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Short communication

First report of *Candidatus* Rickettsia mendelii in *Ixodes brunneus* from the United States

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ABSTRACT

Candidatus Rickettsia mendelii is a novel rickettsial species recently identified in *Ixodes ricinus*. In this study, *Ixodes brunneus* collected from wild birds (n = 77 ticks) or vegetation (n = 4 ticks) in southeastern Virginia were surveyed for rickettsial agents. *Candidatus* Rickettsia mendelii was confirmed in *I. brunneus* through sequencing of the *glt*A and 16S rRNA genes. This is the first report of this rickettsial species in *Ixodes* ticks in North America.

1. Introduction

Candidatus Rickettsia mendelii is a recently reported rickettsial species (Hajduskova et al., 2016) found in *Ixodes ricinus* and most closely related to *Rickettsia bellii*, a member of the rickettsial ancestral group (Philip et al., 1983). Classification of *Candidatus* Rickettsia mendelii was through sequencing of the citrate synthase gene (*glt*A) and 16S rRNA sequencing; these sequences were concatenated and used in constructing a phylogenetic tree (Hajduskova et al., 2016). In a survey for rickettsial agents conducted in the Czech Republic, 4524 *I. ricinus* were collected from various hosts / sources; most of the ticks were from wild birds, but also included ticks from canine hosts and vegetation. Seven of the ticks surveyed were positive for *Candidatus* Rickettsia mendelii: one was collected from vegetation, four from a dog, and two from wild birds (Hajduskova et al., 2016). Since its initial report, *Candidatus* Rickettsia mendelii has been documented in *I. ricinus* in other European countries including Slovakia (Minichova et al., 2017), Ukraine (Rogovskyy et al., 2018), and Poland (Stanczak et al., 2018).

Ixodes brunneus is not considered a primary vector of any human pathogens; as such, this tick species has not been studied in detail for human pathogenic agents. In the United States, *I. brunneus* is a host-specific black-legged tick that feeds exclusively on birds during all life stages (Bishopp and Trembley, 1945). In the southeastern United States, *I. brunneus* has been associated with avian paralysis, a phenomenon observed when female ticks release toxins from their salivary glands while feeding. This toxin can sometimes result in a fatal paralysis of the avian host (Luttrell et al., 1996). Previously, it has been reported that *I. brunneus* can harbor *R. rickettsii* (Clifford et al., 1969). In the study reported here, *I. brunneus* were collected from wild birds and vegetation in southeastern Virginia and tested for the presence of *Rickettsia* spp. This study was part of a larger effort to assess the role that birds play in the movement and maintenance of tick-borne bacteria in southeastern Virginia.

2. Material and methods

2.1. Tick collection

Seventy-seven *I. brunneus* ticks, representing all three life stages, were from wild birds caught at various sites in southeastern Virginia through mist netting from 2012 to 2014. Four *I. brunneus* larvae were flagged from vegetation in 2016.

2.2. Tick DNA extraction

All adult ticks were cut bilaterally; one half of the tick was extracted using a DNeasy Blood and Tissue Extraction Kit (Qiagen, Valencia, CA) while the other half was stored at −80 °C for additional analysis, if required. All nymphal and larval ticks were extracted whole due to their small size. Samples were pulverized using 1.0-mm glass beads using a Mini-Beadbeater-1 (BioSpec Products, Inc., Bartlesville, OK, USA). Cells were lysed using lysis buffers; the DNA was column purified and eluted according to kit manufacturers' guidelines.

