

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

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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.

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ABSTRACT

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 2.7×10^{-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 =

51) 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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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

| | |
|-----------------|---------------------------|
| nt | nucleotide |
| bp | base pair |
| C | degrees celsius |
| cm ² | square centimetres |
| x g | times gravity |
| rpm | revolutions per minute |
| OD | optical density |
| nmol | nanomole |
| pmol | picomole |
| mol | mole |
| V | volts |
| g | gram |
| mg | milligram |
| hr | hour |
| min | minutes |
| sec | seconds |
| L | litre |
| ml | millilitre |
| µl | microlitre |
| M | molar |
| mM | millimolar |
| mg/ml | milligrams per millilitre |
| U/ul | Units per microlitre |
| U | Units |

PUBLICATIONS AND CONFERENCES

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

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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

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