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Original article

Detection of a novel *Rickettsia* (Alphaproteobacteria: Rickettsiales) in rotund ticks (*Ixodes kingi*) from Saskatchewan, Canada

Clare A. Anstead, Neil B. Chilton*

Department of Biology, University of Saskatchewan, Saskatoon, SK, Canada S7N 5E2

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ABSTRACT

A novel *Rickettsia* was detected in the rotund tick, *Ixodes kingi* Bishopp, 1911, based on comparative DNA sequence analyses of 4 genes; the rickettsial-specific 17-kDa antigen gene, citrate synthase gene (*gltA*), the outer surface membrane protein A gene (*ompA*), and the 16S rRNA gene. The rickettsiae in *I. kingi* differed in nucleotide sequence from those of other *Rickettsia* species by 5.8–18.3% for the 17-kDa gene, 0.9–13.9% for *gltA*, 5.5–22.8% for *ompA*, and 0.9–1.6% for the 16S rRNA gene. Phylogenetic analyses of the sequence data revealed that this putative new species of *Rickettsia*, provisionally named *Candidatus* Rickettsia kingi, does not belong to the spotted fever group or typhus group of rickettsiae, but represents a sister taxon to *R. canadensis* and *Candidatus* Rickettsia tarasevichiae. This novel *Rickettsia* was found in 60 of the 87 (69%) ticks examined, which included all feeding life cycle stages of *I. kingi*. Although adult *I. kingi* occasionally parasitize dogs and humans, it remains to be determined if this *Rickettsia* is pathogenic to these host species.

Introduction

Rickettsiae are obligate intracellular bacteria that are transmitted to vertebrates by arthropod vectors that include ticks, fleas, lice, and mites (Fournier and Raoult, 2009; Merhej and Raoult, 2011). These Alphaproteobacteria are the causative agents of disease (e.g., spotted fever and typhus) in many parts of the world (Fournier and Raoult, 2009; Merhej and Raoult, 2011). There are at least 30 recognized species of *Rickettsia*, 19 of which are considered human pathogens (Merhej and Raoult, 2011). A number of other putative species of *Rickettsia* have also been proposed based on sequence differences in 2 or more genes (e.g., Almeida et al., 2011; Izzard et al., 2009; Pacheco et al., 2011; Phan et al., 2011; Shpynov et al., 2003). Historically, the genus *Rickettsia* has been divided into the spotted fever group (SFG), the typhus group (TG), *R. canadensis*, and *R. bellii* (Fournier and Raoult, 2009). The SFG contains the majority of species within the genus, while *R. typhi* and *R. prowazekii* are the members of the TG (Fournier and Raoult, 2009). The TG is associated primarily with lice and fleas, whereas *R. canadensis*, *R. bellii*, and the SFG (except for *R. akari* and *R. felis*) use ixodid ticks as vectors (Fournier and Raoult, 2009; Merhej and Raoult, 2011).

In North America, at least 6 species of *Ixodes* (i.e., *I. scapularis*, *I. pacificus*, *I. cookei*, *I. dentatus*, *I. brunneus*, and *I. texanus*), all of which are known to parasitize rodents (Allan, 2001; Bishopp and Trembley, 1945; Kolonin, 2007), have been shown to contain SFG rickettsiae (Allan, 2001; Anderson et al., 1986; Billings et al., 1998; Clifford et al., 1969; Magnarelli et al., 1985; Phan et al., 2011). The rotund tick, *Ixodes kingi*, is also a common parasite of rodents (i.e., murids, heteromyids, geomyids, and sciurids), as well as other vertebrates, in western North America (Allan, 2001; Bishopp and Trembley, 1945; Gregson, 1971; Salkeld et al., 2006). Although rotund ticks are known to be vectors of several pathogens, including *Coxiella burnetii*, the causative agent of Q fever, and *Francisella tularensis*, the causative agent of tularemia (Sidwell et al., 1964; Thorpe et al., 1965), there are no published reports of rickettsiae in *I. kingi*. In this paper, we report the discovery of a new species of *Rickettsia* in all feeding life cycle stages of *I. kingi* from a locality in central Saskatchewan, Canada.

