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RESEARCH

Evidence for *Ixodes holocyclus* (Acarina: Ixodidae) as a Vector for Human Lyme Borreliosis Infection in Australia

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ABSTRACT. *Ixodes holocyclus* (Acarina: Ixodidae) and *Ixodes cornuatus* (Acarina: Ixodidae) are two tick species found in the more densely populated areas of Australia and are known to be the cause of the neurotoxic disease tick paralysis in humans and mammals. Borreliosis otherwise known as Lyme disease is an emerging infectious disease in humans in Australia. *Borrelia burgdorferi* sensu stricto (Spirochaetales: Spirochaetaceae) and sensu lato are closely related spirochetal species that are the causative agents of Lyme disease in humans. Clinical transmission of this tick-borne disease can be identified in several but not all cases by a characteristic rash known as erythema migrans. However, there has been no study of the tick vectors of this infection in Australia. We used morphological and molecular techniques to identify unequivocally the ticks on the patients of this study to be *I. holocyclus* and then show the presence of *B. burgdorferi* sensu stricto infection in erythema migrans biopsies. *I. holocyclus* has not previously been associated with erythema migrans or Lyme disease. Two patients presented to the lead author's medical practice with erythema migrans in mid and late 2012. The morphology and cytochrome oxidase 1 and ITS2 genes of the two ticks were studied. The skin at the attachment site was sampled by central biopsy for both real time and endpoint *Borrelia* polymerase chain reaction (PCR) analysis and subsequent sequencing. Morphologically, the two ticks were either *I. holocyclus* or *I. cornuatus*. Molecular studies and nucleotide sequencing revealed that both ticks were *I. holocyclus*. Real time and endpoint PCR on the central tissue biopsy samples returned positive results for *B. burgdorferi* DNA. Our results are evidence for transmission of *B. burgdorferi* sensu stricto species to humans by the tick *I. holocyclus* in Australia. *I. holocyclus* is commonly associated with human tick bites on virtually the entire eastern coastline of Australia.

Key Words: *Ixodes holocyclus*, *Borrelia burgdorferi*, Australia, Lyme disease, erythema migrans

Lyme disease is an increasing health burden on the Australian community requiring wider diagnostic recognition (Mayne 2011). The infection is caused by *Borrelia burgdorferi* sensu stricto (Spirochaetales: Spirochaetaceae) and *B. burgdorferi* sensu lato species. The associated vectors causing transmission in Australia need to be firmly identified. We have reported previously the endemic presence of Lyme disease in Australia by positive serological and molecular testing of blood samples taken from symptomatic patients who have never been abroad (Mayne 2011) and subsequently on the positive polymerase chain reaction (PCR) results for *Borrelia* sp. in erythema migrans tissue taken by central biopsy (Mayne 2012). For more than 20 yr, ongoing debate has ensued regarding the possibility of Lyme disease in Australia. There is contradiction in studies with reference to both vectors and *Borrelia* sp. in Australia and establishing the potential of specific tick species to transmit these pathogens (Piesman and Stone 1991, Russel et al 1994, Mayne 2011, Mayne 2012, Radolf et al. 2012). It is important to draw this controversy to a closure finding vectors, providing proof of borreliosis infection in humans and identifying the hosts involved in the tick life cycle. In this study, two patients presented in 2012 with erythema migrans and attached ticks, which appeared to be *Ixodes holocyclus* (Acarina: Ixodidae). The objective of this study was to analyze naturally obtained ticks, confirm their identification, and determine whether *Borrelia* sp. could be isolated or identified from these patients. Lesions and ticks were photographed. The ticks were sent for morphological identification and to a further site for molecular testing. Central skin biopsies were taken and forwarded for molecular analysis. We report here that the ticks were identified as either *I. holocyclus* (Neumann 1899) or

Ixodes cornuatus (Roberts 1960) and that two tissue samples tested positive for *Borrelia* sp.

