

ORIGINAL ARTICLE

Prevalence of the Bacterium *Coxiella burnetii* in Wild Rodents from a Canadian Natural Environment Park

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Impacts

- *Coxiella burnetii* is a zoonotic bacterium that can cause Q fever that has been reported in agricultural settings, yet little is known regarding prevalence in wildlife.
- We detected relatively high prevalence in many species of rodents in a natural environment park.
- Ecotourists and park visitors may be at risk for acquiring Q fever.

Keywords:

Algonquin Provincial Park; *Coxiella burnetii*; reservoir species; small mammals; wildlife disease; zoonotic

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Summary

Zoonotic diseases impact both wild and domestic animal populations and can be transmitted to humans through close contact with animal species. Reservoir species acting as vectors are major traffickers of disease. Rodents contribute to the transmission of *Coxiella burnetii* although little is known about its prevalence in wild animal populations. DNA was extracted from genital swabs collected from woodland jumping mice, deer mice, Southern red-backed voles, Eastern chipmunks, North American red squirrels, as well as Southern and Northern flying squirrels collected from Algonquin Park, Canada. The presence of *C. burnetii* was determined through real-time PCR. All species sampled had some prevalence of infection, except Eastern chipmunks, indicating wild rodents in Algonquin Park are reservoirs for *C. burnetii*. Emerging zoonotic diseases are linked to increasing globalization. Contact amongst individuals increases as crowding, habitat loss and fragmentation increase within wild spaces. Parks often act as a last refuge for wildlife but may also be an important transmission zone of wildlife disease to humans. Investigations that attempt to discover wild reservoir species of zoonotic disease are critically important to understanding the risk of pathogen exchange between wild and human populations.

Introduction

Reservoir species are epidemiologically connected populations or environments where a pathogen can be maintained and passed to a target species (Cleaveland et al., 2001; Daszak et al., 2001, 2004; Blancou et al., 2005; Cutler et al., 2010). About 60% of emerging human pathogens are zoonotic; passed between animals and humans; and about 70% originated in wildlife populations (Daszak et al., 2001, 2004; Cutler et al., 2010). Currently, there are few field studies attempting to identify unknown agents that have the potential to develop into zoonotic diseases (Daszak et al., 2004), yet knowledge of potential zoonotic

pathogens and their transmission ecology in reservoir species populations is critical to preserving public health and livestock economies (Burgdorfer et al., 1963; Daszak et al., 2004; Cutler et al., 2010; Nituch et al., 2011) as well as preventing adverse effects on wildlife conservation (Cleaveland et al., 2001). *Coxiella burnetii*, bovine tuberculosis (*Mycobacterium bovis*) and Nipah virus are examples of zoonotic pathogens that have caused death and economic loss through the infection of humans and domestic animals (Cleaveland et al., 2001; Daszak et al., 2001; Brook and McLachlan, 2006).

Identifying corridors of transmission where pathogens have the ability to move directly between wild hosts and

humans without a domestic carrier is a rising concern for public health and management policies (reviewed in Aguirre and Starkey, 1994; Aguirre et al., 1995). Of particular interest is the interface between wildlife and humans that often occurs in the context of both international and local eco-tourism and jobs where humans can come in close contact with infected wildlife (wildlife research and disease management) (Bengis et al., 2004; Cutler et al., 2010; Cascio et al., 2011). Activities such as camping, hiking or engaging in outdoor sports in natural areas can result in exposure to a variety of zoonoses transmitted through water, parasites or aerosolization (Bengis et al., 2004; Cutler et al., 2010).

Underlying these issues related to pathogen exposure is the fact that variation in host behaviour can have profound consequences for pathogen transmission. For example, highly social species or species with promiscuous mating systems may be at particular risk to be carriers and vectors of parasites and pathogens (Altizer et al., 2003). Thus, risks for transmission of zoonoses to humans may be variable depending on the social behaviour of the host species.

