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Molecular surveillance of piroplasms in ticks from small and medium-sized urban and peri-urban mammals in Australia

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ABSTRACT

Natural landscape alterations as a consequence of urbanisation are one of the main drivers in the movements of wildlife into metropolitan and peri-urban areas. Worldwide, these wildlife species are highly adaptable and may be responsible for the transmission of tick-borne pathogens including piroplasms (*Babesia*, *Theileria* and *Cytauxzoon* spp.) that cause piroplasmosis in animals and occasionally in humans. Little is known about piroplasms in the ticks of urban wildlife in Australia. Ticks from long-nosed bandicoots (*Perameles nasuta*; n = 71), eastern-barred bandicoots (*Perameles gunnii*; n = 41), northern-brown bandicoots (*Isoodon macrourus*; n = 19), southern-brown bandicoots (*Isoodon obesulus*; n = 4), bandicoot sp. (n = 2), flying foxes (*Pteropus* sp.; n = 3), black rats (*Rattus rattus*; n = 7), bush rats (*Rattus fuscipes*; n = 4), brushtail possums (*Trichosurus vulpecula*; n = 19), ringtail possums (*Pseudocheirus peregrinus*; n = 12), short-eared possums (*Trichosurus caninus*; n = 6), possum sp. (*Trichosurus* sp.; n = 8), and red foxes (*Vulpes vulpes*; n = 12) were analysed using piroplasm-specific 18S primers and Sanger sequencing. Seven *Ixodes tasmani* ticks from long-nosed bandicoots and bandicoots sp., three *I. tasmani* ticks and one *Ixodes holocyclus* tick from brushtail possums, and one *Haemaphysalis longicornis* tick from a red fox were positive for piroplasms. New genotypes, with sequences sharing 98% nucleotide similarities with *Theileria* sp. K1 detected in a burrowing bettong (*Bettongia lesueur*), were identified from bandicoot ticks. New genotypes were detected in ticks from brushtail possums, which shared 98% similarity with a *Babesia* sp. (JQ682877) previously identified in marsupials. *Theileria orientalis* was identified in the *H. longicornis* tick from the red fox. *Babesia* and *Theileria* spp. in the ticks parasitizing bandicoots and brushtail possums clustered closely with respective *Babesia* and *Theileria* clades derived from Australian marsupials. This represents the first detection of piroplasms in ticks parasitizing brushtail possums and a red fox in Australia.

1. Introduction

Deforestation, habitat fragmentation, and increases in human populations associated with urbanisation inevitably decrease the natural flora and fauna biodiversity (Mackenstedt et al., 2015). While some wildlife species remain urbanophobes, some have emerged as urban adapters or exploiters, and over the years have become familiar inhabitants of our towns and cities. There are various factors that favour the urban-adaptation of certain wildlife species, including the availability of anthropogenic food resources (Oro et al., 2013), shelter (Parris and Hazell, 2005), and reduction in threats from natural predators (Bateman and Fleming, 2012). Constant supplies of season-independent resources enable many successful urban-adapted wildlife species to attain higher population densities compared to their rural counterparts (Bradley and Altizer, 2007). Consequently, the growth in populations of competent hosts, reservoirs and amplifiers of vector-borne pathogens, increases the prevalence of those pathogens and the frequency of human-wildlife interactions, potentially leading to higher rates of zoonotic disease transmission (Bradley and Altizer, 2007).

Although many mammal populations have declined as a consequence of landscape alterations (Baker and Harris, 2007), others have benefitted, especially from the creation of urban environments. In Australia, the most common mammals in urban areas include native brushtail possums (*Trichosurus vulpecula*), ringtail possums (*Pseudocheirus peregrinus*) (Hill et al., 2007), and bandicoots (*Isoodon* and *Perameles* spp.) (Fitzgibbon et al., 2011); as well as introduced wildlife such as European red foxes (*Vulpes vulpes*) (Marks and Bloomfield, 1999). In addition, bats (*Pteropus* sp.) and rats (*Rattus* spp.) also reside

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in Australian metropolitan areas (Plowright et al., 2011; Tait et al., 2014; Banks and Smith, 2015). Individuals of these species make use of urban areas either exclusively or by nesting in remnant bushland and regularly visiting nearby urban habitats (Harper, 2005).

