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Detection of '*Candidatus* Ehrlichia khabarensis' in rodents and ticks removed from rodents in British Columbia, Canada

Muhammad G. Morshed[a,b], **Andrias Hojgaard**[c], **Min-Kuang Lee**[a], **Lynn M. Osikowicz**[c], **Lars Eisen**[c,*]

[a]British Columbia Centre for Disease Control Public Health Laboratory, Vancouver, B.C., V5Z 4R4, Canada

[b]Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, B.C., V6T 2B5, Canada

[c]Division of Vector-Borne Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, 3156 Rampart Road, Fort Collins, CO, 80521, United States

Abstract

'*Candidatus* Ehrlichia khabarensis' was first described from rodents and insectivores in the Far East territory of Khabarovsk on the Russian Pacific Coast. Here we report the detection of DNA from this microorganism in rodents and fed ticks collected from rodents in British Columbia, Canada in 2013–2014. '*Candidatus* Ehrlichia khabarensis' was detected in (i) a female *Ixodes angustus* tick collected from a *Peromyscus maniculatus*; (ii) a female *Dermacentor andersoni* tick collected from a *Perognathus parvus*; (iii) a pool of 2 larval *Ixodes pacificus* ticks collected from a single *P. maniculatus*; and (iv) a pool of 3 nymphal *I. pacificus* ticks collected from a single *P. maniculatus*. Three of these four rodents (2 *P. maniculatus* and 1 *P. parvus*) with infected ticks also had evidence of '*Candidatus* Ehrlichia khabarensis' in at least one tissue type. The infected *P. maniculatus* and *Ixodes* ticks came from the Vancouver area in western British Columbia and the *P. parvus* and *Dermacentor* tick from an inland site in central British Columbia. Although it remains to be determined whether '*Candidatus* Ehrlichia khabarensis' has any negative impacts on wildlife, domestic animals or humans, we note that all three tick species found to contain the DNA of this microorganism are known to bite humans. Future detection of this microorganism either in ticks collected from rodents and allowed to molt to the next life stage prior to being tested, or from host-seeking ticks, is required to determine if it can survive the tick's molt after being ingested via an infectious blood meal.

Keywords

British Columbia; '*Candidatus* Ehrlichia khabarensis'; *Dermacentor*; *Ixodes*; Rodent

[*]Corresponding author. evp4@cdc.gov (L. Eisen).

1. Introduction

Humans commonly experience tick bites in British Columbia, the westernmost province of Canada, and the Pacific Coast States of Washington, Oregon and California to the south in the United States (Aenishaenslin et al., 2017; Xu et al., 2019). One human-biting tick of special concern is the western blacklegged tick, *Ixodes pacificus*, which is the primary vector to humans in far western North America of causative agents of Lyme disease, *Borrelia burgdorferi* sensu stricto (s.s.), and anaplasmosis, *Anaplasma phagocytophilum*, as well as the suspected vector of the relapsing fever spirochete, *Borrelia miyamotoi* (Mak et al., 2010; Eisen et al., 2017; Xu et al., 2019). In the far west, *B. burgdorferi* s.s. and *A. phagocytophilum* are maintained in natural cycles involving *Ixodes* vector ticks and small mammal reservoirs, particularly rodents (Brown et al., 2006; Morshed et al., 2015; Eisen et al., 2017). We reported previously on the results of environmental surveillance in 2013–2014 for *B. burgdorferi* s.s. in ticks and rodents, predominantly deer mice (*Peromyscus maniculatus*), collected from 12 sites in southern British Columbia (Morshed et al., 2015). In that study, *I. pacificus*, *Ixodes angustus*, *Ixodes soricis* and *Dermacentor andersoni* ticks removed from rodent hosts were tested for presence of *B. burgdorferi* s.s. at the British Columbia Public Health Microbiology and Reference Laboratory. To confirm test results for *B. burgdorferi* s.s. and expand the testing to include two other notable rodent-associated and *Ixodes*-borne human pathogens in North America – *A. phagocytophilum* and *Babesia microti* – a subset of DNA extracts were re-tested at the United States Centers for Disease Control and Prevention (CDC), Fort Collins, Colorado (Morshed et al., 2015).