Materials and methods

For this study, a total of 87 *I. kingi* (3 females, 1 male, 2 nymphs, and 81 larvae) were collected from northern pocket gophers (*Thomomys talpoides*) trapped near Clavet, Saskatchewan (Anstead and Chilton, 2011). Total genomic (g) DNA was extracted from each tick as described by Dergousoff and Chilton (2007). The presence of rickettsiae in ticks was determined by nested (n)-PCR targeting a 434-bp fragment of the rickettsial-specific 17-kDa antigen

* Corresponding author at: Department of Biology, University of Saskatchewan, 112 Science Place, Saskatoon, SK, Canada S7N 5E2. Tel.: +1 306 966 4407; fax: +1 306 966 4461.
E-mail address: neil.chilton@usask.ca (N.B. Chilton).

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gene using primers 17k-5 and 17k-3 (first phase) and then primers 17KD1 and 17KD2 (second phase) (Heise et al., 2010). All PCRs were conducted in 25-μl volumes with 2 μl of gDNA template used in phase 1, and 1 μl of purified amplicon (using the protocol of Dergousoff and Chilton, 2012) from phase 1 used as the template for phase 2. Negative (i.e., no gDNA) controls were included in each set of reactions. The PCR conditions used were: 95 °C for 5 min, 35 cycles of 95 °C for 60 s, 58 °C for 60 s, and 72 °C for 60 s, and a final cycle of 72 °C for 5 min (for phase 1), and 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 30 s, and a final cycle of 72 °C for 5 min (for phase 2). Amplicons were subjected to electrophoresis on SYBR® Safe-stained 1.5% agarose-TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) gels and their banding patterns were visualized by UV transillumination. Single-strand conformation polymorphism (SSCP) analysis (Gasser et al., 2006) was then used as a pre-screen to examine for genetic variation before selecting representative amplicons for DNA sequencing. Amplicons of the 17-kDa gene for *R. peacockii* and *R. montanensis* from *Dermacentor andersoni* and *D. variabilis* (respectively) were used on gels as mobility controls. Five amplicons of the rickettsiae in *I. kingi* were purified and subjected to automated DNA sequencing using primers 17KD1 and 17KD2 in separate reactions.

Four additional genetic markers were used to characterize the rickettsiae in *I. kingi*. Part (382 bp) of the citrate synthase gene (*gltA*) was amplified from the gDNA of 12 rickettsial-infected ticks using primers *Rp*CS.877p and *Rp*CS.1258n (Regnery et al., 1991) and the conditions used by Dergousoff et al. (2009). Then, 532 bp of the outer membrane protein A gene (*ompA*) was amplified from the gDNA of 3 rickettsial-infected ticks using primers *Rr*190.70p and *Rr*190.602n (Regnery et al., 1991). The same PCR conditions were used as for *gltA*, except that the number of amplification cycles was increased to 30. Part (556 bp) of the 16S rRNA gene of 2 rickettsial-infected ticks was amplified using primers 16S-Rick-F1 (5′-TGGCTCAGAACGAACGCTATCGG-3′) and 16S-Rick-R2 (5′-ACCTCTACACTAGAAATTCCATCA-3′) and the following conditions: 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and then 72 °C for 5 min. Amplicons of *gltA*, *ompA*, and the 16S rRNA gene derived from the gDNA of 2 or 3 rickettsial-infected ticks were purified and subjected to DNA sequencing as described above. An attempt was made to amplify ~800 bp of the outer membrane protein B gene (*ompB*) from the rickettisae in *I. kingi* using primers 120-2788 and 120-3599 and the PCR conditions of Roux and Raoult (2000), except that the number of cycles was increased to 35, and the annealing temperature raised from 50 °C to 52 °C. The gDNA of *R. peacockii*-infested *D. andersoni* were included as positive controls in the PCR analyses.

BLAST searches (GenBank) were performed on the DNA sequences of each gene to determine the genetic similarity of the rickettsiae in *I. kingi* to the different taxa within the genus *Rickettsia*. The DNA sequences of the rickettsiae in *I. kingi* were aligned manually with those of other *Rickettsia* species, and phylogenetic analyses were performed separately on the sequence data of each gene using the neighbor joining (NJ) and maximum parsimony (MP) methods in PAUP (Swofford, 2003). For the MP analyses of the 17-kDa gene, *gltA*, and the 16S rRNA gene, characters were treated as unordered and were equally weighted; alignment gaps were treated as 'missing' characters, and the sequences of *R. bellii*, *Ehrlichia ruminantium*, and *Orientia tsutsugamushi* were used as the outgroups (respectively). Midpoint rooting was used in the MP analysis of the *ompA* sequence data. Exhaustive searches with TBR branch swapping were used to infer the shortest trees. The length, consistency index excluding uninformative characters, and the retention indices of each most parsimonious tree were recorded. Bootstrap analyses (1000 replicates for the NJ analyses and 100 replicates for MP analyses) were conducted to determine the relative support for clades in the consensus trees.

