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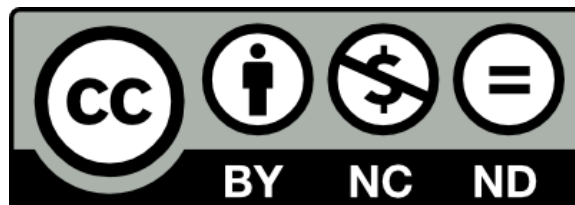
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First report of *Trypanosoma vegrandis* in koalas (*Phascolarctos cinereus*)

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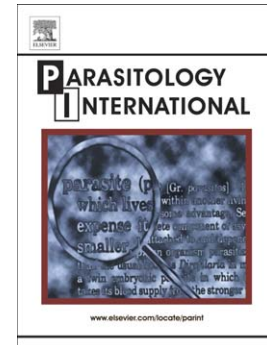
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Short Communication**First report of *Trypanosoma vegrandis* in koalas (*Phascolarctos cinereus*).**

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Abstract

The present study describes the first report of *Trypanosoma vegrandis* in koalas using morphology and sequence analysis of the 18S rRNA gene. The prevalence of *T. vegrandis* in koalas was 13.6% (6/44). It is likely that the small size of *T. vegrandis* (<10 µm in length), coupled with the difficulties in amplifying DNA of this parasite in mixed infections using trypanosome generic primers, are the reason why this organism has not been identified in koalas until now. This study highlights the importance of further research comprising a larger sample size to determine the prevalence of *T. vegrandis* in koalas as well as its potential impacts upon this marsupial species' health.

Keywords: Koalas; *Trypanosoma vegrandis*; 18S rRNA; microscopy

Trypanosomes are flagellated blood parasites transmitted primarily by haematophagous arthropods, which are able to infect all classes of vertebrates. Relatively little is known about trypanosome species infecting Australian mammals with only eight species formally identified to date, three of them (*Trypanosoma irwini*, *T. copemani* and *T. gilletti*) in the koala (*Phascolarctos cinereus*) [1-3].

The known geographical range of *T. vegrandis* currently includes Western Australia (WA) and New South Wales (NSW) and its host range includes the woylie/brush-tailed bettong (*Bettongia penicillata*), western grey kangaroo (*Macropus fuliginosus*), southern brown bandicoot (*Isodon obesulus*), tammar wallaby (*Macropus eugenii*), western quoll (*Dasyurus geoffroyi*), black flying-fox (*Pteropus alecto*), little red flying-fox (*Pteropus scapulatus*), Gould's wattled bats (*Chalinolobus gouldii*) and a lesser long-eared bat (*Nyctophilus geoffroyi*) [4-8]. *Trypanosoma vegrandis* is believed to be the smallest trypanosome species formally described from mammals (8.3 µm in average length and 1.3 µm in average width) [6].

The koala (*Phascolarctos cinereus*), despite being an Australian icon, continues to experience alarming declines in numbers, leading to localized extinctions. It is currently classified as vulnerable to extinction in New South Wales (NSW) under the NSW Threatened Species Conservation Act 1995 No 101 as Schedule 2 'Vulnerable species' [9], and in Queensland (Qld) under the Qld Nature Conservation (Wildlife) Regulation 2006 as Schedule 3 'Vulnerable wildlife' [10]. Previous research has shown that trypanosomes, in particular *T. gilletti*, were associated with indicators of koala ill-health and non-survival [11], and therefore further research on the identification, prevalence and clinical impacts of *Trypanosoma spp.* upon wild koala populations is important [11]. In the present study we report, for the first time, the identification of *T. vegrandis* in the koala using morphological and molecular analysis.

A total of 44 blood samples were collected during routine clinical procedures from koalas that presented to the Australia Zoo Wildlife Hospital (AZWH) at Beerwah, Qld between December 2010 and December 2011, most of which originated from south-east Qld or northern NSW. The sampling was conducted under Murdoch University Animal Ethics Committee permit number W2284/09.