Ticks are one of the most competent arthropod vectors that transmit a vast number of pathogenic bacteria, haemoparasites and viruses that significantly affect the health of human, livestock and companion animals worldwide (Dantas-Torres et al., 2012). Ticks are responsible for a variety of emerging zoonotic diseases and wildlife are often the amplifying hosts for these pathogens (Colwell et al., 2011). The encroachment of wild animals into urban areas as a result of urban development, brings wildlife and their ticks into close proximity with humans, consequently introducing possible spill-over of tick-borne pathogens (Banks and Smith, 2015).

A range of haemoparasites, i.e. piroplasms, have been identified and described in Australian native wildlife (Paparini et al., 2012), and in addition, unnamed species of piroplasm have been detected in brushtailed bettongs (*Bettongia penicillata*), burrowing bettongs (*Bettongia lesueur*), and bandicoots (Paparini et al., 2012; Barbosa et al., 2017). In general, however, knowledge about the piroplasms in Australian wildlife ticks remains scarce and their zoonotic significance is far from being understood at the current time. There have been increasing reports of tick-associated illnesses in Australian humans, including a case of babesiosis reported in an Australian patient who lived in a peri-urban area and had encountered tick bites, but who had not travelled to endemic countries (Senanayake et al., 2012). This emphasises the importance of understanding and examining every aspect of tick-borne disease (TBD), particularly the potential for wildlife ticks to act as reservoirs for human disease.

Urbanised wildlife contribute to the importance and health impact of TBD worldwide, both as transporters of ticks, as well as acting as reservoirs of TBD pathogens (Bradley and Altizer, 2007; Pfäffle et al., 2013; Rizzoli et al., 2014). The occurrence of wildlife in urban and peri-urban settings is becoming more evident (Harper, 2005), increasing the chances of human-wildlife interactions and the potential for human illness via tick bites. Hence, further research into TBD's in wildlife that reside in urban areas together with their ticks is essential. The objective of the present study was to conduct a survey of ticks parasitizing small and medium-sized mammalian wildlife species that are most common in urban environments in Australia, and to identify and characterise any piroplasms present.

2. Materials and methods

2.1. Ethics statements

This study was conducted under the compliance of the *Australian Code for the Responsibility Conduct of Research (2007)* and *Australian Code for the Care and Use of Animals for Scientific Purposes, 2013*. Tick collection was carried out opportunistically with the approval from the Murdoch University Animal Ethics Committee.

2.2. Sample collection and tick identification

Ticks from 18 long-nosed bandicoots (*P. nasuta*; n = 71), two eastern-barred bandicoots (*P. gunnii*; n = 41), five northern-brown bandicoots (*I. macrourus*; n = 19), four southern-brown bandicoots (*I. obesulus*; n = 4), two bandicoot sp. (n = 2), three bat sp. (*Pteropus* sp.; n = 3), three black rats (*R. rattus*; n = 7), four bush rats (*R. fuscipes*; n = 4), seven brushtail possums (*T. vulpecula*; n = 19), six ringtail possums (*P. peregrinus*; n = 12), two short-eared possums (*T. caninus*; n = 6), two possum sp. (*Trichosurus* sp.; n = 8), and one red fox (*V. vulpes*; n = 12) were sampled by veterinarians, wildlife rescuers, and the public from urban and peri-urban areas across Australia. Ticks were preserved in 70% ethanol before shipment to Murdoch University and the species was subsequently identified using morphological keys (Roberts, 1970).

