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Research Brief

Molecular confirmation of the first autochthonous case of human babesiosis in Australia using a novel primer set for the beta-tubulin gene

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HIGHLIGHTS
- We design new primers for the amplification of Babesia microti beta-tubulin gene.
- We reconstruct the phylogeny of the first isolate of B. microti from Australia.
- The first Australian isolate of B. microti shows homology to known genotypes.

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ABSTRACT

In 2012, the first autochthonous Australian case of human babesiosis was reported, after microscopic examinations of blood samples revealed intra-erythrocytic parasites in a hospitalized 56 year-old man from NSW, who died in 2011 (Senanayake et al., 2012). Independent molecular analyses carried out in Australia and the USA, identified Babesia microti at the 18S ribosomal RNA (18S rRNA), and the beta-tubulin (β-tubulin) gene loci. Here we present the details of a novel PCR-based assay for the β-tubulin gene that was developed, during the original study, to corroborate the results obtained from the analysis of the 18S rDNA. The complete phylogenetic reconstruction, based on the two loci sequenced from the Australian clinical isolate, is also shown here for the first time.

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1. Introduction

Protozoa of the genus Babesia (Piroplasmida, Apicomplexa) are thought to be the second most common blood-borne parasites of mammals after the trypanosomes (Telford III and Maguire, 2006). Over 100 species have been identified, infecting many mammalian and some avian species (Schnittger et al., 2012; Uilenberg, 2006). Babesia spp. are the aetiologic agents of babesiosis, a tick-transmitted disease causing a significant global socio-economically burden in humans, livestock and companion animals.

Human babesiosis is a globally emerging zoonosis. Clinical features mainly include: fever, fatigue, headache, chills, neck stiffness,
accompanied by haemolytic anaemia, and possibly organ failure. With exceptions, human babesiosis is caused predominantly by *Babesia microti* (in the USA) and *B. divergens* (in Europe), but, prior to 2012, has never been found in Australia (Hunfeld et al., 2008).

The first Australian case of human babesiosis was reported in 2012, after microscopic examination of blood samples from a hospitalized 56-year-old man from NSW (who died in 2011), revealed intra-erythrocytic parasites (Senanayake et al., 2012). Traditionally, the extensive genetic variability, at the 18S ribosomal RNA (18S rRNA), and beta-tubulin (β-tubulin) loci, has been exploited to develop diagnostic molecular tests and reconstruct piroplasm phylogeny (Cacciò et al., 2000; Schnittger et al., 2012). During the 2012 Australian investigation, these two markers were successfully utilised, but the details of the molecular analyses were not included in the original report (Senanayake et al., 2012). Similarly, although BLAST searches and preliminary analyses conducted suggested a strong similarity between the Australian genotype and other variants available in GenBank, detailed phylogenetic reconstructions (including trees) were not presented (Senanayake et al., 2012).

Thus, the present paper deals with the description of the molecular confirmation of the first diagnosis of babesiosis in Australia, and the development of a novel PCR-based assay at the β-tubulin locus, to corroborate the results obtained from the analysis of the 18S rDNA, and reconstruct the phylogeny of the isolate.

### 2. Material and methods

#### 2.1. Beta-tubulin primer design

Iterative alignments and phylogenetic reconstructions, based on several known *B. microti* sequences available from GenBank (*n* = 50), were carried out using Clustal W (Larkin et al., 2007). MEGA 5 (Tamura et al., 2011), and Geneious 7.0.4 (Biomatters Ltd., NZ). Two consensus sequences were generated based on two clusters (namely I and II) of highly similar β-tubulin genotypes, and five novel PCR primer sets were designed using Primer3 (Rozen and Skaletsky, 2000) (Fig. 1, and Table 1 primers).

#### 2.2. Molecular analyses

Genomic DNA was isolated from whole blood/EDTA (100 μL), using the MasterPure Purification Kit (Epicentre Biotechnologies, USA), and resuspended in 50 μL of TE buffer. Three blood samples were collected from the patient within a week. Sequencing of the piroplasm-specific 18S rDNA complete locus was achieved, independently, by two laboratories in USA and Australia, using various sets of PCR primers, as previously described (Senanayake et al., 2012).