Thin-blood smears were also made at the time of blood collection and stained with Wright-Giemsa stain (Hematek® Stain Pak) within 5 minutes of blood collection. Digital light micrograph images of any trypomastigotes observed in blood films were taken at $\times 1000$ magnification. Morphological measurements (total length, breadth, kinetoplast to anterior (KA), kinetoplast to nucleus (KN), posterior to kinetoplast (PK) and free flagellum (FF) of the trypomastigotes were made using Image-Pro Express software (Media Cybernetics, U.S.A.) and the means and standard errors were calculated. Morphological features were measured and compared with available morphological measurements for trypanosomes infecting Australian marsupials.

For molecular analysis, whole genomic DNA was extracted from 200 μ l of koala blood using a MasterPure™ DNA Purification Kit (EPICENTRE® Biotechnologies, U.S.A.). DNA was eluted in 35 μ l of TE buffer and stored at -20°C until use. A range of molecular techniques using *T. vegrandis*-specific primers and generic *Trypanosoma sp.* primers (i.e. primers designed to amplify a range of trypanosomatids) were used to detect both *T. vegrandis* and co-infecting trypanosome species. An approximately 350 bp fragment of *T. vegrandis* 18S rDNA was amplified using a *T. vegrandis*-specific nested PCR with the forward primer TVEF and reverse primer TVER and the internal primers, TVIF and TVIR as previously described [5]. Preliminary results however revealed that these primers also amplified *T. gilletti*, presumably due to the close genetic similarity between these two species. For this reason, an alternative PCR methodology was developed to amplify *T.*

vegrandis from a sample (Timbo) that was positive by microscopy for *T. vegrans*, but which was co-infected with *T. gilletti*, as initially only *T. gilletti* could be amplified using the *T. vegrans*-specific nested PCR. Therefore, an approximately 900 bp fragment of the 18S rRNA gene was amplified using primers S823F and S662 sourced from Maslov et al. [12], as external primers according to McInnes et al. [2]. The PCR product from these external primers was run on a 2% agarose gel stained with SYBR safe (Invitrogen, USA). The gel band was excised and purified using an in-house filter tip method as previously described [13] and 1 µl of gel-purified PCR was used in a secondary reaction with internal primers TVIF and TVIR [5]. In order to detect co-infections with other trypanosome species, all samples that were positive for *T. vegrans* using the *T. vegrans*-specific primers were also screened by nested PCR using generic Trypanosoma sp. primers (TRY927F, TRY927R, SSU561F and SSU561R) that amplify an approximately 927 bp fragment of *Trypanosoma* 18S rDNA as previously described [14].

All positive *T. vegrans*-specific PCR products were purified using the filter tip method previously described [13] and sequenced in both directions using the *T. vegrans*-specific internal forward TVIF and reverse TVIR primers [5] with an ABI PrismTM Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, U.S.A.) on an Applied Biosystem 3730 DNA Analyzer. For amplicons generated using the universal trypanosome primers, sequencing was conducted using the internal PCR primers SSU561F and SSU561R [14].

Using light microscopy, the morphology of *T. vegrans* in blood films from the koala, represented short stumpy lanceolate shaped trypanosomes, with no undulating membranes (Fig. 1a and b). The trypanosomes generally lacked a clearly defined kinetoplast and nucleated region and were observed as polymorphic. The posterior end was long and drawn out and tapered into a point. The anterior end was more rounded and contained a short

free flagellum. The measurable morphological dimensions of the two trypanosomes in the koala from Figure 1a ranged in total length between 5.8 - 8.29 μm and in breadth between 0.9 - 1.3 μm . The measurable morphological dimensions of the larger trypanosome, which contained a faintly stained nuclear region (Fig.1b) was 9.98 μm in total length, 1.79 μm in breadth, 6.91 μm from posterior to nucleus, 2.85 μm from nucleus to anterior and 4.17 μm in free flagellum.

The PCRs conducted on the 18S rRNA locus using *T. vegrandis*-specific internal primers confirmed the presence of *T. vegrandis* in 6 out of 44 koalas examined, a total prevalence of 13.6%. Six partial 18S rDNA sequences (222 – 283bp) were obtained from each of the positives and submitted to GenBank under the following accession numbers (KP271047- KP271052). Phylogenetic analysis was conducted using maximum likelihood (ML) in MEGA 6 (<http://megasoftware.net/>). Two isolates (Timbo and K55) exhibited 100% similarity to *T. vegrandis* isolate G7 (KC753536) and two (K9 and K10) were 100% identical to *T. vegrandis* isolate G6 (KC753535), both of which were from woylies. Two isolates (K37 and K59) grouped within the *T. vegrandis* clade but exhibited 3 and 4 single nucleotide polymorphisms (SNP's) from *T. vegrandis* isolate G7 (KC753536), respectively (data not shown).