Coxiella burnetii is a highly virulent (Sawyer et al., 1987; McQuiston and Child, 2002; Woldehiwet, 2004), obligate intracellular parasite of the bacterial order *Legionellales*, which include mostly pathogenic species that cause Legionnaire's disease, and other pneumonia (Kruszewska and Tylewska-Wierzbanska, 1993; Willems et al., 1993; Arricau-Bouvery and Rodolakis, 2005). *Coxiella burnetii* differs from other *Legionellales*, in that the bacterium shows more resistance to antibiotics and environmental stressors, such as desiccation, low pH and high temperature, UV radiation, sonication, pressure (>50 000 psi; 1 psi = 6.89 kPa) and oxidative stress (Baca and Paretzky, 1983; Norlander, 2000; McQuiston and Child, 2002; Woldehiwet, 2004; Arricau-Bouvery and Rodolakis, 2005; Raoult et al., 2005). As a result of its long-term environmental stability, extremely low infectious dose (ID₅₀ approaching one organism), aerosol infectious route, a history of weaponization by various countries and the ease of producing large quantities of infectious material, *C. burnetii* was classified as a category B bioterrorism agent (Madariaga et al., 2003). *Coxiella burnetii* infects a wide variety of vertebrate (horses, pigs, cows, goats, sheep, deer, mice, squirrels, rabbits, cats, dogs, camels, reptiles and birds; Baca and Paretzky, 1983; Woldehiwet, 2004) and arthropod hosts (particularly tick species; Willems et al., 1993; Kruszewska and Tylewska-Wierzbanska, 1993; McQuiston and Child, 2002; Woldehiwet, 2004; Kim et al., 2005; Hussein et al., 2008; Cutler et al., 2010). Rodents, in particular, may play a major role in the transmission from wildlife to domestic animals and humans (Burgdorfer et al. 1963; Yadav et al.,

1979; Baca and Paretzky, 1983). Domestic animals, both livestock and pets, are considered the main source of human infection (McQuiston and Child, 2002; Woldehiwet, 2004; Perugini et al., 2009). In humans, *C. burnetii* infection is diagnosed as Query fever, or Q fever, and is often transmitted through contact with domestic livestock (e.g. inhalation of contaminated aerosols, urine, faeces, milk or contact with infected livestock such as sheep, goats, and cattle; Burgdorfer et al. 1963; Enright et al., 1971; Baca and Paretzky, 1983; Berri et al., 2000). Q fever may be asymptomatic or present with acute symptoms including fever, myalgia, headaches and endocarditis (Baca and Paretzky, 1983; Willems et al., 1993; Woldehiwet, 2004; Perugini et al., 2009). In non-human animals, the disease is generally asymptomatic and referred to as coxiellosis (Aitken, 1989; McQuiston and Child, 2002; Woldehiwet, 2004). However, infection of female livestock may cause placental infection, abortion, still-born or weak offspring (Sawyer et al., 1987; Aitken, 1989; McQuiston and Child, 2002; Woldehiwet, 2004; Kim et al., 2005; Perugini et al., 2009).

Q fever has been of recent interest because of a series of outbreaks in Europe, most associated with domestic animals including goats (e.g. Karagiannis et al., 2009; Sprong et al., 2012). In particular, a recent outbreak of Q fever in the Netherlands has brought the threat of *C. burnetii* infection to the attention of public health officials (Van der Hoek et al., 2012). Infection of domestic and wild animals by *C. burnetii* is known to occur in Canada but its distribution, prevalence and transmission dynamics are not well known (Marrie et al., 1993). While *C. burnetii* has been reported in rodents, little is known regarding this pathogen and the risk of transmission in North American wildlife (but see Marrie et al., 1993). *Coxiella burnetii* is not controlled by public health or animal care regulations and is not reportable in most provinces, suggesting that occurrence could be higher than previously assumed (Lang, 1989). As part of a larger study examining bacterial infections of the reproductive tract in natural populations of rodents, we tested for the presence of *C. burnetii* and assessed its prevalence among several species of rodents in a highly visited natural environment park.