Moreover, as part of an ongoing effort at CDC to develop metagenomics-based capacity for broad detection of known pathogenic microorganisms in tick samples, leftover DNA extracts from ticks collected in 2013 were pooled and processed for metagenomics analysis broadly targeting genera of bacteria and protozoan parasites which include human pathogens carried by *I. pacificus* or *Ixodes scapularis* ticks in North America: initially *Anaplasma* (*A. phagocytophilum*), *Babesia* (*B. microti*) and *Borrelia* (*B. burgdorferi* s.s., *Borrelia mayonii* and *B. miyamotoi*), but subsequently expanded to also include *Ehrlichia* (*Ehrlichia muris eauclairensis*). Here we describe how this resulted in the detection in rodents and ticks collected from rodents in British Columbia of '*Candidatus* Ehrlichia khabarensis', which previously was reported from rodents (the northern red-backed vole, *Myodes rutilus*, and the grey red-backed vole, *Myodes rufocanus*) and an insectivore (the common shrew, *Sorex araneus*) in the Far East territory of Khabarovsk on the Russian Pacific Coast (Rar et al., 2010, 2015).

2. Materials and methods

2.1. Sample sources and DNA extraction

Ticks removed from rodents in our previous study in British Columbia, Canada were grouped by species and life stage recovered from an individual rodent on a given date (Morshed et al., 2015). In some cases this was a single tick whereas in other cases multiple ticks (up to 5) of the same species and life stage were grouped. As described previously (Morshed et al., 2015), single ticks or tick groups were homogenized and DNA extracted at the British Columbia Public Health Microbiology and Reference Laboratory using the

QIAGEN Blood and Tissue DNA Extraction Kit (Qiagen, Hilden, Germany). The initial part of the work presented here included 48 DNA extracts representing ticks removed from rodents in southern British Columbia in 2013. Overall, these 48 DNA extracts were based on 166 ticks of the following species and life stages: *I. pacificus*, 96 larvae, 42 nymphs and 2 adults; *I. angustus*, 5 larvae, 3 nymphs and 1 adult; *D. andersoni*, 6 larvae and 10 nymphs; and 1 adult *Ixodes* sp. tick. Subsequent work (see Section 2.4) included additional DNA extracts from ticks recovered from rodents as well as rodent tissues (blood, ear, kidney, liver, heart and spleen), similarly extracted at the British Columbia Public Health Microbiology and Reference Laboratory, using the QIAGEN Blood and Tissue DNA Extraction Kit.

2.2. DNA amplification of Anaplasma, Babesia, Borrelia and Ehrlichia, and subsequent sequencing

Subsamples (3 μl) from the 48 initial tick DNA extracts described in section 2.1 were combined into a single DNA extract pool. PCR primers were generated to amplify an approximately 900 bp DNA amplicon of *Borrelia* spp. and *Anaplasma* spp. *16S rDNA*, and an approximately 1100 bp DNA amplicon of *Babesia* spp. *18S rDNA* (Table 1). The PCR primers were chosen after aligning the *16S rDNA* sequence for *B. burgdorferi* s.s. (NC_001318), the *16S rDNA* sequence for *A. phagocytophilum* (CP000235) and the *18S rDNA* sequence for *B. microti* (AB243680), using the blast function on the United States National Institutes of Health website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). PCR reactions were performed in 50 μl with 25 μl 2x Sso Advanced (BioRad, Hercules, CA, USA), 5 μl tick pool extract, 2.5 μl primers (10 nM stock) and 17.5 μl H_2O. Cycling conditions consisted of 98 °C for 2 min to denature DNA followed by 40 cycles of 98 °C for 10 s, 55 °C for 10 s and 72 °C for 1 min, ending with a 5 min incubation at 72 °C, using a C1000 Touch thermal cycler (BioRad).