The universal trypanosome nested PCR by Noyes et al. [14] revealed that 3 isolates (K10, K55, K59), were also positive for *T. irwini* and mixed chromatograms were obtained for samples K9 and K37. The presence of *T. gilletti* was confirmed in isolate Timbo, which had originally been detected as positive for this trypanosome species using the *T. vegrandis* species-specific nested PCR as previously reported [5].

This is the first report of *T. vegrandis* in koalas, a finding that was confirmed using morphology and molecular analysis. In the present study, a clearly defined kinetoplast could not be observed in the trypomastigotes probably due to the small size of *T. vegrandis*, which

can limit an accurate identification of internal structures, when compared to larger trypanosome species. However, the images and morphological dimensions presented in this study are clearly similar to the *T. vegrandis* trypomastigotes described by Thompson et al. [6]. Although the isolate (Timbo) examined by microscopy had a mixed *T. vegrandis*/*T. gilletti* infection, the trypanosomes measured are much too small to be *T. gilletti*, which has been speculated to measure 47.1 μm in length and 1.2 μm in breath [3]. The identity of *T. vegrandis* was also confirmed using sequence analysis of the 18S rRNA gene, which clearly showed that the koala-derived isolates grouped within the *T. vegrandis* clade.

Mixed trypanosome infections are common in koalas [11] and it has been reported that the use of generic PCR to detect trypanosome infections can mask the presence of less abundant genotypes [5,11]. Initial attempts to amplify *T. vegrandis* using primers that were reported to be specific to *T. vegrandis* [5], amplified *T. gilletti*, which is phylogenetically most closely related to *T. vegrandis* [4,5]. As a result of this, an alternative methodology was developed to amplify *T. vegrandis* using a generic trypanosome external primer set [12], cutting out the resultant PCR band from the gel and then using 1 μl of the purified gel slice with the *T. vegrandis* specific primers [5], for a second round of PCR. The method worked well and may be useful for amplifying *T. vegrandis* from other mixed infections. In the present study, screening of the *T. vegrandis* positive isolates with the universal trypanosome nested PCR by Noyes et al. [14], provided evidence of mixed infections involving *T. vegrandis* and one or more trypanosome species (*T. gilletti* or *T. irwini*). This finding highlights the importance of the use of *T. vegrandis* specific primers and sequencing the products for identification of this parasite species in naturally infected koalas.

The PCR prevalence of *T. vegrandis* in koalas (13.6%) reported in the present study was at the lower end of prevalence estimates of *T. vegrandis* previously reported in woylies (14% - 46%) [5, 15-16] and various other marsupial (up to 32%) [5] and bat species (88.9%)

[8]. In koalas, *T. vegrandis* was less prevalent than *T. irwini* (71.1%) and *T. gilletti* (21.5%), but more prevalent than *T. copemani* (4.4%) [3]. The group of animals tested in the present study comprised presumably sick or injured koalas presenting to the AZWH and therefore may not reflect the prevalence in the wild population.

It is likely that the small size of *T. vegrandis* (<10 µm in length), coupled with the difficulties in amplifying this parasite in mixed infections using generic trypanosome primers, are the reason why the parasite has not been identified until now in koalas. This is the first time that *T. vegrandis* species-specific primers have been used on blood from this host. Further investigations comprising a larger sample size and random sampling are required to determine and compare the prevalence more accurately. Differences in prevalence could also be due to different locations, hosts, sensitivity of molecular tests used and the capacity of Australian trypanosomes to migrate to different organs in the host [5].

The observation that *T. vegrandis* is present in marsupials and bats from WA and NSW [4-8], marsupials in the Northern Territory (Barbosa et al., unpublished data) and now in koalas from Qld suggests that this parasite species has a wide distribution in Australia. Of the eight native trypanosomes formerly described, only *T. copemani* and *T. vegrandis* species have been identified in multiple hosts, with the chronic effects of *T. copemani* infections possibly linked with the decline of woylie populations [5,16]. The current findings add more evidence to the fact that trypanosomes in Australian marsupials comprise a heterogeneous community, with low levels of host specificity and no evidence of restricted geographical distribution [1,4,5,14-17].

