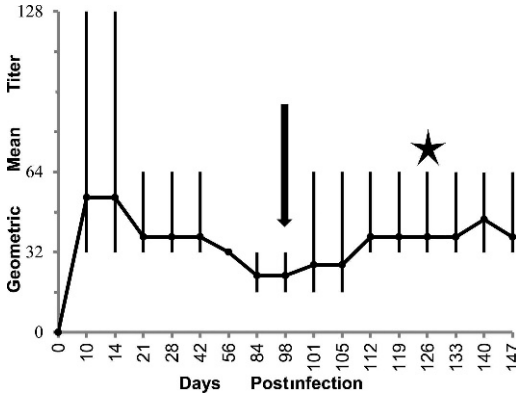


FIGURE 1. Geometric mean titers as measured by passive hemagglutination against *Yersinia pestis* F1 antigen for coyotes (*Canis latrans*) exposed intradermally to *Y. pestis*. Error bars represent the range of antibody titers for coyotes (n=4) in this group. The arrow designates rechallenge at 98 days postinfection. The star designates a second rechallenge at 126 days postinfection.

there was no change in anti-F1 titers over 2 additional wk (Fig. 1).

DISCUSSION

The serologic response to F1 capsule antigen in coyotes exposed by ingestion was similar to that described in previous studies using canids. Domestic dogs (*Canis lupus familiaris*) offered *Y. pestis*-infected rat viscera showed peak geometric mean titers (GMT) of 1:2,048 at day 10, which gradually declined to 1:256 by day 90 and subsequently remained stable through day 330 (Rust et al., 1971). In another study, a number of wild carnivores, including coyotes, were orally inoculated with *Y. pestis*, but inoculation dose and antibody titers were not reported, making it difficult to compare to other studies. Nonetheless, the timing of the antibody response reported was similar, with first detection at 8–14 days and a peak in titers between 20 days and 30 days that later declined to undetectable levels by 6–8 mo (Barnes, 1982).

Previously reported trials using oral inoculation have demonstrated that the probable route of entry for *Y. pestis* is via

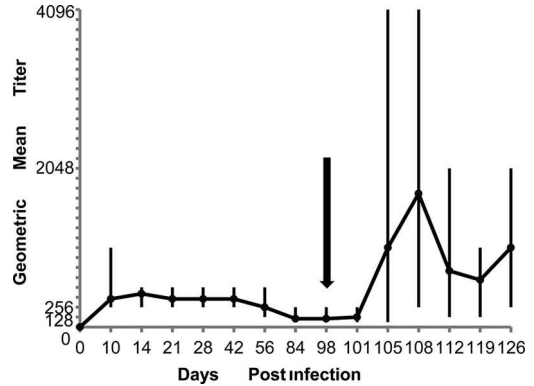


FIGURE 2. Geometric mean titers as measured by passive hemagglutination against *Yersinia pestis* F1 antigen for coyotes (*Canis latrans*) exposed orally to *Y. pestis*. Error bars represent the range of antibody titers for coyotes (n=4) in this group. The arrow designates rechallenge at 98 days postinfection.

palatine tonsil/pharyngeal exposure (Rust et al., 1972; Chen et al., 1976; Butler et al., 1982). In our study, individual variation in consumption of infectious material was observed; some mice were fully consumed whereas others were mouthed or partially ingested, and we speculate that some of the variability in antibody titer within the group may be due to differences in the dose and exposure to *Y. pestis* within the oral cavity. These results are similar to those presented for oral infection in swine (*Sus scrofa*) (Marshall et al., 1972) and raccoons (*Procyon lotor*) (Shepherd, 1973). This variable dose exposure may also explain the differences noted in antibody response between the coyotes in this study and the dogs infected via ingestion of infected rat viscera (Rust et al., 1971).

The low dose of organism inoculated ID in our study was designed to approximate a dose delivered by a single flea bite, and the amplitude of antibody production noted in these animals differed somewhat from previous studies. These differences were likely due to variations in route of exposure and dose of inoculum. Two dogs inoculated with *Y. pestis* SC at 10³ or 10⁷ cfu had reported peak GMT of 1:1,024, which persisted at 1:128 thru day 330

(Rust et al., 1971); further comparison to this study is limited because individual titers were not available. Domestic ferrets (*Mustela putorius furo*) inoculated SC with $\leq 10^3$ organisms failed to produce an antibody response to F1 (Williams et al., 1991). Likewise, a raccoon inoculated ID with 10^4 organisms produced a peak antibody titer of only 1:16 (Shepherd, 1973), and multiple rat (*Rattus*) species inoculated SC with serial dilutions of *Y. pestis* (10^2 to 10^7 cfu) failed to produce a consistent antibody response at doses $< 10^4$ cfu. In addition, rats in the same study lacked an anamnestic response after low-dose rechallenge, but mounted a significant antibody response with subsequent moderate- to high-dose rechallenges (Chen and Meyer, 1974).