2.3. Tick identification

Each tick was identified using molecular methods, as morphological identification is often inconclusive with immature stages and engorged ticks (Anderson et al., 2004). *Ixodes brunneus* was identified using a PCR-amplified 454-bp segment of the 16S mitochondrial RNA gene

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(Macaluso et al., 2003; Wright et al., 2014). Each PCR reaction (final volume 25 µL) consisted of 12.5 µL EconoTaq PLUS 2X Master Mix (Lucigen, Middleton, WI), 2 µL of each primer (10 µM), 3.5 µL of nuclease-free water, and 5 µL DNA. The thermocycler protocol included a denaturing step at 95 °C for 3 min, followed by 30 cycles of: 95 °C for 30 s, 52 °C for 45 s, and 72 °C for 60 s; the reaction finished with an extension step at 72 °C for 7 min. The 16S amplicons were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (ABI- ThermoFisher Scientific, Carlsbad, CA) with analysis on an ABI 3130*xl* genetic analyzer. Ticks were identified to species by comparison with known tick 16S gene sequences using NCBI Blast (http://blast.ncbi.nlm.nih.gov). No template negative controls were included in PCR reactions.

2.4. *Rickettsia spp.* identification

Two real-time PCR assays were used: one amplifying a portion of the rickettsial 17-kDa gene to detect the presence of *Rickettsia* spp. in the ticks; the other amplified a portion of the *omp*B gene specific to *R. parkeri* (Jiang et al., 2012). Each rickettsial 17-kDa gene real-time PCR reaction (final volume 25 µL) consisted of 12.5 µL EconoTaq PLUS 2X Master Mix (Lucigen, Middleton, WI), 1.25 µL of each primer (10 µM), 1 µL of probe (10 µM), 2 µL $MgCl_2$ (25 mM), 2 µL of nuclease-free water, and 5 µL DNA. Each *R. parkeri omp*B real-time PCR reaction (final volume 25 µL) consisted of 12.5 µL EconoTaq PLUS 2X Master Mix (Lucigen, Middleton, WI), 1.75 µL of each primer (10 µM), 1 µL of probe (10 µM), 2.5 µL $MgCl_2$ (25 mM), 0.5 µL of nuclease-free water, and 5 µL DNA. The thermocycler protocol included a pre-hold step of 50 °C for 2 min, a denaturing step of 95 °C for 2 min, followed by 45 cycles of: 95 °C for 15 s and 60 °C for 30 s. Using the rickettsial 17-kDa-specific assay (Jiang et al., 2012), ticks positive for *Rickettsia* spp. (Cq ≤ 35 cycles) were detected. Depending on availability of DNA, rickettsia-positive ticks were further examined by amplification and sequencing of one or more of *omp*A, *omp*B, *sca*4, *glt*A, and 16S rRNA genes (Table 1). No template negative controls were included in PCR reactions. Sequences were initially analyzed using Geneious software (Kearse et al., 2012) version R11 (http://www.geneious.com, Biomatters Inc., New Zealand) and compared using NCBI Blast (http://blast.ncbi.nlm.nih.gov) to identify the rickettsial species.

3. Results

The sequence of the 16S mitochondrial RNA gene confirmed 81 ticks collected from wild birds and vegetation were *I. brunneus*. The rickettsial 17-kDa real-time PCR indicated 44 of 66 (67%) ticks tested positive for rickettsial DNA; we were not able to test the remaining 15 because the DNA was of poor quality due to DNA degradation, or the samples had already been used for other experiments. Five of the 66 ticks had *Candidatus* Rickettsia mendelii as determined by sequencing of the rickettsial *glt*A gene, based on a BLAST alignment to identify the species. A 790-bp partial *glt*A fragment (Accession number MH458574) was 99.9% (789 of 790-bp) identical to the sequence of *Candidatus* Rickettsia mendelii (Hajduskova et al., 2016). We amplified the rickettsial 16S rRNA gene from 3 of the 5 ticks. Some of the sequencing chromatograms contained multiple overlapping peaks, but BLAST analysis of clean single read (one direction) fragments of 627–745 bp were > 99% identical to *Candidatus* Rickettsia mendelii. A 627-bp fragment of the 16S gene from one isolate, sequenced in both directions, was 99.2–99.7% identical to the three *Candidatus* R. mendelii 16S genes in GenBank (Accession numbers: KJ882316, KJ882317, KJ882318); the closest match to any *Rickettsia* sp. other than *Candidatus* Rickettsia mendelii was < 98%. We were not able to amplify *omp*A, *omp*B, or *sca*4 from any of the 5 tick samples that were positive for *Candidatus* R. mendelii; similar results were reported by Hajduskova et al. (2016). Using *R. parkeri* species-specific real-time PCR analysis (Jiang et al., 2012), *R. parkeri* was detected in 19 of the 81 (23.5%) ticks. Amplified portions of the *glt*A and *omp*A genes were sequenced, in one direction, from two ticks for each gene. The closest similarity for the sequence was to the respective *R. parkeri* genes. For the *glt*A gene, similarity was 100% over at least 225bp, while for *omp*A it was > 98% over more than 475bp. The rickettsia present in the other 17-kDa positive ticks was not able to be determined due to lack of DNA or inability to obtain sequence data.