The sequencing library was generated using the Ion Xpress Plus Fragment Library kit (Thermo Fisher, Grand Island, NY, USA) and amplicon DNA from the single combined tick DNA extract pool described above. The DNA library was sequenced using the Ion PGM template OT2 200 kit, Ion 316 Chip and the Ion Torrent PGM system according to manufacturer protocols (Thermo Fisher). Processed sequences were imported into CLC Genomics Workbench (Qiagen, Valencia, CA, USA) where sequences were filtered, trimmed and contigs were built using the De Novo Assembly function. Although the selected primers were generated to amplify *Anaplasma*, *Babesia* and *Borrelia*, they also were found to amplify non-specific *16S*/*18S rDNA*, presumably representing other microorganisms present in the ticks.

Because *I. scapularis*, which is closely related to *I. pacificus*, is a vector of *E. muris eauclairensis* in the Upper Midwest of the United States (Pritt et al., 2017; Eisen and Eisen, 2018), we further analyzed the sequencing contigs for the presence of *Ehrlichia* spp. using the megablast function from the National Institutes of Health blastn suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This analysis indicted presence of *Ehrlichia* DNA within the large pool of tick DNA extracts, specifically a 785 bp contig (GenBank accession number: MK952145) with 100% identity to the *16S* ribosomal RNA gene of '*Candidatus* Ehrlichia khabarensis' strain m3 (KR063138).

2.3. Development of a PCR assay for detection of 'CandidatusEhrlichia khabarensis'

For further identification in tick or rodent samples of the *Ehrlichia* indicated by the metagenomics analysis, we employed a PCR assay targeting the *Ehrlichia groEL* gene and using previously described primers (Bell and Patel, 2005; Table 1). PCR reactions were performed in 20 μl with 10 μl Sso Advanced SYBR Green supermix (BioRad), 400 nM of each primer (forward and reverse), 5 μl of DNA sample and H_2O to a final volume of 20 μl. Touch-down PCR was performed on a LightCycler 480II (Roche, Indianapolis, IN, USA) with cycling conditions of 3 min at 98 °C to denature DNA followed by 45 cycles of 98 °C for 5 s, with touch-down annealing temperatures starting from 65 °C and decreasing 1 °C each cycle until 55 °C for 10 s, followed by 72 °C for 30 s. After 45 cycles of the touch-down PCR reaction, a melting curve analysis was performed with the following conditions: 95 °C for 1 min, 40 °C for 30 s and 70 °C for 30 s followed by an increase to 95 °C with a Ramp Rate of 0.02 °C/s and 25 acquisitions/°C. PCR products from the touch-down PCR were extracted from a 2% agarose gel using Freeze 'N Squeeze DNA extraction columns (BioRad) and sequenced using the BigDye terminator cycle sequencing kit (Thermo Fisher). For rodent tissues we performed the same PCR as described above except we did not include a melting curve analysis, but instead analyzed the PCR product on a 2% agarose gel and sequenced positive samples using the BigDye terminator cycle sequencing kit.

The primers described by Bell and Patel (2005) were originally designed to identify *A. phagocytophilum, Ehrlichia chaffeensis* and *Ehrlichia ewingii* in a Fluorescence Resonance Energy Transfer (FRET) PCR assay. The touch-down PCR assay described above identified a PCR product, from one (13MT101) of the initial 48 DNA extracts that made up the larger pool described in Section 2.1., with a melting point of 79.1 °C. This PCR product was distinct from that of control DNA generated from cultured *Ixodes*-borne pathogens: *A. phagocytophilum* USG3 strain (82.5 °C) and *E. muris eauclairensis* Wisconsin strain (80.0 °C). After isolating the PCR product with the melting point of 79.1 °C, we identified a product (GenBank accession number: MK956955) that is 351 bp long and has 99.1% identity (348/351) with the *groEL* gene of '*Candidatus* Ehrlichia khabarensis' strain m3 (KR063139). This melting curve analysis was subsequently used for detection of '*Candidatus* Ehrlichia khabarensis' in additional DNA extracts from ticks and rodent tissues as outlined in section 2.4.

