Emerging Canine Tick-borne Diseases in Australia and Phylogenetic Studies of the Canine Piroplasmida

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This thesis is presented for the degree of Doctor of Philosophy of Murdoch University

2006
I declare that this thesis is my own account of my research and contains as its main content, work that has not previously been submitted for a degree at any tertiary education institution.

Ryan Jefferies
Canine tick-borne diseases are an emerging problem within Australia and throughout the world. This thesis investigates Babesia gibsoni and Anaplasma platys infections in dogs in Australia and also explores the evolutionary relationships and taxonomy of the canine piroplasm species and the members of the order Piroplasmida.

A nested PCR-RFLP assay was developed for the detection and differentiation of the canine piroplasm species and was found to have a high detection limit, capable of detecting a $2.7 \times 10^{-7}$% parasitaemia or the equivalent of 1.2 molecules of target DNA. Detection of piroplasm DNA applied to Whatman FTA® classic cards using nested-PCR was found to have a lower detection limit than when using DNA extracted from whole blood but higher than IsoCode® Stix or QIAamp extraction from filter paper based techniques. The nested PCR-RFLP assay was further evaluated for the detection of B. gibsoni infection in dogs being exported from Australia to New Zealand and compared to the current screening methods, the Immunofluorescent Antibody Test (IFAT) and microscopy. Of 235 dogs screened, 11 were IFAT positive, 1 was microscopy positive and 3 were PCR positive for B. gibsoni, highlighting the discordance that exists between various detection techniques. Replacing microscopic examination of blood smears with PCR-RFLP is suggested for screening dogs entering New Zealand, in addition to revising the current IFAT cut-off titre to minimize false positive results. The first case of B. gibsoni in New South Wales is also reported.

A study was also conducted to further investigate the recent discovery of B. gibsoni in Australia and the association of this infection with American Pit Bull Terriers in an epidemiological study. Both American Pit Bull Terriers (n = 100) and other dog breeds (n =
were screened for *B. gibsoni* using IFAT and PCR-RFLP. A questionnaire was also completed by each dog owner regarding the husbandry and habits these dogs. Fourteen dogs were positive for *B. gibsoni* using IFAT and/or PCR-RFLP and all were American Pit Bull Terriers. Dogs that were male and/or were bitten by or were biters of other American Pit Bull Terriers were statistically more likely to be *B. gibsoni* positive, thus suggesting that blood-to-blood transmission may contribute to the spread of this disease.

Experimental *B. gibsoni* infections were established *in vivo* to investigate the efficacy of combined atovaquone and azithromycin therapy and to determine the detection limits of PCR, IFAT and microscopy during various stages of infection. While atovaquone and azithromycin produced a reduction in circulating parasite levels, it did not cause total eradication, and possible drug resistance also developed in one dog. PCR was found to be most useful in detecting early and acute stage infections, while IFAT was most useful during chronic and acute infections. Microscopy is suggested to be only useful for detecting acute stage infections. This study also describes the detection of *B. gibsoni* in tissue samples during chronic infection for the first time, suggesting possible sequestration of this parasite.

*Anaplasma platys* has also only recently been reported in Australia and the distribution, molecular-characterisation, pathogenesis, co-infection with *Babesia canis vogeli* and treatment of infection with doxycycline were investigated. For the first time, *A. platys* is reported in Western Australia, Queensland and Victoria, with each isolate found to be genetically identical on the basis of the 16S rRNA gene. No correlation could be established between *A. platys* infection and the development of clinical signs or pathogenesis and definitive treatment using doxycycline could not be determined.

Isolates of canine piroplasms from various geographical locations worldwide (n = 46), including Australia were characterised on the basis of multiple gene loci to explore the distribution, genetic variation and possible phylogeographical relationships of these species.
Separate genotypes of *B. canis vogeli*, *B. canis canis* and *B. gibsoni* are suggested and may be correlated to different geographical origins. Characterization of *B. canis vogeli*, *B. canis canis* and *B. canis rossi* on the basis of the HSP 70 gene and *B. gibsoni* on the basis of the ITS 1, 5.8S rRNA gene and ITS 2 is described for the first time. Elevation of each of the *B. canis* subspecies, with the exclusion of *B. canis presentii*, to separate species is also proposed.

The current paraphyly and taxonomic confusion associated with the members of the order Piroplasmida led to a review of the phylogenetic and taxonomic status of this group of organisms. Phylogenetic relationships are determined using 18S rRNA gene, 5.8S rRNA gene, HSP 70 gene and combined loci analyses. Rearrangement of the Piroplasmida into three families, including the new family Piroplasmidae is proposed, in addition to the establishment of two new genera, the *Piroplasma* (Patton, 1895) and the *Achromaticus* (Dionisi, 1899). Other proposed schemes of classification and the limitations of phenotypic characteristics in taxonomic classification within the Piroplasmida are also discussed.
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Funding for aspects of this study was kindly provided by the Australian Companion Animal Health Foundation. Greatful acknowledgment is also given to the Australian Society for Parasitology for providing financial assistance for travel to the Annual Scientific Conference in Hobart and for the travel award that enabled me to attend the IX European Multicolloquium of Parasitology in Valencia, Spain

I am indebted to my supervisors, Associate Professors Peter Irwin and Una Ryan, who have provided me with regular support, endless ideas and tireless encouragement. Thanks Peter for your veterinary expertise, constant enthusiasm, integral research network, field trips and of course, the occasional midnight drug shift. Your mentoring and motivation has been way beyond that expected of a supervisor. Thanks Una for your kind and caring nature, positive praise and for teaching me so much of my technical knowledge. You are inspiring as a molecular biologist and as someone who really knows how to have fun and ‘dance like nobodies watching’.

