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Short communication

Australian penguin ticks screened for novel *Borrelia* species

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ABSTRACT

Lyme borreliosis (or Lyme Disease) is an emerging threat to human health in the Northern Hemisphere caused by tick-borne bacteria from the *Borrelia burgdorferi* sensu lato (Bbsl) complex. Seabirds are important reservoir hosts of some members of the Bbsl complex in the Northern Hemisphere, and some evidence suggests this may be true of penguins in the Southern Hemisphere. While the Bbsl complex has not been detected in Australia, a novel *Borrelia* species ('*Candidatus* Borrelia tachyglossi') was recently sequenced from native ticks (*Ixodes holocyclus* and *Bothriocroton concolor*) parasitising echidnas (*Tachyglossus aculeatus*), suggesting unidentified borreliae may be circulating amongst native wildlife and their ticks. In the present study, we investigated whether ticks parasitising little penguins (*Eudyptula novaehollandiae*) harbour native or introduced *Borrelia* bacteria. We chose this penguin species because it is heavily exploited by ticks during the breeding season, lives in close proximity to other potential reservoir hosts (including native wildlife and migratory seabirds), and is known to be infected with other tick-borne pathogens (*Babesia*). We screened over 230 penguin ticks (*Ixodes* spp.) from colonies in south-eastern Australia, and found no evidence of *Borrelia* DNA. The apparent absence or rarity of the bacterium in south-eastern Australia has important implications for identifying potential tick-borne pathogens in an understudied region.

1. Introduction

Lyme borreliosis (LB) is a multi-organ inflammatory illness of humans that is the most common and widely distributed vector-borne disease in the temperate regions of the Northern Hemisphere (Middleton et al., 2016). LB is caused by spirochaetes of the *Borrelia burgdorferi* sensu lato (Bbsl) complex transmitted by ticks, predominantly in the genus *Ixodes* (Biesiada et al., 2012; Middleton et al., 2016), and leads to disorders of the skin, joints, heart and neurological system (Biesiada et al., 2012; Hercogová, 2015; Halperin, 2016). Late symptoms can include painful radiculitis, arthritis, carditis, meningitis, encephalitis, palsy (Biesiada et al., 2012; Hercogová, 2015; Halperin, 2016), and possibly progressive dementia and chronic fatigue syndrome (Ballantyne, 2008; Minkoff, 2016), although the last remains a matter of contention (Halperin, 2015, 2016).

An increasing number of people bitten by ticks in Australia are presenting with similar symptoms to those of LB (Chalada et al., 2016). These reports have sparked considerable debate over the causative agent, triggering a Senate Inquiry (Senate Community Affairs Committee Secretariat, 2016) and raising the profile of tick-borne diseases nationwide. Studies to date have failed to detect any members of the Bbsl complex in Australia (Wills and Barry, 1991; Russell et al., 1994) or establish native human-biting ticks, such as *Ixodes holocyclus* (Australian paralysis tick), as competent Bbsl vectors (Piesman and Stone, 1991). The current consensus is that the Bbsl complex is not present in Australia and that Australian Lyme-like illness is probably caused by an unidentified microorganism transmitted by native ticks (Wills and Barry, 1991; Russell et al., 1994; Gofton et al., 2015a,b; Senate Community Affairs Committee Secretariat, 2016).

Natural vertebrate reservoir hosts are integral to maintaining cycles of infection, in that they carry pathogens but are often asymptomatic themselves (Chambert et al., 2012; Voordouw et al., 2015). Hosts that form large, spatially and temporally predictable aggregations (e.g. packs, colonies or herds) and exhibit considerable long-distance movements are of particular epidemiological interest, due to the high potential for pathogen spread. Seabirds are important reservoir hosts for some members of the Bbsl complex, most notably *Borrelia garinii* vectored by the generalist seabird tick *Ixodes uriae* (Olsén et al., 1995; Gylfe et al., 2001; Duneau et al., 2008; Gómez-Díaz et al., 2010; Lobato et al., 2011). Over 60 seabird species are parasitised by this tick