2.4. PCR-based detection of 'CandidatusEhrlichia khabarensis' in ticks and rodent tissues

All tick DNA extracts screened for '*Candidatus* Ehrlichia khabarensis' with the PCR-based melting curve analysis described in Section 2.3 originated from our previous study on ticks infesting rodents in British Columbia (Morshed et al., 2015). Testing included (i) the 48 individual DNA extracts making up the larger pool described in Section 2.1 and (ii) 232 additional DNA extracts from ticks recovered from rodents. In some cases, DNA extracts were generated from a single tick whereas in other cases multiple ticks (up to 5) of the same species and life stage were grouped. Overall, the 280 DNA extracts tested for presence of '*Candidatus* Ehrlichia khabarensis' were based on 733 ticks of the following species and life stages: *I. pacificus*, 455 larvae, 187 nymphs and 11 adults; *I. angustus*, 17 larvae, 9 nymphs

and 2 adults; *I. soricis*, 1 nymph and 1 adult; *D. andersoni*, 33 larvae, 15 nymphs and 1 adult; and 1 adult *Ixodes* sp. tick.

As a follow-up, we also screened DNA from tissues of selected rodents for '*Candidatus* Ehrlichia khabarensis', as described in Section 2.3. In the case of four individual rodents (3 *P. maniculatus* and 1 Great Basin pocket mouse, *Perognathus parvus*) from which ticks infected with '*Candidatus* Ehrlichia khabarensis' were recovered, we tested all available tissue types: blood, ear, kidney, liver, heart and spleen. We also tested the livers, a productive tissue based on the report by Rar et al. (2015), from an additional 132 rodents (121 *P. maniculatus* and 11 *P. parvus*) that did not harbor infected ticks but were collected from or near to the same sites as rodents that did produce infected ticks (Burnaby Mountain, West Vancouver and Kelowna/Okanagan).

2.5. Phylogenetic analysis

All *groEL* sequences from the study were used in a BLAST (http://www.ncbi.nlm.nih.gov/blast) search of the NCBI GenBank database. A phylogenetic analysis of these *groEL* sequences and similar GenBank reference sequences, including other tick-borne *Ehrlichia* species known to occur in North America or elsewhere (Bakken and Dumler, 2015; Biggs et al., 2016; Cabezas-Cruz et al., 2016), was then performed with MEGA7 software (Kumar et al., 2015). The sequences were trimmed, aligned using the CLUSTAL W algorithm, and a tree was constructed using the Maximum Likelihood method. Reference sequences included in the phylogenetic analysis are shown in Table 2.

2.6. Regulatory compliance

As previously described by Morshed et al. (2015), the rodent tissues and ticks recovered from rodents used in this study came from animals captured and euthanized in accordance with animal care protocols approved by the University of British Columbia, Canada.

3. Results and discussion

Out of the 280 tested DNA extracts from ticks recovered from rodents, 4 (1.4%) were found to be positive for '*Candidatus* Ehrlichia khabarensis' (Table 3, Fig. 1). One of the positive samples represented a single female *I. angustus* tick collected from a *P. maniculatus* mouse trapped at the Burnaby Mountain site in western British Columbia in 2013. A second positive sample represented a single female *D. andersoni* tick collected from a *P. parvus* trapped at the Okanagan site in central British Columbia in 2014. The final two positive samples both represented *I. pacificus* ticks collected from *P. maniculatus* mice trapped in West Vancouver in western British Columbia in 2013: one of these samples represented 2 larval *I. pacificus* ticks and the other sample represented 3 nymphal *I. pacificus* ticks. Of the four rodents from which infected ticks were recovered, two of the *P. maniculatus* and the *P. parvus* had at least one tissue type testing positive for '*Candidatus* Ehrlichia khabarensis' (Table 4, Fig. 1). No individual tissue type was positive across all 3 infected rodents (Table 4). Preliminary screening of liver samples from an additional 132 rodents (121 *P. maniculatus* and 11 *P. parvus*) without evidence of infection in recovered feeding ticks, but collected from the same or nearby sites as the 4 rodents yielding infected ticks, did produce

another three positive samples, all from *P. maniculatus* mice (Table 4). Collectively, our data suggest that '*Candidatus* Ehrlichia khabarensis' has a wide geographic distribution in British Columbia and that *P. maniculatus* mice are commonly infected with this microorganism.