Many thanks are due to my overseas collaborators who have provided me with important samples and expertise, which have made this PhD project possible. These people include, Yeoh Eng Cheong, Lucia O’Dwyer, Angel Criado-Fornelio, Robert Puentespina, Michael Goodlet, Brad Easton, Nalinika Obeyesekere, Cynthia Lucidi, Graciela Oliver, Gad Baneth, Linda Jacobson, Monika Zahler, Akos Mathe, Gabor Foldvari, Edward Breitschwerdt, Barbara Hegarty, Adam Birkenheuer, Sue Shaw and Martin Kenny. Thanks also to Sandra, Myles and Salim for providing me with accommodation while overseas.
Many thanks are also due to John Jardine and all the staff at Vetpath Laboratories, who conducted IFA testing and collected numerous samples for this research. To Lynne Chambers, the RAAF in Darwin, Patrick Drury, Sue Jaensch, Carl Muhlnickel, staff at IDEXX laboratories and Louise Jackson. Thanks also to Mark Lewis and all the American Pit Bull Terrier owners who contributed blood samples and questionnaire information.

To all the people at Murdoch University who have helped with various aspects of this project; Ian Robertson, Francis Brigg, Andy Thompson, Russ Hobbs, Rebecca Traub, Clare Constantine, Marion Macnish, Simon Reid, Zablon Njiru, Phil Clark and the staff at clinical pathology and the animal isolation house.

A special acknowledgement is given to my experimental dogs, Yum Yum, Pitti Sing and Peep Bo. Rest in peace.

Thank you also to everyone who has shared an office or lab bench-space with me, especially Chee Kin, Jeremy, Bong, Jill, Michael, Nicolai, Mark, Josh, Clare, Jo, Susannah, Carolyn and Celia. And to my PhD buddy Natalie, thanks for all those chats (and bitch sessions!) in the department corridors and I must confess, I owe you a carton of beer!

To my family Peter, Kate and Mal, and especially Jane, who has always helped me get through the rough times. To Linda and Francois, thanks for the many quiet beers and games of pool at the Seaview and all the other fun times we have had together! To the fantastic Meredith and Kim, thank you for being such wonderful friends and enduring the good, bad and just plain crazy! And to Andrew (aka couch boy), you’re an absolute star!

This thesis is dedicated to Alice Mary Paisley-Kerr for cultivating my thirst for knowledge - you are an inspiration.
ABBREVIATIONS AND UNITS

Abbreviations

ANOVA  univariate analysis of variance
AQIS  Australian Quarantine and Inspection Service
BSA  bovine serum albumin
CICT  canine infectious cyclic thrombocytopenia
DMSO  dimethyl sulphoxide
DNA  deoxyribonucleic acid
dNTP  deoxynucleotide triphosphate
EDTA  ethylenediaminetetraacetic acid
ELISA  enzyme linked immunosorbent assay
FTA  Flinders Technology Associates
HCT  haematocrit
HGB  haemoglobin
HSP  heat shock protein
ICZN  International Code of Zoological Nomenclature
IFAT  immunofluorescent antibody test
ITS  internal transcribed spacer
LAMP  loop-mediated isothermal amplification method
MAFNZ  Ministry of Agriculture and Forestry, New Zealand
MCV  mean cell volume
MPV  mean platelet volume
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PCV  packed cell volume
PLT  platelet number
Q-PCR  quantitative polymerase chain reaction
RCC  red cell count
RFLP  restriction fragment length polymorphism
RNA  ribonucleic acid
rRNA  ribosomal ribonucleic acid
sp.  species (singular)
spp.  species (plural)
TP  total protein
UV  ultraviolet light
WBC  white blood cell count
### List of Units

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Publications

The following publications have been drafted for submission:

**Jefferies R.,** Ryan U.M. and Irwin P.J. Development of a nested PCR-RFLP for the detection and differentiation of the canine piroplasm species and its use with filter paper-based technologies

**Jefferies R.,** Ryan U.M., Jardine J.E, Broughton D.K. and Irwin P.J. Detection of Babesia gibsoni infection in dogs travelling from Australia to New Zealand


**Jefferies R.,** Ryan U.M., J. Jardine and Irwin P.J Experimental Babesia gibsoni infection for the assessment of atovaquone and azithromycin therapy and the detection limits of PCR during various stages of infection

**Jefferies R.,** Ryan U.M., Chambers L., Robertson I.D. and Irwin P.J. Anaplasma platys and Babesia canis vogeli infections in military German Shepherd dogs from northern Australia.


**Jefferies R.,** Ryan UM, O’Dwyer LH., Oliver G. and Irwin PJ. Further molecular characterisation of Babesia canis isolates from South America

**Jefferies R.,** Ryan UM, Jacobson L, Baneth G, Mathe A, and Irwin PJ. Proposed re-classification of the Babesia canis subspecies, including elevation of each to a species level of classification.
Jefferies R., Ryan U.M. and Irwin P.J. A review of the taxonomic status of the order Piroplasmida

Abstracts in conference proceedings