The novel β-tubulin primer sets were used to amplify 1 μL of genomic DNA from the patient. A gradient PCR protocol, with six temperature steps, and the following amplification conditions, were used in a G-Strom thermal cycler (G-Strom, UK): 94 °C – 5 min; then 40 cycles of: 94 °C – 30 s, 55–62 °C – 30 s, 72 °C – 1 min; followed by 72 °C – 7 min and 4 °C – hold. PCR products were run on a 1% agarose gel containing SYBR Safe Gel Stain (Invitrogen, USA), and visualised with a dark reader trans-illuminator (Clare Chemical Research, USA). Each reaction (25 μL) included: 0.25 mM of each primer, 1.5 mM MgCl₂, and 0.002 U Kapa Taq (Kapa Biosystems, USA). PCR products corresponding to the expected length were excised from the gel, purified using a MO BIO UltraClean DNA purification kit (MO BIO Laboratories, USA) and sequenced using an ABI Prism Terminator Cycle Sequencing kit (Applied Biosystems, USA), on an Applied Biosystem 3730 DNA Analyzer. Sequences obtained were submitted to GenBank with Acc. No. JX417370 (18S rDNA), and JX417371 (β-tubulin).

#### 2.3. Phylogenetic reconstruction

Phylogenetic analyses by the Maximum Likelihood (ML) method (500 replicates), were performed in MEGA 5 (Tamura et al., 2011), and by PhyML (Guindon et al., 2010) on the Phylogeny.fr platform (Dereeper et al., 2008). Nucleotide substitution models were chosen based on the Bayesian Information Criterion (BIC), calculated in MEGA 5. All positions containing gaps and missing data were eliminated.

For 18S rDNA, the evolutionary history was inferred based on the HKY85 model (Hasegawa et al., 1985). A discrete Gamma distribution was used to model evolutionary rate differences among

Fig. 1. Binding sites of the novel β-tubulin primers. Map of the binding sites for the novel primer sets designed. Two clusters, namely I and II (*n* = 15 and 6 sequences), including highly similar variants of the β-tubulin gene available from GenBank, were chosen to generate a consensus sequence (one for each cluster), and design the novel PCR primer sets. Base numbers refer to the alignment used during primer design.

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sites (6 categories (+G, parameter = 0.15)). The analysis involved 24 nucleotide sequences, and 907 total positions in the final dataset.

For β-tubulin, the evolutionary history was inferred based on the Tamura 3-parameter model (Tamura 1992). A discrete Gamma distribution was used to model evolutionary rate differences among sites (6 categories (+G, parameter = 0.45)). The analysis involved 33 nucleotide sequences, and 781 total positions in the final dataset. Codon positions included were 1st + 2nd + 3rd + Noncoding.

2.4. Specificity and sensitivity testing

A specificity test was carried out using 1 μL of genomic DNA, extracted from various frozen animal bloods (n = 16). Screened animal hosts included: mice (n = 2; *B. microti* positive by microscopy), woylies (*Bettongia penicillata ogilbyi*; n = 10; *Babesia* sp. positive by PCR/microscopy), a boodie (*Bettongia lesueurii*; n = 1; *Theileria* sp. positive by PCR/microscopy), and dogs (n = 3; *Babesia* sp. positive by microscopy).

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Table 1

Oligonucleotides for the molecular detection of *Babesia microti* β-tubulin gene, designed during this study. The column “cluster” refers to the two major groups of sequences (arbitrarily named cluster I and II, with no systematic validity), which were identified while designing the β-tubulin primers. For each oligonucleotide the melting temperature (Tm) is indicated, while product length refers to the amplicon product, expected for each primer pair.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Primer name</th>
<th>Primer pair ID</th>
<th>Sequence (5’→3’)</th>
<th>Tm</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>BMTub2</td>
<td>104F</td>
<td>A</td>
<td>TGGACCTAGACCTGGTACCATGG</td>
<td>58.97</td>
</tr>
<tr>
<td></td>
<td>BMTub2</td>
<td>855R</td>
<td>B</td>
<td>TGCTCTCTGCTGCTGAGGAAGCA</td>
<td>60.24</td>
</tr>
<tr>
<td></td>
<td>BMTub2</td>
<td>190F</td>
<td>C</td>
<td>TGCCATAGTGGACGAGGCTTGGTT</td>
<td>57.02</td>
</tr>
<tr>
<td></td>
<td>BMTub2</td>
<td>601R</td>
<td>D</td>
<td>TGCCATAGTGGACGAGGCTTGGTT</td>
<td>57.02</td>
</tr>
<tr>
<td></td>
<td>BMTub2</td>
<td>496F</td>
<td>E</td>
<td>TGCCATAGTGGACGAGGCTTGGTT</td>
<td>57.02</td>
</tr>
<tr>
<td>II</td>
<td>BMTub1</td>
<td>71F</td>
<td>F</td>
<td>GGACCTAGACCTGGTACCATGG</td>
<td>58.97</td>
</tr>
<tr>
<td></td>
<td>BMTub1</td>
<td>917R</td>
<td>G</td>
<td>TGCTCTCTGCTGCTGAGGAAGCA</td>
<td>60.24</td>
</tr>
<tr>
<td></td>
<td>BMTub1</td>
<td>73F</td>
<td>H</td>
<td>TGCCATAGTGGACGAGGCTTGGTT</td>
<td>57.02</td>
</tr>
<tr>
<td></td>
<td>BMTub1</td>
<td>789R</td>
<td>I</td>
<td>TGCCATAGTGGACGAGGCTTGGTT</td>
<td>57.02</td>
</tr>
</tbody>
</table>