Further *groEL* sequencing and cluster analysis confirmed that the positive tissues and ticks recovered from the rodents clustered together with '*Candidatus* Ehrlichia khabarensis' strain m3 (KR063138) (Fig. 1). Positive samples from the West Vancouver and Burnaby Mountain sites in western British Columbia, whether rodent tissues or ticks collected from the rodents, had 100% sequence identity, whereas the positive rodent tissue and tick collected from that rodent from the Okanagan site in central British Columbia had a 3 bp mismatch (99.1% identity) when compared with the samples from western British Columbia.

To the best of our knowledge, we present the first record of '*Candidatus* Ehrlichia khabarensis' from British Columbia (and North America). However, a few previous studies have suggested that an uncharacterized *Ehrlichia* may be present in ticks or animals in British Columbia, including female *I. pacificus* ticks, wild ungulates and cattle (Magnarelli et al., 1995; Gajadhar et al., 2010; Lobanov et al., 2012). As '*Candidatus* Ehrlichia khabarensis' originally was described from the Far East territory of Khabarovsk on the Russian Pacific Coast (Rar et al., 2010, 2015), it is perhaps not surprising to find it along the Pacific Coast of Canada. Although it remains to be determined whether '*Candidatus* Ehrlichia khabarensis' has any negative impacts on wildlife, domestic animals or humans, we note that all three tick species (*D. andersoni*, *I. angustus* and *I. pacificus*) found to contain the DNA of this microorganism in British Columbia are known to bite humans (Merten and Durden, 2000; Xu et al., 2018). The detection of '*Candidatus* Ehrlichia khabarensis' in *I. pacificus* is of most concern as (i) this is the primary vector to humans of tick-borne pathogens in far western North America and (ii) the closely related *I. scapularis* can acquire, maintain and transmit another *Ehrlichia* species, *E. muris eauclairensis* (Karpathy et al., 2016; Lynn et al., 2017).

In this study, all ticks testing positive for '*Candidatus* Ehrlichia khabarensis' were removed from rodents and most of these rodents also showed evidence of infection in their tissues. Although it seems likely that the ticks acquired infection while feeding on the rodents, suggesting that *P. maniculatus* and *P. parvus* may serve as reservoirs for '*Candidatus* Ehrlichia khabarensis', we cannot rule out the possibility that the ticks already were infected prior to feeding. Moreover, detection of '*Candidatus* Ehrlichia khabarensis' either in ticks collected from rodents and then allowed to molt to the next life stage prior to being tested or from host-seeking ticks is required to determine if this microorganism can survive the tick's molt after being ingested via an infectious blood meal. Should this occur, then additional studies would be required to experimentally demonstrate transmission of '*Candidatus* Ehrlichia khabarensis' by the tick species of interest.