Methods

Study sites and species

Samples were collected during the summer 2009, from populations of Southern Red-backed voles (*Clethrionomys gapperi*), Northern flying squirrel (*Glaucomys sabrinus*), Woodland jumping mice (*Napaeozapus insignis*), Deer mice (*Peromyscus maniculatus*), Eastern Chipmunks (*Tamias striatus*) and North American red squirrels

(*Tamiascurius hudsonicus*) located along the Highway 60 corridor and on a fixed plot at the Wildlife Research Station (WRS) in Algonquin Provincial Park, Ontario (45°30'N, 78°40'W). Algonquin Provincial Park is large wilderness area heavily used by campers, backpackers and canoeists. The Park reports >350 000 camper nights and >200 000 day – use visitors in 2010 (Ontario Parks 2011). Sampling of rodent populations occurred along trap lines that were located in a variety of habitats; hardwood forests, mixed forests and low-lying bog areas. Southern flying squirrels (*Glaucomys volans*) were sampled from a separate population located northwest of Peterborough, Ontario, at the James McLean Oliver Ecological Centre (44°34'25"N, 78°29'36"W).

Field methods

At the WRS, North American red squirrels and Northern flying squirrels were sampled over 23 ha of mixed conifer and deciduous forest using Tomahawk Model 102 live traps (Tomahawk Live Trap Co., Tomahawk, WI, USA). Traps were set 40 m apart in a line of 10 traps, each equipped with a sheet of aluminium covering the top and sides and polyester bedding inside to discourage predators and to reduce exposure of trapped animals to inclement weather (Gorrell and Schulte-Hostedde, 2008). Traps were baited (10 g mixture of peanut butter and oats with a thin slice of apple), set at dawn 5–6 days each week and checked twice daily (early and late morning).

Woodland jumping mice, Meadow jumping mice, red-backed voles, Eastern chipmunks and Deer mice were trapped using sunflower seeds soaked in water and Sherman and Longworth traps (Rogers Manufacturing Co., Kelowna, BC, Canada) set at 10 m intervals in lines of 20 (two per site) on the ground along the Highway 60 corridor. Traps were set overnight once a week, and animals were processed early in the morning.

Southern flying squirrels were trapped throughout the 38-ha Oliver study area with Tomahawk model 102 live traps baited with sunflower hearts. Between 20 and 67 traps were placed on shelf brackets approximately 2 m high in trees, set at sunset, and checked between one and four times per evening.

Sample collection

Trapped individuals were transferred into a canvas handling bag to be sexed, aged, weighed (Pesola scale; mass \pm 0.1 g) and issued numbered ear tags (model 1005-1; National Band and Tag, Newport, KY, USA) for future identification. Genital swabs were collected using PurFybr polyester swabs (ultra fine, aluminium shaft, 0.25" diameter; PurFybr Inc., Munster, IN, USA). Wearing gloves, the

swab was removed from the packaging and directly rotated 20 times over the genital area. The tip of the swab was then cut directly into a sterilized Eppendorf tube (1.5 mL) and stored at -20°C for future analysis.

DNA extraction

DNA was extracted from the swabs using the Qiagen DNeasy Blood and Tissues Kit according to the manufacturer's protocols (Qiagen Inc., Mississauga, ON, Canada) with the following modifications as specified by the Animal Health Laboratory, University of Guelph. The initial buffer and proteinase-K were added directly to the tube containing the swab. The swab was left in the tube during incubation for 20 min at 56°C , and samples were vortexed (G-560; Scientific Industries, Bohemia, NY, USA) for 15 s every 10 min. The solution was removed from the tube, which was then centrifuged (accuSpin Micro 17; Thermo Fisher Scientific, Nepean, Canada.) at 17 000 g for 1 min to obtain any remaining solution. For the final elution, 50 μL of the final buffer was added, and the tube was vortexed and centrifuged for 1 min at 10 000 g. The final step was repeated, and the final eluent (100 μL) was transferred to a sterile 0.5-mL microcentrifuge tube labelled with the sample number and the individual's ear tag numbers and stored at -20°C . Total DNA yields were measured spectrophotometrically using a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA) to ensure sufficient DNA had been extracted.