The impact of *T. vegrandis* on koala health is currently unknown. A previous study in koalas reported that infection with trypanosomes was significantly associated with indicators of koala ill-health and non-survival [11]. In that study, *T. vegrandis* was not detected in the koalas but this is due to the fact that it was conducted before *T. vegrandis* had been formally

described by Thompson et al. [6] and also because species-specific internal primers to *T. irwini*, *T. copemani* and *T. gilletti* only were used for screening [11]. Further research is required to determine the prevalence and potential impacts of *T. vegrandis* and mixed trypanosome infections on koala health.

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Figure 1a and b. Light photomicrograph of the trypomastigote stages of *T. vegrans* in a Modified Wright's stained blood film from a koala (isolate Timbo). Scale bars represent 10 μ m.

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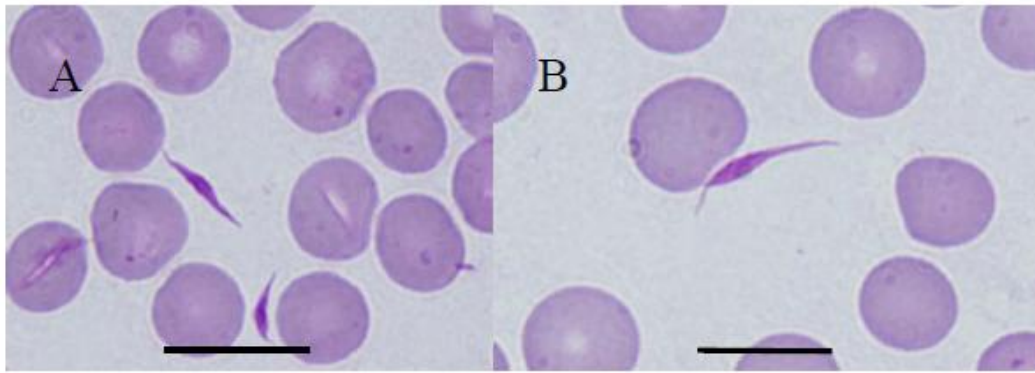
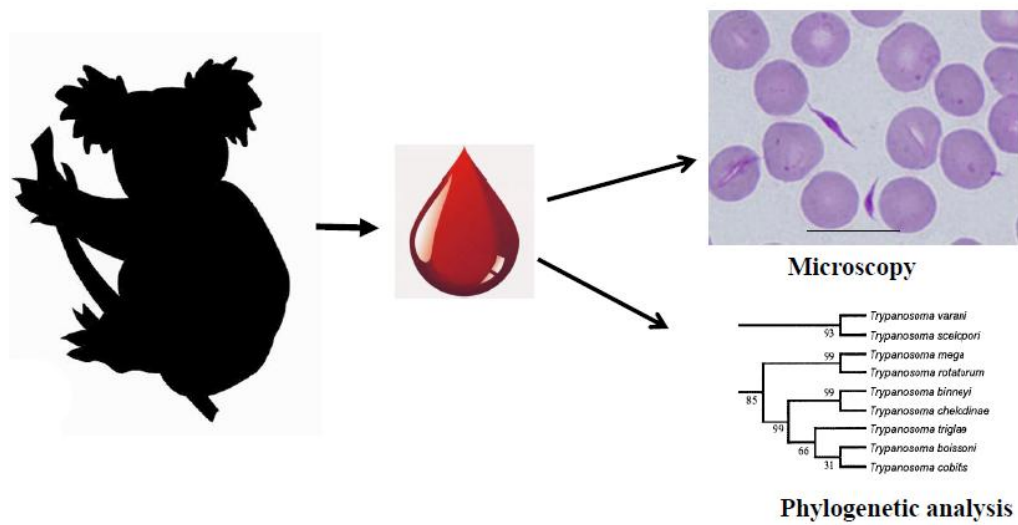


Figure 1

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Graphical abstract

First report of *Trypanosoma vegrandis* in koalas (*Phascolarctos cinereus*).



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Highlights

- First report of *Trypanosoma vegrandis* in koalas
- Prevalence of *T. vegrandis* in koalas was 13.6% (6/44)
- Potential health impact on koalas discussed

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