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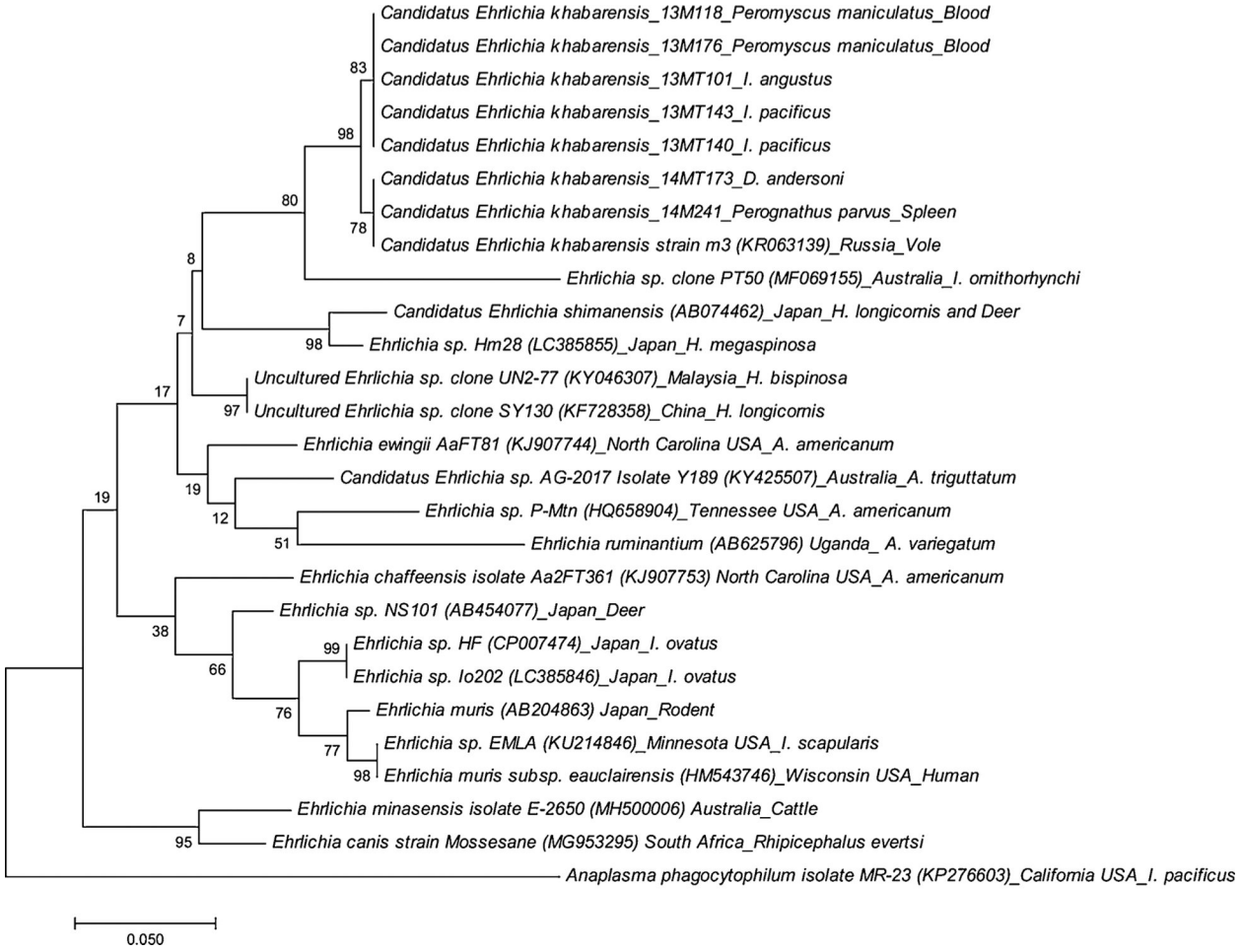

Fig. 1.

Phylogenetic relationships among *Ehrlichia* species and *Anaplasma phagocytophilum* derived from analysis of *groEL* gene sequences.

Table 1

Primers included in the study.

Primers	Target	Sequence 5′—3′	Reference
494-F	*16S/18S rDN*[a]	TGACGGGCRGTGTGTACAA	This study
495-R	*16S/18S rDNA*[a]	GTGCCAGCAGCCGCGGTAA	This study
Esp-F	*Ehrlichia groEL* gene	TACTCAGAGTGCTTCTCAATGT	Bell and Patel (2005)
Esp-R	*Ehrlichia groEL* gene	GCATACCATCAGTTTTTTCAAC	Bell and Patel (2005)

[a]Aligned sequence shared by *16S rDNA* for *B. burgdorferi* s.s. (NC_001318) and *A. phagocytophilum* (CP000235), and *18S rDNA* for *B. microti* (AB243680).

Table 2

Characteristics of reference *groEL* sequences included in the phylogenetic analysis.