Materials and Methods

Erythema migrans lesions on the patients were photographed using a Canon 350 EOS digital SLR camera, Figs. 1 and 2. A DermLite II pro dermoscope was then used to inspect the ticks at 10× magnification. Macroscopically, each appeared to be a nymph tick of *I. holocyclus*, which is colloquially referred to as the grass tick, whereas the adult form is called the paralysis tick. Using a step down ring to attach the dermoscope, each tick was further imaged, Figs. 3 and 4. The ticks were forwarded to the University of Newcastle for formal morphological identification and then subsequently to Parasitology Section at the University of Queensland and University of Sunshine Coast. Molecular studies to identify the tick species were performed using sequences of mitochondrial (cytochrome oxidase subunit I, COX1) and nuclear DNA (internal transcribed space 2, ITS2; Song et al. 2011). Briefly, Genomic DNA was extracted from half the body of the tick sample. COX1 and ITS2 regions were amplified by PCR. Fragments of COX1 gene (~800 bp) and ITS2 (~700 bp) were amplified using the primer pairs HCO2064/HCO1215 and ITS865/ITS105, respectively, and the amplicons were purified and sequenced. The sequences of the COX1 and ITS2 fragments were aligned with COX1 haplotypes and ITS2 genotypes of *I. holocyclus* and *I. cornuatus*, respectively. A central skin punch biopsy was taken from the erythema migrans at the attachment site on both patients. Tissue samples were forwarded to Australian Biologics in Sydney, Australia, for *B. burgdorferi* detection by PCR

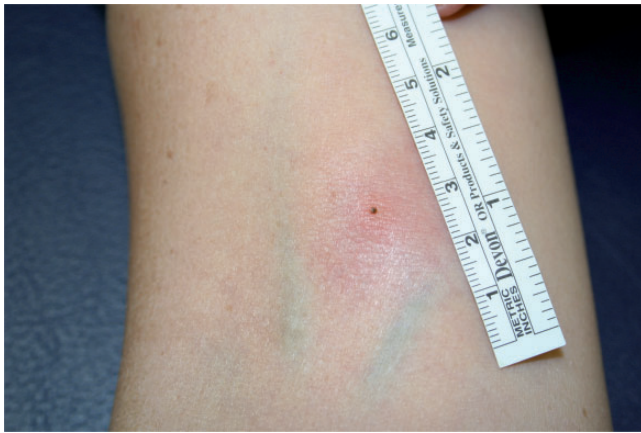


Fig. 1. Erythema migrans with tick attached centrally.



Fig. 2. Erythema migrans with tick just removed.

using the Eco Real-Time PCR system from Illumina San Diego USA, with software version 3.0.16.0. DNA was extracted from the tissue. The two samples were analyzed in duplicate with positive and negative controls using primers and probe AB-B1 (proprietary to Australian Biologics) for the *Borrelia* 16S rRNA gene. The thermal profile for both analyses involved incubation for 2 min at 50°C, polymerase activation for 10 min at 95°C, then PCR cycling for 40 cycles of 10 s at 95°C and 45 s at 60°C. The positive control used was an ATCC *B. burgdorferi* genomic control. Validation of the assay was produced by use of external sequencing and through participation in quality assurance programs for the detection of *Borrelia* by PCR with Quality Control for Molecular Diagnostics in Glasgow, UK. The extracted tissue DNA was then used in a *Borrelia* sequencing endpoint PCR assay targeting the 16S RNA gene using 12.5 µl of Go-Taq (Promega), 1.5 µl of both forward and reverse primers, 4.5 µl H₂O and 5.0 µl of DNA in a total volume of 25 µl. Primers are proprietary to Australia Biologics. Cycling conditions were an initial denaturation of 94°C for 4 min, then 94°C for 60 s, 60°C for 60 s, and 72°C for 60 s for 45 cycles with a final extension of 10 min at 72°C. PCR products were analyzed by standard 2.5% agarose gel. The amplicon was eluted from the gel and purified. The amplicon was 286 bp and was sequenced at the Australian Genome Research Facility in Sydney, Australia.

