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Molecular detection and characterisation of *Babesia* and *Theileria* in Australian hard ticks

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ABSTRACT

Babesia and *Theileria* are intraerythrocytic protozoans of the phylum Apicomplexa. These species are capable of infecting wild and domestic animals and have historically caused great economic loss in the agricultural industry. In recent years human babesiosis has been deemed an emerging zoonosis in North America, Europe and Asia. The first locally acquired case of babesiosis in Australia, caused by *Babesia microti*, was reported in March 2012. A number of native *Babesia* and *Theileria* species have been identified in Australian marsupials, however their associated tick vectors and threat to human health is unknown. In the present study DNA was extracted from 1154 ticks collected from across Australia. PCR was used to amplify a *Babesia* and *Theileria*-specific partial region of the 18S ribosomal RNA gene. Positive samples were sequenced and phylogenetic analysis was performed. Twenty-nine sequences were obtained from ticks belonging to the genera *Ixodes*, *Haemaphysalis* and *Bothriocroton*. The sequences were closely related to *Babesia macropus,* and *Theileria* recently identified in marsupials and monotremes. Bayesian inference and maximum likelihood methods showed that Australian *Babesia* and *Theileria* species form monophyletic groups.

1. Introduction

Piroplasms are intraerythrocytic protozoal parasites of the phylum Apicomplexa, order Piroplasmorida. Two important genera of the Piroplasmorida are *Babesia* and *Theileria*. *Babesia* and *Theileria* spp. infect a wide range of wild and domestic animals, and include agriculturally and economically important pathogens. Piroplasms are transmitted by species of the hard tick family Ixodidae (Mans et al., 2015). Barker et al. (2014) list 56 species of hard ticks found in Australia with species from five genera, *Ixodes*, *Haemaphysalis*, *Rhipicephalus*, *Amblyomma* and *Bothriocroton*.

The *Theileria* species, *T. annulata* and *T. parva* cause significant disease in cattle and are endemic in Africa. Locally endemic to Australia, species of the *T. orientalis* group (*T. buffeli*, *T. sergenti* and *T. orientalis*) also cause bovine theileriosis. The vector of theileriosis in Australia is *Haemaphysalis longicornis* (Hammer et al., 2015; Kamau et al., 2011). Bovine piroplasmosis is also caused by *B. bovis* and *B. bigemina*. Bovine babesiosis was first recognised in Australian cattle in Far North Queensland in the late 19th Century (Mackerras, 1959). The parasites were believed to have been brought into Northern Australia via traffic of infected cattle from Java (Indonesia) to Darwin, Northern Territory (Australia) in 1872 (Mackerras, 1959). *Rhipicephalus australis* is an established vector of these species (Bock et al., 2004). Bovine piroplasmosis in Australia is controlled via a vaccination program.

Human babesiosis is considered to be an emerging zoonosis where a small group of species have been identified as causes of illness. Clinically relevant species include *B. microti*, *B. divergens* and *B. duncani* (Vannier and Krause, 2012). Human babesiosis is described as having a Holarctic distribution with infection prevalent in North America, Europe and Asia. Vectors include members of the genus *Ixodes.* Disease presents with symptoms similar to malaria, including, but not limited to, fever, chills and lethargy. In severe disease haemolytic anaemia may occur. Disease severity depends on the immune status of the patient and the species of *Babesia* involved. The first locally acquired case of human babesiosis in Australia was reported in 2012 where the causative organism was identified as *B. microti* (Senanayake et al., 2012). At present, *B. microti* has not been reported in Australian ticks.

Piroplasms have been identified, via morphological and molecular methods, in a wide range of native Australian animals (Table 1). Piroplasms resembling *Babesia* sp. have also been described in a Proserpine rock wallaby (*Petrogale persephone*), a brown antechinus (*Antechinus stuartii*), a Bynoe's gecko (*Heteronotia binoei*), a stern ctenotus (*Ctenotus severus*), a spotted python (*Bothrochilus maculosus*), a kookaburra (*Dacelo novaeguineae*), a Brahminy kite (*Haliastur indus*), a pied butcherbird

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Table 1
Piroplasms recorded in Australia prior to this study.