In the present study, the ID route was chosen to mimic natural exposure via flea bite. Previous experimental inoculation studies used the SC route rather than an ID route, and little is known about the difference in pathogenesis or antigen presentation between exposure routes. The dose administered was similar to that reported in the literature for flea transmission (Jarrett et al., 2004; Lorange et al., 2005); however, the inoculum did not contain components of flea saliva which may have immunomodulatory effects (Andersen et al., 2007; Vadyvaloo et al., 2010). Recent microarray analyses compared the transcriptome of *Y. pestis* recovered from infected fleas with that of the organism grown in culture or recovered from infected rat lymph nodes. Differences in gene upregulation suggest that bacterial growth within the flea midgut results in characteristics that are different than in vitro cultures and in vivo propagation (Vadyvaloo et al., 2010). This is further characterized by the observation that a decrease in phagocytosis of *Y. pestis* by murine macrophages and human neutrophils was noted after passage through a flea. These findings suggest that the flea midgut environment may prepare *Y. pestis* for its exposure to, and evasion of, the

mammalian innate immune response (Jarrett et al., 2004; Vadyvaloo et al., 2010).

The substantial variation in antibody titers noted between oral and ID inoculation groups is likely a reflection not only of route of exposure but also of differences in dose, and possibly *Y. pestis* characteristics related to cultivation (i.e., 28 C vs. 37 C). The inoculum used for ID exposure was grown at 28 C, and therefore, it is reasonable to assume that *Y. pestis* was presented to the immune system with hexa-acylated lipopolysaccharide, which likely triggers the TLR4 response and induces a localized proinflammatory response, both of which might result in the rapid clearance of the organism by an efficient, localized, innate immune response. To investigate the extent to which the lack of response in the ID group was dose related, animals in this group were rechallenged a third time; two animals were inoculated ID with 10^4 cfu and two animals were offered infected mice (data not shown). Those receiving a fourth ID inoculation at 10^4 cfu (approximating 10–20 flea bites), demonstrated a 10–20-fold titer increase, with peak titers reaching 1:512 and 1:1,024. Even more pronounced, but in keeping with high responses to oral inoculation, the two animals receiving a final oral exposure reached titers of 1:8,192 and 1:16,384 respectively. These findings do not specifically address effects of inoculation route but strongly suggest a dose-dependent antibody response.

The minimal change in histology, antibody titers, and acute phase proteins, suggests that the ID inoculation used in this study resulted in a localized proinflammatory response. It is suspected that *Y. pestis* experienced rapid local clearance by phagocytes within the dermis. Although antigen presentation did occur as noted by the production of anti-F1 antibody, *Y. pestis* was not recovered by culture or visualized in regional lymph nodes. Persistence of *Y. pestis* inoculated by naturally infected fleas in the dermis and its

associated evasion of the innate immune response may result in production of higher antibody titers.

Limitations of this study include lack of data to assess duration of antibody titers over multiple seasons. The assumption is that in endemic areas coyotes will have continued exposures throughout a season which is why emphasis was placed on rechallenge antibody titer measurements rather than duration of antibody persistence. This study had a small sample size per group, which included mostly males. Previous serologic assessments to *Y. pestis* in rodents and coyotes indicate that there may be differences in the level and duration of antibody production between sexes (Willeberg et al., 1979; Jones et al., 2000). Based on our results, further characterization of the potential effects of dose and flea-induced changes to *Y. pestis* on antibody production may be warranted. Nonetheless, our data provide a baseline from which to interpret serologic titers collected from free-ranging coyotes. These data may be helpful in refining the current epidemiologic models that use coyote serosurveillance data to predict human risk in plague-endemic areas (Lowell et al., 2009; Brown et al., 2011). High antibody titers should indicate that exposure to *Y. pestis* is likely recent or recurrent. However, further evaluation is necessary in the interpretation of low to moderate antibody titers to differentiate waning titers from low-dose exposures. In addition, these results suggest that the use of a standard fourfold or greater titer change between acute and convalescent titers to confirm canid *Y. pestis* infection may not be clinically sound. It is possible that isotype analysis of anti-F1 antibodies will enable better determinations of acute, convalescent, and distant-past infections in sentinel coyotes and better assessment of disease occurrence and associated human risk.

ACKNOWLEDGMENTS

We are grateful to Jeret Benson, Angela Bosco-Lauth, Paul Gordy, Elisa French, Airn

Tolnay, and Natalie Cleton, who provided assistance with animal care; Nicole Marlenee for technical assistance; James zumBrunnen for the statistical analysis; Sarah Bevins for the graphics; Christopher Paddock for the immunohistochemistry; Terry Spraker for his histologic expertise; and two anonymous reviewers for their helpful edits. The procedures used in the housing, handling, and sampling of coyotes were approved by the Institutional Animal Care and Use Committee at Colorado State University (protocol 10-1751). Funding for this project was provided by USDA-APHIS-WS-National Wildlife Disease Program and Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention and Biomedical Research Training Grant (T32 NRSA 7072-9). The authors extend special thanks to the USDA-APHIS-WS-NWRC-Predator Field Station for providing training, support, and the captive-bred coyotes used in this study. The opinions and assertions made in this report are solely those of the authors and do not represent those of the CDC or USDA-APHIS. Use of trade or product names does not imply endorsement by the US government.

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Submitted for publication 21 February 2013.

Accepted 19 April 2013.