Real-time PCR identification of *Coxiella burnetii*

A real-time PCR protocol for the detection of *C. burnetii* was obtained from Kim et al. (2005). The primers, targeting the IS1111 insertion sequences of *C. burnetii*, consisted of trans-f (5'-GGGTAAAACGGTGAACAACA-3'), trans-r (5'-ACAACCCCGAATCTCATTG-3') and an internal probe trans-p (5'-AACGATCGCGTATCTTTAACAGCGCTTG-3') (Sigma-Aldrich, Oakville, Canada). The probe was labelled with the reporter dye 5-carboxyfluorescein (FAM) on the 5' end and the quencher dye N', N', N', N-tetramethyl-6carboxyrhodamine (TMR) on the 3' end. The total q-PCR volume was 25 μL and consisted of 12.5 μL iQSupermix (Bio-Rad Laboratories, Hercules, CA, USA), 1.2 μL each of trans-f, trans-r and trans-p, 6.9 μL DEPC water and 2 μL DNA template. Real-time PCRs were run using the Chromo 4 Real-time PCR Detection System (Bio-Rad). The positive control was obtained from cow placental tissue; from an abortion confirmed as caused by *C. burnetii* (C_t value = 9.63, concentration 2.09×10^{10} copies/ μL ; Animal Health Laboratory, University of Guelph), and DEPC water was used in place of sample DNA as a negative control. Amplification was carried out with an initial

denaturation time of 10 min at 95°C, then 40 cycles of the following: 15 s at 94°C for denaturation, 30 s at 55°C for primer annealing and extension at 72°C for 30 s. Cycling was followed with a melt curve from 55 to 95°C to test the purity of the products. The presence and abundance of *C. burnetii* was confirmed in samples showing positive amplification by comparing positive signals to curves from of the *C. burnetii* control. A 10-fold dilution series of *C. burnetii* was made from 10¹⁰ to 10³ copies/μL. To confirm the qPCR product identity, a sub-set was sent for sequencing at Génome Québec at McGill University (Québec, Canada), and resulting partial sequences matched against the NCBI non-redundant database using BLASTn (Altschul et al., 1990).

Statistical analyses

Infection prevalence, 95% confidence intervals and pairwise Fisher's exact tests to compare the inter-specific prevalence of *C. burnetii* were performed using the software Quantitative Parasitology 3.0 (Reiczigel and Rózsa, 2005; Budapest). We adjusted α values using a sequential Bonferroni correction because of the series of pairwise comparisons (Rice, 1989). Intraspecific infection prevalence for individual sex and age was compared using Fisher's exact test from Statistica 9.0 (Statsoft Inc., Tulsa, OK, USA). Statistical significance was accepted at $\alpha = 0.05$.

Results

A total of 230 individuals from seven species (Woodland jumping mice, $N = 30$; deer mice, $N = 71$; Southern red-backed voles, $N = 18$; Eastern chipmunks, $N = 12$; North American red squirrels, $N = 63$; Northern flying squirrels, $N = 24$; Southern flying squirrels, $N = 12$) were tested for *C. burnetii* using real-time quantitative PCR (q-PCR). Total DNA yields varied between 2 and 67.5 ng/μL. Positive signals of *C. burnetii* were compared to standard curves of the *C. burnetii* control, indicating most infection levels were on the order of 10³ copies/μL of DNA. The presence of *C. burnetii* was confirmed from the positive samples (Fig. 1) with all samples that were sequenced matching *C. burnetii*. The closest sequence matches were clones and isolates from goats and human infections by *C. burnetii* from a recent unpublished study of South American animals (Mares-Guia et al., 2011).