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(Dietrich et al., 2011), and as most are highly migratory, global transmission of *Borrelia* has occurred, followed by diversification within seabird colonies (Olsén et al., 1995; Gylfe et al., 2000; Gylfe et al., 2001; Gómez-Díaz et al., 2011). *Borrelia* species associated with both LB and relapsing fever (RF) borreliae have now been found in penguins (Gauthier-Clerc et al., 1999; Yabsley et al., 2012; Schramm et al., 2014) suggesting they are reservoir hosts of the bacteria in the Southern Hemisphere. Thus far, however, only penguins in the sub-Antarctic and Antarctic regions have been investigated for the presence of *Borrelia* DNA.

In Australia, the roles of native ticks and of wildlife reservoir hosts in the cycling of tick-borne pathogens are well documented. For example, Australian ticks are known to transmit *Coxiella* and *Rickettsia* species that can cause illness in humans (Stenos et al., 2003; Cooper et al., 2013; Graves and Islam, 2016; Oskam et al., 2017). Although research aiming to identify the causative agent(s) of Australian Lyme-like illness remains in its infancy, recent studies have used advanced genetic techniques to screen Australian ticks for tick-borne pathogens (Cooper et al., 2013; Gofton et al., 2015a,b; Graves et al., 2016; Loh et al., 2016; Oskam et al., 2017). To date, four borreliae have been identified in Australia, including two introduced with domestic animals (*Borrelia theileri* and *Borrelia anserina*), and two native species (*Borrelia queenslandica* – though this species remains unconfirmed – and '*Candidatus* Borrelia tachyglossi') (Gofton et al., 2015a,b; Chalada et al., 2016; Loh et al., 2016, 2017). *Borrelia theileri*, *B. anserina* and *B. queenslandica* had been identified by the end of the 1960s and cause borreliosis in animals (in cattle, poultry, and rodents respectively). These species have never been associated with Lyme-like illness in humans, despite an attempt to infect a human volunteer with one of the species (Chalada et al., 2016). '*Candidatus* B. tachyglossi' was only recently sequenced from ticks (*I. holocyclus* and *Bothriocroton concolor*) parasitising echidnas (*Tachyglossus aculeatus*) (Gofton et al., 2015a,b; Loh et al., 2016, 2017). Research has yet to establish whether the echidna is a reservoir host for the bacterium, whether *I. holocyclus* and *B. concolor* are vectors, or whether the bacterium can be transmitted to humans. Although '*Candidatus* B. tachyglossi' is closely related to the RF and reptile-associated (REP) *Borrelia* groups, it forms its own clade within the genus *Borrelia* and has unknown pathogenic consequences (Loh et al., 2017).

Little penguins (*Eudyptula novaehollandiae*) are native to Australia and are heavily parasitised by *Ixodes* ticks (*I. eudyptidis* and *I. kohlsi*) when breeding. The penguins are also known to harbour *Babesia* spp., which is a protozoan parasite that causes piroplasmosis in vertebrates, and is a common co-infection partner of *B. burgdorferi* in North America (Dunn et al., 2014; Diuk-Wasser et al., 2016; Walter et al., 2016). To date, there has only been one human babesiosis fatality due to the tick-borne protozoan, *Babesia microti* (Senanayake et al., 2012), which is genetically distinct from the *Babesia* species described in little penguins. Phillip Island Nature Reserve (Victoria, Australia) represents the largest colony of little penguins, and is also home to a range of other iconic native Australian animals, including echidnas and koalas (Phillip Island Nature Parks, 2015). At least 10 species of ticks from four genera are known to parasitise echidnas, and five of these tick species also exploit other animals and humans (see Fig. 1). Furthermore, *Bothriocroton* ticks known to exploit echidnas have recently been found in penguin burrows at Phillip Island Nature Park (K. L. Moon pers. obs.), suggesting echidnas and penguins on the island may share parasites and associated pathogens (see Fig. 1). The island is also visited annually by migratory seabirds including short-tailed shearwaters (*Ardenna tenuirostris*), which breed in considerable numbers (Phillip Island Nature Parks, 2014). Despite the potential for the presence of a native *Borrelia* species (due to associations with native Australian wildlife), and the presence of *B. garinii* (due to associations with migratory seabirds), no study has previously investigated whether borreliae are cycling in Australian penguin colonies. We screened over 230 *Ixodes* ticks from penguin hosts at Phillip Island for borreliae, representing the first large-scale assessment of the presence of *Borrelia* spp. DNA in ticks from south-eastern Australia.