All ticks that were positive for *Candidatus* R. mendelii were *I. brunneus* juveniles: 3 nymphs and 2 larvae (Table 2). Two nymphs were collected from the same bird, a hermit thrush (*Catharus guttatus*) in Zuni, one nymph from a tufted titmouse (*Baeolophus bicolor*) in Portsmouth, one larva from a hermit thrush in Chesapeake, and one larva flagged from vegetation in Cape Charles.

4. Discussion

This is the first report of *Candidatus* Rickettsia mendelii in a tick population within the United States. In the Czech Republic study, *Candidatus* Rickettsia mendelii was determined to be phylogenetically unique based on the sequence of the *glt*A gene and 16S rRNA; other rickettsial genes: *omp*A, *omp*B, and *sca*4 could not be detected (Hajduskova et al., 2016). Therefore, no sequence data are available for the *omp*A, *omp*B, and *sca*4 genes of *Candidatus* Rickettsia mendelii for comparison. Likewise, we were unable to amplify or sequence these three genes from the *I. brunneus* infected with *Candidatus* Rickettsia mendelii. It is unknown why we were unable to amplify *omp*A, *omp*B, and *sca*4 in *Candidatus* Rickettsia mendelii, but it could be attributed to the absence of these genes or a high degree of sequence heterogeneity in this *Rickettsia* sp. We were, however, able to amplify and sequence rickettsial genes (*omp*A and *glt*A) from other 17-kDa-positive and *R. parkeri*-positive *I. brunneus.* Results suggested the presence of other *Rickettsia* spp. in these ticks and, in some cases, co-infections (multiple peaks in sequencing chromatograms) with two different *Rickettsia* sp.

Table 1
Primers used to amplify rickettsial genes. These primers were used to amplify outer membrane protein A (*omp*A), outer membrane protein B (*omp*B), gene D (*sca*4), citrate synthase (*glt*A), and 16S rRNA.

Gene	Fragment Size	Primers	Reference
*omp*A	540-bp	190-FN1 5′- AAGCAATACAACAAGGTC - 3' 190-RN1 5′- TGACAGTTATTATACCTC - 3"	(Paddock et al., 2004)
*omp*B	1895-bp	RompB11F 5'- ACCATAGTAGCMAGTTTTGCAG - 3' Romp1902R 5'- CCGTCATTTCCAATAACTAACTC - 3'	(Jiang et al., 2005)
*sca*4	928-bp	D1F 5'- ATGAGTAAAGACGGTAACCT - 3' D928R 5'- AAGCTATTGCGTCATCTCCG - 3'	(Jiang et al., 2005)
*glt*A	830-bp	CS239 5'- GCTCTTCTCATCCTATGGCTATTAT - 3' CS1069 5'- CAGGGTCTTCGTGCATTTCTT - 3'	(Labruna et al., 2004)
16S rRNA	757-bp	Ric 5'- TCTAGAACGAACGCTATCGGTAT - 3' Ric Rt 5'- TTTCATCGTTTAACGGCGTGGACT - 3'	(Nilsson et al., 1997)
16S rRNA	1385-bp	Ric 5'- TCTAGAACGAACGCTATCGGTAT - 3' Ric U8 5'- TGCGTTAGCTCACCACCTTCAGG - 3'	(Nilsson et al., 1997)

Table 2
Collection locations of *Ixodes brunneus* ticks, infected with *Candidatus* Rickettsia mendelii, from various sites across southeastern Virginia.