Piroplasm	Location	Host	Ticks present	Reference
Theileria tachyglossi	Townsville, QLD Moss Vale, NSW	Echidna (*Tachyglossis aculeatus*)	*Bothriocroton undatum*	Backhouse and Bolliger (1957); Mackerras (1959); Priestly (1915)
Babesia tachyglossi	Mosman, NSW	Echidna (*Tachyglossis aculeatus*)	*Bothriocroton hydrosauri*	Backhouse and Bolliger (1959), (1957)
Babesia thylacis	Innisfail, QLD	Southern-brown bandicoot (*Isoodon obesulus*)	None recorded	Mackerras (1959)
Theileria peramelis Mackerras	Mt Nebo, QLD	Southern brown bandicoot (*Isoodon obesulus*) Long-nosed bandicoot (*Perameles nasuta*) Long-nosed potoroo (*Potorous tridactylus*)	*Ixodes* sp. *Haemaphysalis* sp.	Mackerras (1959)
Theileria ornithorhynchi	Interlaken, TAS Brookfield, QLD Innisfail, QLD Shoalhaven River, NSW Kangaroo River, NSW Moorong, NSW Wynyard, TAS	Platypus (*Ornithorhynchus anatinus*)	*Ixodes ornithorhynchi*	Collins et al. (1986); Kessell et al. (2014); Mackerras (1959); Paparini et al. (2015)
Theileria brachyuri	Bald Island, WA	Quokka (*Seotnix brachyurus*)	None recorded	Clark and Spencer (2007)
Theileria penicillata	Avon Valley, WA	Brush-tailed bettong (*Bettongia penicillata*)	None recorded	Clark and Spencer (2007)
Theileria fuliginosa	Boyup Brook, WA	Western grey kangaroo (*Macropus fulignosis*)	None recorded	Clark and Spencer (2007)
Theileria gilberti	Albany, WA	Gilbert's potoroo (*Potorous gilbertii*)	*Ixodes australiensis Ixodes fecialis*	Lee et al. (2009)
Babesia sp.	Dwellingup, WA	Brush-tailed bettong (*Bettongia penicillata*)	None recorded	Paparini et al. (2012)
Theileria sp.	Kanyana, WA	Boodie (*Bettongia lesueur*)	None recorded	Paparini et al. (2012)
Babesia macropus	Norwell, QLD Calamvale, QLD Townsville, QLD Mid-North Coast, NSW	Eastern grey kangaroo (*Macropus giganteus*) Agile wallaby (*Macropus agilis*)	*Haemaphysalis* sp.	Dawood et al. (2013); Donahoe et al. (2015)
Babesia sp.	Maria Island, TAS	Little penguin (*Eudyptula minor*)	Argasidae sp. Ixodidae sp.	Vanstreels et al. (2015)

(*Cracticus nigrogularis*) and a pied currawong (*Strepera graculina*) (O'Donoghue and Adlard, 2000). In the majority of cases the tick vector of these piroplasms remains unclear.

Typically, piroplasm infection manifests as a subclinical infection in wild animals with reported clinical cases being associated with physiological stress (Schnittger et al., 2012). For instance, anaemia associated with high parasitaemia caused by *Babesia* sp. has been observed in the brown antechinus (*Antechinus stuartii*) and may contribute to the described total mortality in males observed following mating (Barker et al., 1978). Further, Dawood et al. (2013) and Donahoe et al. (2015) reported clinical infection in Eastern grey kangaroos and agile wallabies in eastern Australia where stress likely resulted from capture, restraint and translocation, in addition to a potential lack of maternal antibodies and acquired immunity due to hand-rearing in tick-free environments. In addition, Kessell et al. (2014) describe fatal anaemia in the presence of *T. ornithorynchi* in an orphaned juvenile platypus which was determined to have yet been weaned. The zoonotic potential of these species of *Babesia* and *Theileria* is undetermined.

This study aimed to detect piroplasms in Australian ticks using polymerase chain reaction (PCR) targeting a partial region of the 18S ribosomal RNA gene.

2. Materials and methods

2.1. Sample collection

1163 ticks were collected by the general public, wildlife carers, veterinarians and the authors from native wildlife, domestic animals and livestock, and human hosts, from across Australia. Ticks were collected from 2012 to 2016. Ticks were photographed and allocated a unique identification number. Ticks were identified morphologically using a dichotomous key (Barker and Walker, 2014; Roberts, 1970). Ticks which could not be identified to genus level were excluded from further analysis. Data including collection location, host and engorgement status was also recorded. Where no host was applicable, ticks were recorded as questing. Ticks were stored at −20 °C until required.

2.2. DNA extraction

The surface of each tick was dipped three times in 70% (w/v) ethanol and rinsed in distilled water. Ticks were homogenised using a Geno/Grinder bead mill with a 5 mm stainless steel bead. Nymphal and adult ticks were homogenised individually while larval ticks were pooled and homogenised in groups of 10–20 ticks. Genomic DNA was then extracted using the DNeasy Blood and Tissue Kit (Qiagen, Australia) according to the manufacturer's instructions for animal tissue, with a 16 h initial lysis step. DNA extraction blank controls consisting of sterile PBS were included. Extracted DNA was stored at −20 °C until required.

2.3. Molecular analyses

Tick DNA was screened with a nested set of primers targeting a 431 bp region of the piroplasm 18S gene as previously described (Kim et al., 2013). Positive samples were subjected to an additional nested PCR targeting a 900 bp region of the 18S gene using first round primers designed against piroplasms during this study, and second round primers described by Jefferies et al. (2007). All PCR reactions were performed in a T100 Thermal Cycler (Bio-rad, Australia). All PCR reactions contained a negative control consisting of sterile molecular grade water to control for contamination. Primer sequences used in this study can be found in Table 2.

First and second round screening reactions contained 1X ThermoPol Buffer, 1 mM $MgCl_2$, 200 µM dNTPs, 200 nM of each primer, 1.25 U *Taq* DNA Polymerase (New England Biosciences, Australia), 5 µL of extracted DNA and molecular grade water to a total volume of 25 µL.