Results

The Ticks. Macroscopic and microscopic analysis at Newcastle and Queensland universities confirmed morphologically that the specimens appeared to be nymphs of the *I. holocyclus* tick. Morphologically, they were either *I. holocyclus* (Neumann 1899) or *I. cornuatus* (Roberts 1960). Molecular analysis showed that the fragments of the tick

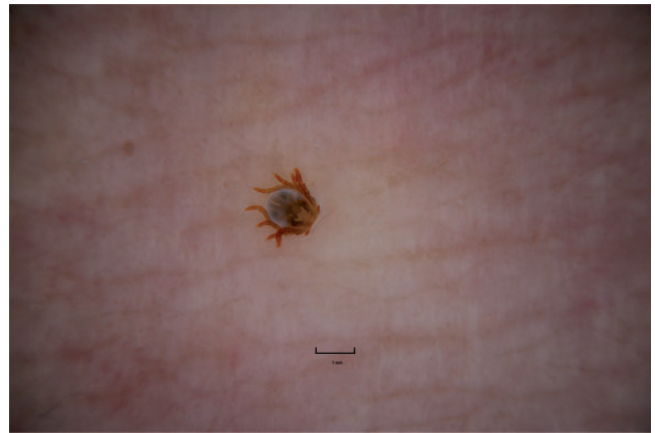


Fig. 3. Dermoscopic view of apparent *I. holocyclus* nymph tick still attached.

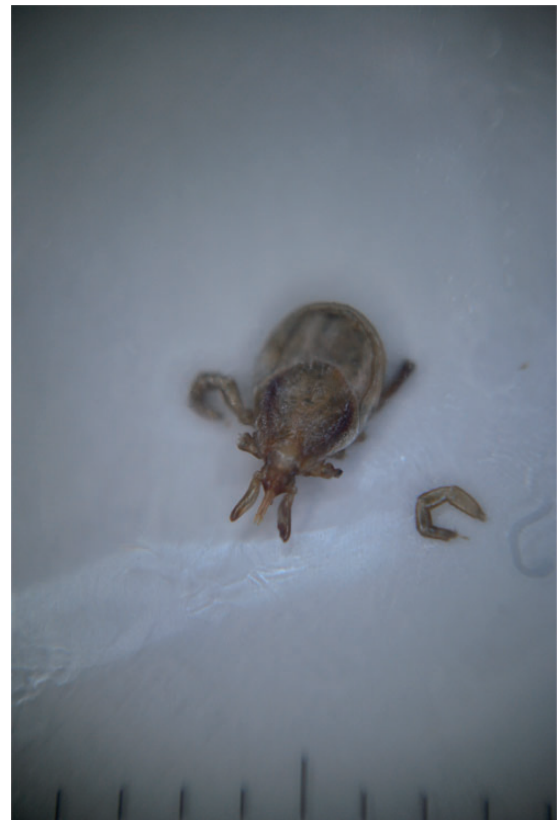


Fig. 4. Dermoscopic view of apparent *I. holocyclus* nymph tick just detached.

samples are in the clade that was identified morphologically as *I. holocyclus* for both patients' ticks.

The Skin Biopsy PCRs—Real Time. Real-time PCR of the biopsied tissue sent to Australian Biologics in Sydney for molecular analysis was positive for *Borrelia* sp. DNA in both samples.

The Skin Biopsy PCRs—Endpoint. No product could be produced for patient A. Tissue endpoint PCR for patient B-CH1277 gave a positive result for *Borrelia* sp. DNA. The sequence produced for B-CH1277 was then submitted for Basic Local Alignment Search Tool (BLAST) inquiry at National Institutes of Health, National Center for Biotechnology Information as previously described (Mayne 2012). BLAST analysis indicates that it is best matched with *Borrelia* sp., with

Table 1. Blast results showing the top four alignments at 99% Ident and very low *E* of 2.0-81