Woodland jumping mice and deer mice had significantly higher prevalence than almost all species (Prevalence – 83.3% and 76.1%, respectively) (Fig. 2). *Coxiella burnetii* was not detected in eastern chipmunks. Prevalence between adult females, adult males, juvenile females and juvenile males was also compared within each species. In Woodland jumping mice, adult males had higher

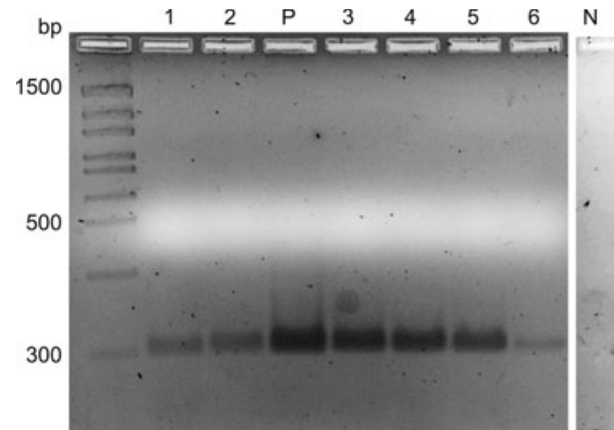


Fig. 1. Electrophoresis gel confirming the presence of *Coxiella burnetii*. qPCR products amplified from total DNA of rodent genital swabs. Lanes 1 through 6, total DNA from swabs, P, positive control (DNA of positive *C. burnetii* infection), N, negative control (lane image taken from separate gel). The bands represent the positive screen from samples containing fragments of the IS1111 sequence of *C. burnetii*.

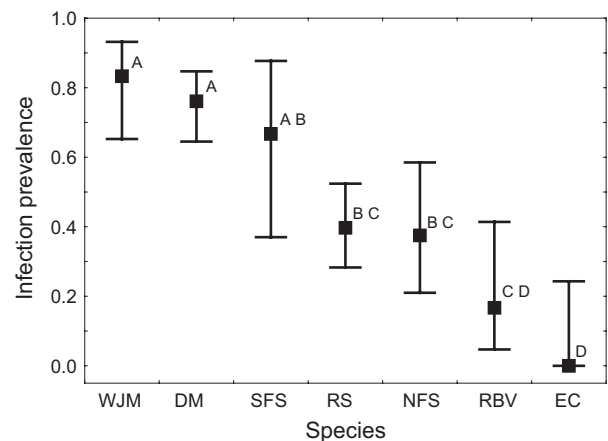


Fig. 2. Prevalence of infection with *Coxiella burnetii* of seven rodent species in Algonquin Park, Ontario, Canada (WJM – woodland jumping mouse ($n = 30$), DM – deer mouse ($n = 54$), SFS – southern flying squirrel ($n = 12$), RS – red squirrel ($n = 63$), NFS – northern flying squirrel ($n = 24$), RBV – red-backed vole ($n = 18$), EC – eastern chipmunk ($n = 12$)). Species with the same letter beside their whisker are not significantly different from each other based on Fisher's Exact Tests ($P > 0.05$). Error bars represent 95% confidence limits as calculated by software Quantitative Parasitology 3.0 (Reiczigel and Rózsa, 2005; Budapest).

rates of infection than adult females ($\chi^2 = 13.588$, $P < 0.001$). All other comparisons were non-significant.

Discussion

Our study provides evidence of *C. burnetii* infection across six of seven sampled rodent species. Variation in

prevalence among the seven species was wide, ranging from 0% to >80%. Prevalence varied across species but differed between sexes in only one case. Adult male woodland jumping mice had higher prevalence of infection than adult females, but otherwise no sex or age differences were detected. These results are in sharp contrast to Pluta et al. (2010) who found no evidence of *C. burnetii* infection in rodents in Germany but confirm an earlier study that positively detected *C. burnetii* in a small sample of deer mice and kangaroo rats in Utah, USA (Stoenner and Lackman, 1960). Given the resulting patterns from our study, two major issues become apparent. First, what is the basis for the species differences in infection prevalence among the rodents sampled? Second, is there a risk for transmission from these species to the visiting public in a heavily used natural environment park?