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Fig. 2. Phylogenetic analysis of the 18S rDNA gene. Phylogenetic analysis of the 18S rDNA gene obtained with the Maximum Likelihood (ML) method (500 replicates). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The sequence analysed during this study [gi | 407316676 *B. microti* FB (human) AUSTRALIA] is underlined and in bold.
Most of the marsupial samples (7 woylies and the boodie) had been certified as piroplasm-positive during a previous study (by PCR/sequencing) (Paparini et al., 2012). The remaining samples were selected for the specificity test, based on unpublished microscopic screenings of blood films, which had revealed the presence of possible piroplasm-like inclusions. Based on the performance in the preliminary trials, and the potential informative value of the expected amplicon (847 bp), primer set D (BMTub1|71F/BMTub1|917R; Table 1) was selected.

A sensitivity test was carried out starting from two independent 1:10 serial dilutions of one of the patient’s genomic DNA preparations (piroplasm-positive; neat concentration: 40 ng/µL). DNA was diluted down to 4 pg/µL (i.e., 0.0001X), and each replicate serial dilution amplified with all 5 primer pairs. For each 25 µL PCR reaction, 1 µL of DNA (neat or diluted) was used.

For both tests, thermal cycling conditions were the same as in the gradient PCR test, except that annealing temperature was 55 °C.

3. Results

The ML tree based on the partial 18S rDNA fragment obtained at Murdoch University (907 total positions in the final alignment) showed that the Australian human isolate was, genotypically, highly similar to Asian and North American variants, isolated from rodents and humans (Fig. 2). When the full length 18S consensus was successively assembled from contigs independently obtained (lengths range: 154–1767 bp) (Senanayake et al., 2012), the Australian isolate showed 100% homology to the unpublished B. microti isolate Gray (Acc. No. AY693840), isolated from a lab host (hamster).

All the five novel primer sets provided positive amplification from the patient’s DNA, particularly at the lowest range of annealing temperatures (e.g., 55.1–55.6 °C) (data not shown). The five partial β-tubulin sequences obtained (318–798 bp), were assembled into a consensus sequence of 798 bp. This was used to confirm the morphological and 18S rDNA-based identifications of B. microti from the patient’s blood (Senanayake et al., 2012).
4. Discussion

As with any novel molecular assay, the reaction conditions, reagent’s concentration, and thermal cycling protocol implemented during this study, may require further validation when used in combination with other piroplasm isolates. Despite this, the PCR assays developed provided highly efficient amplification of a partial fragment of the β-tubulin gene, from a human-derived B. microti strain. Interestingly, from the phylogenetic reconstruction, the Australian clinical isolate was more closely related to the sequences from cluster I, identified during primer design (see Section 2), but the molecular protocol adopted allowed positive amplification, using any set of oligonucleotides (i.e., also those designed based on the consensus sequence obtained from cluster II).

This shows that the primers may be used to amplify different genetic variants of human B. microti isolates efficiently. This result is also consistent with the fact that the primers were designed based on an alignment of human isolates; however, screening of additional samples, positive for a broader range of piroplasm taxa, would be recommended. The mouse blood sample, despite having previously tested positive for B. microti by microscopy, was older than 5 years, and possibly unsuitable for further analysis.

The novel β-tubulin primers sets presented in this paper proved useful for confirming the 18S rDNA-based identification of the first case of human babesiosis in Australia. Interestingly, the patient’s history suggested that the infection was locally acquired following a tick bite, because, due to general ill-health, he had not worked or travelled for many years (>40). It thus seemed unlikely that the babesiosis was acquired from overseas. The new molecular tools described in the present report may prove beneficial in unravelling the intriguing questions about the origin and epidemiology of this pathogen in Australia. For example, none of the currently recognised tick vectors of B. microti are known to exist in Australia, and it is suggested that future studies should determine whether such ticks could have become established in enzootic foci within the country, or, alternatively, whether Australian native ticks are competent vectors for this species of piroplasm. Furthermore, with transfusion-transmitted babesiosis (TTB) currently recognised as the most common transfusion-associated infection in the United States (Young et al., 2012), the implications of this finding for the Australian blood transfusion service merit investigation.