Sequences producing significant alignments	Max score	Total score	Query cover (%)	<i>E</i> value	Ident (%)	Accession
<i>B. burgdorferi</i> CA382, complete genome	311	311	69	2.00 E-81	99	CP005925.1
<i>B. burgdorferi</i> N40, complete genome	311	311	69	2.00 E-81	99	CP002228.1
<i>B. burgdorferi</i> ZS7, complete genome	311	311	69	2.00 E-81	99	CP001205.1
<i>B. burgdorferi</i> B31, complete genome	311	311	69	2.00 E-81	99	AE000783.1
<i>B. burgdorferi</i> JD1, complete genome	305	305	69	9.00 E-80	99	CP002312.1
<i>B. garinii</i> BgVir chromosome linear, complete sequence	261	261	67	2.00 E-66	95	CP003151.1
<i>B. garinii</i> NMJW1, complete genome	255	255	67	1.00 E-64	94	CP003866.1
<i>Borrelia bissettii</i> DN127, complete genome	255	255	69	1.00 E-64	94	CP002746.1
<i>B. garinii</i> PBI, complete genome	255	255	67	1.00 E-64	94	CP000013.1

an identity of 99%, and an *E* value of 2.0E-81. Significant alignments are listed in Table 1 with accession numbers.

Discussion

The ticks in this study were identified as *I. holocyclus* both morphologically and by molecular analysis. *Borrelia* sp. DNA was detected in both patient tissue samples demonstrating transmission from the ticks. Further, in one patient, we have identified the strain as *B. burgdorferi* sensu stricto. In Australia, there are two published reports of human Lyme disease, the first on erythema migrans (McCrossin 1986) and the second on a locally acquired human *B. burgdorferi* infection (Hudson et al. 1998). At the time of the McCrossin study in 1986, only serological diagnostic tests were available. The Hudson study in 1998 described a cultured isolate of *Borrelia garinii* identified by molecular testing from one patient who had traveled overseas, but due to the length of time since overseas travel (17 mo) likely acquired the infection in Australia. Sequencing results, however, suggested similarity to a European strain of *B. garinii*, rather than the *B. garinii* species typically described in Asia. Neither of the aforementioned studies identified a precise vector. In Russell et al. (1994), the midgut contents of 279 nymph and 289 adult *I. holocyclus* mostly unfed ticks (82 fed) were examined with no spirochetes found using dark field microscopy and no evidence of *Borrelia* DNA on PCR analysis. Spirochetes within unfed nymphs exist in low numbers and in a poorly understood metabolic state that enables them to endure prolonged periods of nutrient deprivation (Radolf et al. 2012). In this state, the transcription regulators Rrp2–RpoN–RpoS and the hybrid histidine Hk1–Rrp1 pathways are inactive, as are mammalian-phase genes, whereas tick-phase genes are maximally expressed. At the commencement of feeding, this status is reversed with rapid replication and upregulation of OspC production and downregulation of OspA. The mechanics of tick behavior and salivary hypostome spirochete movement from tick to skin confirms the

possibility of borrelial transmission to the host well before the blood meal starts, which is believed to be at about 48 h. Russell et al (1994) found spirochete-like objects in the fed ticks but assumed that these were contaminants from a blood meal. Our study suggests that these tick species can transmit *Borrelia* sp. to humans. Australia has ~75 tick species. The Eastern coastline provides habitat for *I. holocyclus*, and the presence of *I. holocyclus* may be more extensive across central Victoria and Tasmania than previously believed (Song et al. 2011). This species can transmit other human pathogens and also may be the cause of the neurotoxic disease tick paralysis. The total eastern and southern region contains a very high proportion of Australia's human population, who may become infected with such pathogens through the bites of ticks. Surveys on the prevalence of tick-borne pathogens in larvae, nymphs, and adult ticks should be done in these areas.

We used morphological and molecular tools to identify the species of ticks found on patients. We used PCR to amplify *Borrelia* sp. DNA from the patients. *I. holocyclus* is a potential vector for *Borrelia* sp. transmission in Australia. It is widespread in areas where a significant number of humans live, and surveys on the prevalence of *Borrelia* sp. in reservoir species and different tick stages should be done routinely in these areas.

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