Species differences in infection prevalence could be related to different life history traits, particularly social and mating systems. *Coxiella burnetii* is transmitted through contact with aerosols, infected faeces and birth products or through a tick vector, routes that are more effective with frequent contact between individuals (Burgdorfer et al., 1963; Enright et al., 1971; Baca and Paretzky, 1983; Berri et al., 2000). *Coxiella burnetii* can also be transmitted sexually through sperm cells transferred to females (Kruszewska and Tylewska-Wierzbanska, 1993). Thus, species with more promiscuous mating systems could have higher a prevalence of infection, as infected individuals are more likely to come into contact with and infect previously uninfected individuals under this scenario (Lockhart et al., 1996).

No Eastern chipmunks were found to be positive for *C. burnetii*. As a solitary, strongly territorial species intolerant to conspecifics, except briefly during the mating season (Burt, 1972; Banfield, 1974; Forsythe, 1990) risk of exposure through conspecifics may be relatively low. However, chipmunks have a polyandrous mating system, which could contribute to the spread of infection, and thus, the lack of positives may be a result of low statistical power. Interestingly, there was no difference in *C. burnetii* prevalence between flying squirrels, red squirrels and red-backed voles, although their mating and social systems differ. Although similar to the eastern chipmunk in mating system and territorial behaviour, the red squirrel revealed significant infection prevalence (Burt, 1972; Banfield, 1974; Forsythe, 1990; Kruszewska and Tylewska-Wierzbanska, 1993). Northern flying squirrels have a promiscuous mating system but are more social. Group feeding and sharing winter dens to conserve heat could contribute to disease transmission between conspecifics (Burt, 1972; Banfield, 1974; Forsythe, 1990). Red-backed voles are also promiscuous, but are territorial and lead solitary lives during the summer and then aggregate in

shared nests during the winter to conserve heat (Burt, 1972; Banfield, 1974; Forsythe, 1990).

Promiscuous mating habits involving mating with multiple partners may also increase the risk of infection transmission. Deer mice and woodland jumping mice had the highest rates of infection. Both of these species are relatively social, tolerate conspecifics and use communal nests during the winter (Banfield, 1974; Forsythe, 1990). However, deer mice and woodland jumping mice have multiple mating periods and litters per year, while the other rodent species generally only have one or two litters per year (Burt, 1972; Banfield, 1974; Forsythe, 1990). Routes of infection vary across host–parasite relationships and are a major influence in disease prevalence. According to Aitken (1989), the virulence of *C. burnetii* decreases for cycles where ticks are not the main vector. Thus, if ticks are not the main source of infection for some species, the bacterium will be less virulent and may not pass between individuals as readily, decreasing the observed prevalence. Most transmission routes in small mammals are still unknown.

From a public health perspective, it is important to know whether tourists are at risk of acquiring Q fever from the wildlife that may carry the pathogen. In Algonquin Park, there have been no reports of Q fever (B. Steinberg, Algonquin Park biologist, Pers. Comm.), yet Q fever symptoms are often underreported in many jurisdictions in part because of the non-specific nature of clinical symptoms and the lack of clear guidelines in terms of reportage for individuals not associated with the agricultural industry (Lévesque et al., 1995; Comer et al., 2001; McQuiston and Child, 2002). Two factors would appear to be important with respect to a public health risk. First, the transmission of *C. burnetii* is varied, ranging from contact with urine or faeces, through contact with infected birth products or through infected arthropods such as ticks (Pluta et al., 2010; Sprong et al., 2012), although most infections occur through inhalation of infected aerosols or dust (Karagiannis et al., 2009; Sprong et al., 2012). Second, the encounter rate between tourists and infected hosts and their secretions would have to be high enough for elevated risk of infection to occur. Although many of the infected rodent species are nocturnal (e.g. woodland jumping mouse, northern and southern flying squirrel, red-backed vole) and would seem to be less likely to be encountered, tourists would be at risk if they encountered areas in which these animals have nested in or otherwise have left dried urine, faeces or other secretions. For example, species such as deer mice and flying squirrels are known to nest in human dwellings, which could lead to risks of acquiring Q fever by cabin dwellers (Reynolds et al., 2003). Similar risks were found with rodents infected with hanta virus and