2. Materials and methods

2.1. Sample collection

A total of 232 *Ixodes* ticks (representing *I. eudyptidis* and *I. kohlsi* species) from 46 little penguin hosts at Phillip Island, Victoria (38.4899° S, 145.2038° E), and two *Ixodes* ticks from two penguins at Montague Island (New South Wales: 36.2510° S, 150.2270° E), were taken directly from the host animal, or from inside their nest burrows, during the course of regular monitoring activities (Moon et al., 2015). Ticks were immediately placed in 96% ethanol for preservation.

2.2. DNA extraction and analysis

Ticks were sorted into categories based on host individual and life cycle stage (unfed nymphs, fed nymphs, unfed males, unfed females and fed females). Genomic DNA (gDNA) extractions were then carried out as described by Gofton et al. (2015a,b), using a Qiagen DNeasy Blood and Tissue Kit, with specimens from the same host and life cycle stage extracted as one sample, leaving a total of 72 pooled samples.

Three *Borrelia*-genus specific nested PCR assays were conducted, targeting two genes (*flaB* and *gyrB*) as described by Loh et al. (2016, 2017) (see Table 1 for primer details). '*Candidatus* B. tachyglossi' genotype B described in Loh et al. (2016, 2017) was used as a positive control in all assays. Template-free controls and extraction reagent blank controls were included at every step in the assays to rule out the possibility of contamination. Amplicons of expected sizes were excised, purified and sequenced as described by Loh et al. (2016). Aligned sequences were compared to previously detected sequences using a BLAST nucleotide search in GenBank (https://blast.ncbi.nlm.nih.gov/BLAST/).

3. Results

Nested PCR assays resulting in amplicons of the correct length were identified in four samples using the *flaB* fragment 1 primers, three samples using the *flaB* fragment 2 primers, three samples using the *gyrB* primers, and in the positive controls. The PCR products of one sample from Phillip Island had amplicons of appropriate sizes for both the *flaB* (fragment 2) and *gyrB* assays. Nested PCR assays amplified the *Borrelia* genes in all (100%) of our positive controls, whereas none of the template-free controls or extraction reagent blank controls produced bands. All amplifications from penguin tick samples resulted in faint bands relative to the positive controls. PCR products from all 10 amplicons were sequenced using BigDye v.3.1 terminator on an ABI 373096 Capillary Sequencer (Life Technologies, USA). Though some amplicons produced clean sequences, these bore no significant similarity to any existing sequences in GenBank, suggesting that they were the result of non-specific primer binding and amplification. *Borrelia* gDNA was therefore not present in any of the ticks sampled from the Phillip Island or Montague Island penguins.

4. Discussion

Using highly conserved genus-specific housekeeping genes (*flaB* and *gyrB*), we found no genetic evidence for the presence of *Borrelia* in over 230 little penguin ticks from Phillip Island Nature Reserve in Victoria, nor in two ticks from Montague Island in New South Wales. Non-detection does not conclusively demonstrate absence, but our large-scale sampling of the Phillip Island colony strongly suggests that *Borrelia* is either absent or has an extremely low prevalence in little penguin ticks at this site.

Unlike the generalist tick *I. uriae*, which is responsible for the