Tick Species	Tick Life Stage	Location	Source
Ixodes brunneus	Nymph	Blackwater Ecological Preserve in Zuni, Virginia	Hermit thrush, *Catharus guttatus*
Ixodes brunneus	Nymph	Blackwater Ecological Preserve in Zuni, Virginia	Hermit thrush, *Catharus guttatus*
Ixodes brunneus	Nymph	Residential area in Portsmouth, Virginia	Tufted titmouse, *Baeolophus bicolor*
Ixodes brunneus	Larva	Property of Nature Conservancy in Chesapeake, Virginia	Hermit thrush, *Catharus guttatus*
Ixodes brunneus	Larva	Kiptopeke State Park in Cape Charles, Virginia	Vegetation

were possible, although we could not determine this with certainty. *R. parkeri* species-specific real-time PCR amplified *R. parkeri* DNA in some ticks suggesting coinfections of *I. brunneus* with *Candidatus* Rickettsia mendelii and *R. parkeri*. Additionally, some of these ticks were from the same bird, potentially inflating the *R. parkeri* prevalence. Thus, the precise number of individual ticks within our sample set carrying *Candidatus* R. mendelii is unknown.

How and why *Candidatus* Rickettsia mendelii is present in two tick taxa from two isolated geographic regions, separated by the Atlantic Ocean, is unknown. Hajduskova et al. (2016) suggested a symbiotic rather than pathogenic relationship for this organism. It is possible that our identification of this organism in *I. brunneus* is a previously unidentified endosymbiont in ticks from the United States. *Candidatus* Rickettsia mendelii was found in an unfed *I. brunneus* larva flagged from vegetation, suggesting transovarial transmission from an infected adult female tick. A less likely possibility is that the infected nymphs and larvae acquired *Candidatus* Rickettsia mendelii from the bird host. Although birds serve as hosts for many tick species, information on systemic infection by *Rickettsia* spp. in bird blood is limited and with mixed results; some studies have detected *Rickettsia* spp. in bird blood (Berthova et al., 2016; Hornok et al., 2014), others have not (Cohen et al., 2015). Life histories of the hermit thrush and tufted titmouse reveal that the former does not normally migrate outside of North America (Dellinger et al., 2012), while the latter has never been recorded outside of North America (Ritchison et al., 2015). Thus, it would be extremely unlikely these birds traveled to Europe and played host to an infected *I. ricinus* to acquire the bacteria.

It is also possible that an infected *I. ricinus* travelled to North America and infected an *I. brunneus* via sharing of hosts or co-feeding. As far as is known, *I. ricinus* has not been found in North America where other *Ixodes* spp. are established (Rich et al., 1995). One means for *I. ricinus* to travel to North America would be on a host. The two avian species that the infected *I. ricinus* were collected from in the Czech Republic were the common nightingale (*Luscinia megarhynchos*) and the European robin (*Erithacus rubecula*), neither of which migrate outside of Europe (Hajduskova et al., 2016), much less as far as North America.

One further explanation is that *I. ricinus* may have fed on livestock, which could have been transported to North America, although this is also unlikely as there have been no livestock exports historically from the Czech Republic to the United States (Mikulasova, 2016).

The most plausible explanation for our finding of *Candidatus* Rickettsia mendelii in *I. brunneus* is that it is already established in eastern North America and has merely gone undetected. With recent reports on foreign ticks (Rainey et al., 2018) potentially going undetected for many years (Buccino, 2018) it is possible that the presence of unusual bacteria in native ticks may indicate a past or contemporaneous introduction. Our study indicates a need for the identification of both endosymbiont and pathogenic rickettsial species. Understanding interactions between ticks and their microbes can provide insights into pathogen-vector-host activity. The work presented here emphasizes a need for more thorough studies on the movement and occurrence of different rickettsial species as they become established in different geographic regions. Although *Candidatus* R. mendelii is unlikely to be a human pathogen, continued active surveillance provides important information and early warning of potential infectious agents.

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Animal care

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Declaration of Competing Interest

None.

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