Table 1
Closest relative sequences to the partial 17-kDa gene, *gltA*, *ompA* and 16S rRNA gene, sequences of the *Rickettsia* detected in the *I. kingi* from Saskatchewan, Canada.

Gene	*Rickettsia* (Genbank accession no.)	% sequence similarity
17-kDa	*R. canadensis* (CP000409)	94.9 (374 of 394 bp)
	Candidatus R. monteiroi (FJ269036)	92.1 (363 of 394 bp)
	Candidatus R. hoogstraalii (EF629538)	87.8 (346 of 394 bp)
	R. monacensis (EF380355)	87.3 (344 of 394 bp)
gltA	*Candidatus* R. tarasevichiae (EF445981)	99.1 (338 of 341 bp)
	Rickettsia sp. H820 (JF714219)	98.8 (336 of 340 bp)
	R. canadensis (CP000409)	97.9 (334 of 341 bp)
	R. asiatica (AB297808)	96.8 (330 of 341 bp)
	Candidatus R. monteiroi (FJ269035)	96.5 (329 of 341 bp)
	R. helvetica (JQ669952)	96.5 (329 of 341 bp)
ompA	*Rickettsia* sp. H820 (JF714220)	94.5 (464 of 491 bp)
	R. canadensis (CP000409)	86.0 (425 of 494 bp)
	R. tamurae (DQ103259)	86.0 (426 of 496 bp)
	Candidatus R. cooleyi (AF031535)	85.5 (423 of 495 bp)
16S rRNA	rickettsial endosymbiont (AY961085)	99.3 (549 of 553 bp)
	R. massiliae (CP003319)	99.1 (548 of 553 bp)
	Most other SFG[a] *Rickettsia*	98.9 (547 of 553 bp)
	Candidatus R. tarasevichiae[b] (AM418457)	98.8 (398 of 403 bp)
	R. canadensis (CP000409)	98.4 (544 of 553 bp)

[a] SFG, spotted fever group.
[b] Only a partial sequence available for this taxon.

Results

A single band of the expected size (~450 bp) for the partial rickettsial 17-kDa gene was detected on agarose gels for amplicons derived from the gDNA of 60 of the 87 (69%) ticks. These 60 PCR-positive samples represented all feeding life cycle stages of *I. kingi* (i.e., 55 larvae, 1 nymph, and 4 adults). No bands were detected on agarose gels for the negative control (i.e., no gDNA) samples. The banding patterns of all 60 PCR-positive samples on SSCP gels were identical to one another, but differed to those of *R. peacockii* and *R. montanensis* (data not shown). There were no differences in the DNA sequences of 5 representative samples (GenBank accession no. HE647694), but each differed in sequence by 5.8–18.3% (i.e., 21–72 bp) when compared to those of the 17-kDa gene for taxa within the genus *Rickettsia*. A BLAST search of the 394-bp 17-kDa gene sequence of *Rickettsia* in *I. kingi* revealed that it was genetically most similar (94.9%) to the sequence of *R. canadensis* (Table 1). The NJ tree produced from the phylogenetic analysis of the aligned sequence data revealed strong statistical support (98% bootstrap value) for the *Rickettsia* in *I. kingi* representing a sister taxon to *R. canadensis* and *Candidatus* R. monteiroi to the exclusion of members of the SFG and TG (Fig. 1). A MP analysis of the sequence data set (i.e., 100 cladistically informative characters) produced 41 equally most-parsimonious trees (strict consensus tree not shown), with a length of 297, a CI of 0.58, and a RI of 0.72. As with the NJ tree, there was support (71% bootstrap value) for the inclusion of *Rickettsia* in *I. kingi* within a clade that included *R. canadensis* and *Candidatus* R. monteiroi (Fig. 1).

There were no differences in the DNA sequences of *gltA* (GenBank accession no. HE647692) for amplicons derived from the gDNA of 3 rickettsial-infected *I. kingi*. However, they differed in sequence by 0.9–13.9% (i.e., 3–48 bp) when compared to the *gltA* sequences of species of *Rickettsia* available on GenBank. The closest match in sequence was to a sequence of *Candidatus* R. tarasevichiae (Table 1). The NJ tree produced from analyses of the *gltA* sequence data (342 alignment positions) showed that this putative new species of *Rickettsia* formed a clade, with strong bootstrap