2.3. DNA extraction and amplification

The external tick surface was washed with solutions of 10% sodium hypochlorite followed by 70% ethanol. Genomic DNA (gDNA) was extracted using the DNeasy Blood and Tissue kit (Qiagen) in parallel with the extraction reagent blank (EXB) controls (Loh et al., 2016).

The BTF1/BTR1 and BTF2/BTR2 primers, targeting a 850 bp region of the 18S rRNA gene, were used in a nested-PCR as described previously (Jefferies et al., 2007). All PCR reactions were conducted in parallel with EXB and no-template controls, as well as *Babesia gibsoni* gDNA extracted from a canine blood sample as a positive control. Each 25 μL PCR reaction contained 1x Green GoTaq Flexi buffer, 1.5 mM $MgCl_2$, 1 mM dNTPs, 400 nM of each primer, 1.25U of GoTaq DNA polymerase (Promega, Madison, WI, USA), and 2 μL undiluted DNA. A second set of 18S primers, BT18SF1/BT18SR1 and BT18SF2/BT18SR2 that amplify a longer 18S gene fragment (1466 bp), were used for phylogenetic purposes, on the samples positive in the initial screening, following the conditions described in Paparini et al. (2012). Amplified DNA products were visualised on 1% agarose gels containing SYBR safe under a blue light transilluminator. Bands were then excised and purified using an in house filter tip method (Yang et al., 2013) before Sanger sequencing was carried out using each forward and reverse primer at the Western Australian State Agricultural Biotechnology Centre (SABC).

2.4. Phylogenetic analyses

Raw sequences were trimmed using Geneious version 8.1.9 and MAFFT v7.017 was used to construct multiple alignments with previously described piroplasm nucleotide sequences retrieved from GenBank (Katoh et al., 2002; Kearse et al., 2012). Alignments were then refined using MUSCLE (Edgar, 2004). Alignments of 1701 bp and 856 bp were generated for the reconstruction of the phylogenetic tree and the inset trees, respectively. MEGA6 was used to determine the best nucleotide substitution model on the basis of Bayesian Information Criterion (Tamura et al., 2013). Finally, Bayesian analyses were used for phylogenetic reconstruction of the piroplasm 18S trees, with invgamma rate variation, Gamma categories of five, MCMC length of 1,100,000, burn-in length of 10,000, and subsampling frequency of 200 (Huelsenbeck and Ronquist, 2001). *Plasmodium falciparum* (JQ627152) was used as an outgroup. 18S sequenes generated in this study were deposited into GenBank under the accession numbers: MG251435-MG251440.

3. Results

Of the 137 ticks collected from bandicoots, seven ticks (Table 1) were positive for both short and long piroplasm 18S fragments. Of these, five were from *I. tasmani* nymphs from two long-nosed bandicoots, and a single *I. tasmani* female removed from a bandicoot sp., at the Australia Zoo Wildlife Hospital in Queensland (QLD); and a single female *I. tasmani* from a bandicoot sp. from a veterinary clinic in Port Sorell, Tasmania (TAS). Using the shorter 18S gene primers, amplicons were generated in four brushtail possum ticks; comprising one *I. tasmani* female tick from the Royal Botanical Gardens in New South Wales (NSW), and one *I. holocyclus* and two *I. tasmani* ticks from two brushtailed possums at the Australia Zoo Wildlife Hospital, QLD. Among the 12 tick samples from a red fox from Grosevale, NSW, only a single *H. longicornis* nymph was positive.

Sequence analysis identified three unique genotypes in the bandicoot ticks, designated as *Theileria* sp. B16, *Theileria* sp. B43, and *Theileria* sp. B60. *Theileria* sp. B16 was identified in the *I. tasmani* tick from TAS, and shared 97.1% and 99.9% similarity with *Theileria* sp. B43 and *Theileria* sp. B60, respectively (Supplementary Table S1). Genotype