tick-borne relapsing fever (Trevejo et al., 1998; Sinclair et al., 2007).

Thus, while there has been no direct evidence indicating Q fever infection because of interactions with wildlife, it is clear that little is known about the risk of transmission from wildlife in North America (McQuiston and Child, 2002). One particular group that may be at risk from wildlife is trappers, who are exposed to blood and viscera from the animals they trap (Lévesque et al., 1995). In addition, people using cottages or cabins that have been occupied by rodents including deer mice, flying squirrels and red squirrels may also be at risk. Given the relatively high prevalence of *C. burnetii* among many frequently encountered rodents, emphasis on reporting of Q fever should include visitors to wilderness parks and other wild areas. Q fever in humans is not nationally reportable (i.e. not under national surveillance) in Canada, but most provinces and territories of Canada have reporting protocols. Q fever is, however, an 'Annually Notifiable' disease in livestock. Annually notifiable diseases are diseases for which, if a laboratory identifies infection, Canada must submit an annual report to the World Organisation for Animal Health (OIE) indicating their presence within Canada (Canadian Food Inspection Agency – <http://www.inspection.gc.ca/english/anima/dise-mala/guidee.shtml> accessed December 12, 2011). Governments may wish to monitor eco-tourists and others that engage in outdoor recreation for evidence of Q fever, given the apparent prevalence in various rodent populations.

Risk of pathogen exchange within wild populations is elevated with increased crowding. Habitat loss and fragmentation lead to more frequent interactions and therefore increase pathogen exchange between individuals (Cleaveland et al., 2001; Daszak et al., 2001, 2004; Blancou et al., 2005; Cutler et al., 2010). At the community level, contact between species will also increase with higher density, leading to a higher probability that a pathogen will infect a novel species (Cleaveland et al., 2001; Daszak et al., 2001, 2004; Blancou et al., 2005; Cutler et al., 2010). Habitat alteration and land use changes (for example, ecotourism activities as part of a wilderness park) also alter wildlife population dynamics and migration patterns, increasing more potential contact between wildlife carrying zoonotic pathogens and domestic animals and humans (Cleaveland et al., 2001; Daszak et al., 2001, 2004; Blancou et al., 2005; Cutler et al., 2010). To understand the risks for humans that visit wilderness areas, a clearer understanding how transmission occurs between wildlife and humans is necessary. As eco-tourism continues to encroach on wilderness areas, the rate of contact between humans and wildlife will inevitably increase. Furthermore, agriculture farmland is often situ-

ated adjacent to protected park areas increasing the risk of transmission between wildlife and domestic livestock (reviewed in Aguirre et al., 1995). Future inquiries should attempt to determine the potential of transmission between wildlife populations and park patrons that use parks. Few specific investigations have been made on the wild reservoir species for *C. burnetii*, particularly in Canada. Thus, a better understanding of all the potential sources of the disease across a larger geographic range is necessary to understand the risks posed by wildlife for human infection. The potential virulence of these wild populations should also be targeted in future work to understand the risk for acute versus chronic infection by different strains; subsequent differentiation of *C. burnetii* genotypes isolated from rodent populations could be performed using rapid typing (Hornstra et al., 2011) to determine the pathogenicity of these unknown reservoirs of potential Q fever infection in wildlife areas.

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