GenBank accession number	Sequence Name	Source	Location
KR063139	'*Candidates* Ehrlichia khabarensis' strain m3	*Myodes rufocanus*	Khabarovsk Territory, Russia
HM543746	*Ehrlichia muris* subsp. *eauclairensis*	Human	Wisconsin, USA
AB204863	*Ehrlichia muris*	Rodent	Japan
KJ907753	*Ehrlichia chaffeensis* isolate Aa2FT361	*Amblyomma americanum*	North Carolina, USA
KJ907744	*Ehrlichia ewingii* isolate AaFT81	*Amblyomma americanum*	North Carolina, USA
MG953295	*Ehrlichia canis* strain Mossesane	*Rhipicephalus evertsi*	South Africa
MH500006	*Ehrlichia minasensis* isolate E-2650	Cattle	Australia
AB625796	*Ehrlichia ruminantium*	*Amblyomma variegatum*	Uganda
AB074462	'*Candidatus* Ehrlichia shimanensis'	*Haemaphysalis longicornis*	Japan
KU214846	*Ehrlichia* sp. EMLA	*Ixodes scapularis*	Minnesota, USA
HQ658904	*Ehrlichia* sp. P-Mtn	*Amblyomma americanum*	Tennessee, USA
KY425507	'*Candidatus* Ehrlichia sp. AG-2017 'isolate Y189	*Amblyomma triguttatum*	Australia
MF069155	*Ehrlichia* sp. clone PT50	*Ixodes ornithorhynchi*	Australia
CP007474	*Ehrlichia* sp. HF	*Ixodes ovatus*	Japan
LC385855	*Ehrlichia* sp. Hm28	*Haemaphysalis megaspinosa*	Japan
LC385846	*Ehrlichia* sp. Io202	*Ixodes ovatus*	Japan
AB454077	*Ehrlichia* sp. NS101	Deer	Japan
KF728358	Uncultured *Ehrlichia* sp. clone SY130	*Haemaphysalis longicornis*	China
KY046307	Uncultured *Ehrlichia* sp. clone UN2-77	*Haemaphysalis bispinosa*	Malaysia
KP276603	*Anaplasma phagocytophilum* isolate MR-23	*Ixodes pacficus*	California, USA

Table 3

Tick pools testing positive for '*Candidatus* Ehrlichia khabarensis'.

ID for tick DNA extract	Collection site[a]	Collection date	Rodent source of collected ticks			Ticks included in DNA extract			
			ID	Species	Sex	Species	No. larvae	No. nymphs	No. females
13MT101	Burnaby Mountain	15 June 2013	13M118	*Peromyscus maniculatus*	Female	*Ixodes angustus*	0	0	1
13MT140	West Vancouver	12 July 2013	13M176	*Peromyscus maniculatus*	Female	*Ixodes pacificus*	2	0	0
13MT143	West Vancouver	12 July 2013	13M179	*Peromyscus maniculatus*	Male	*Ixodes pacificus*	0	3	0
14MT173	Okanagan	30 July 2014	14M241	*Perognathus parvus*	Female	*Dermacentor andersoni*	0	0	1

[a]Site locations were described previously by Morshed et al. (2015).

Table 4

Rodent tissues testing positive for '*Candidatus* Ehrlichia khabarensis'.

Collection site[a]	Collection date	Rodent			Outcome for tested rodent tissue					
		ID	Species	Sex	Blood	Ear	Kidney	Liver	Heart	Spleen
Burnaby Mountain	15 June 2013	13M118	*Peromyscus maniculatus*	Female	Positive	Negative	Positive	Negative	Negative	Positive
West Vancouver	12 July 2013	13M176	*Peromyscus maniculatus*	Female	Positive	Positive	Positive	Positive	Positive	Negative
West Vancouver	12 July 2013	13M179	*Peromyscus maniculatus*	Male	Negative	Negative	Negative	Negative	Negative	Negative
Okanagan	30 July 2014	14M241	*Perognathus parvus*	Female	Negative	Negative	Negative	Negative	Negative	Positive
Burnaby Mountain	14 June 2013	13M106	*Peromyscus maniculatus*	Male	NT[b]	NT	NT	Positive	NT	NT
West Vancouver	23 July 2014	14M226	*Peromyscus maniculatus*	Male	NT	NT	NT	Positive	NT	NT
Kelowna/ Okanagan	30 July 2014	14M244	*Peromyscus maniculatus*	Male	NT	NT	NT	Positive	NT	NT

[a] Site locations were described previously by Morshed et al. (2015).

[b] NT, not tested.