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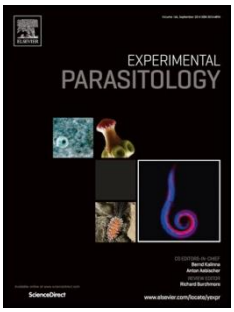

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Author: J.M. Austen, U. Ryan, W.G.F. Ditcham, J.A. Friend, S.A. Reid

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The innate resistance of *Trypanosoma copemani* to human serum

J.M. Austen[a], U. Ryan[a*], W.G.F. Ditcham[a], J.A. Friend[b], S.A. Reid[c]

[a]School of Veterinary and Life Sciences, Murdoch University, South Street, Murdoch, Western Australia, 6150

[b]Department of Parks and Wildlife, 120 Albany Highway, Albany, Western Australia, 6330

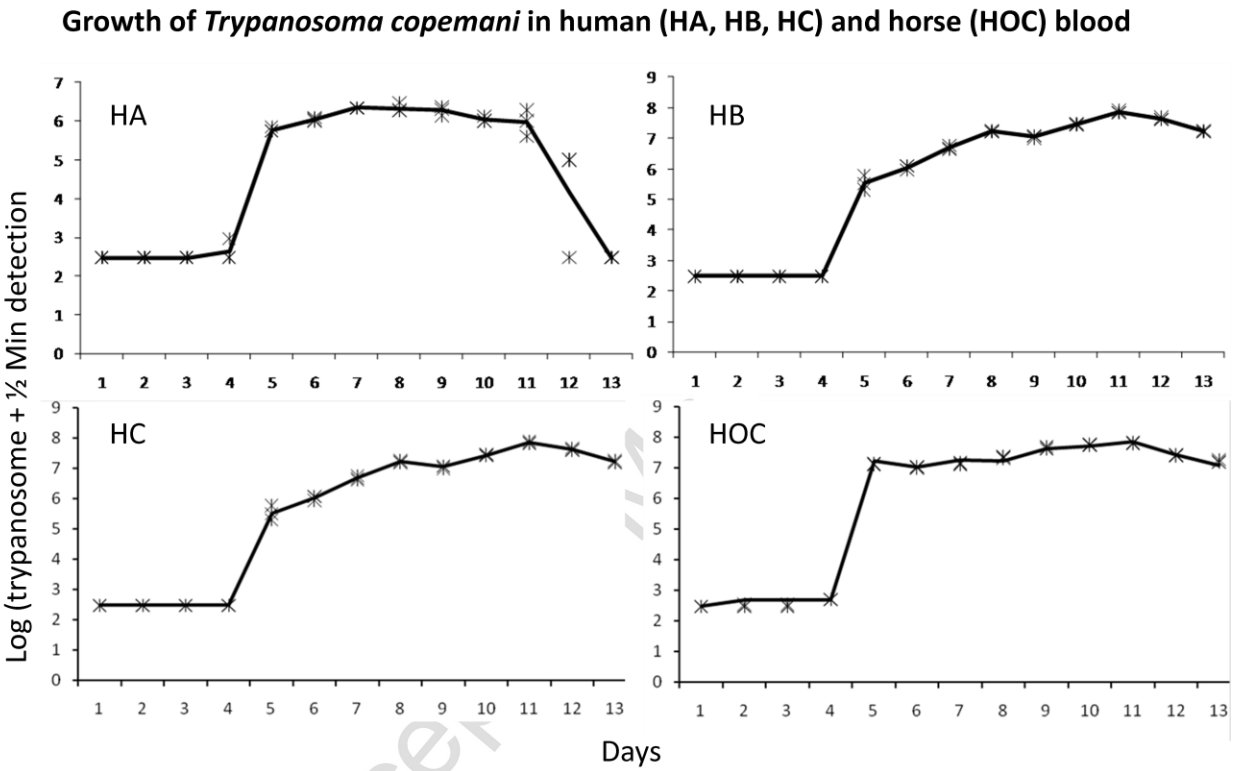
[c]School of Population Health, Faculty of Medicine and Biomedical Sciences, University of Queensland, Herston Road, Herston, QLD, 4006

**Corresponding author. Mailing address: School of Veterinary and Life Sciences, Murdoch University, Murdoch, Western Australia, Australia, 6150. Phone: 61 89360 2482. Fax: 61 89310 4144. E-mail: Una.Ryan@murdoch.edu.au*

Highlights

- Assessed the susceptibility of an Australian marsupial trypanosome to human serum
- Used the blood incubation infectivity test (BIIT)
- *Trypanosoma copemani* has the potential to be infective for humans
- First report of a marsupial trypanosome being potentially infective to humans

Graphical Abstract



Abstract

Trypanosoma copemani is known to be infective to a variety of Australian marsupials. Characterisation of this parasite revealed the presence of stercorarian-like life-cycle stages in culture, which are similar to *T. rangeli* and *T. cruzi*. The blood incubation infectivity test (BIIT) was adapted and used to determine if *T. copemani*, like *T. cruzi* and *T. rangeli*, has the potential to grow in the presence of human serum. To eliminate any effects of anticoagulants on the complement system and on human high density lipoprotein (HDL), only fresh whole human

blood was used. *Trypanosoma copemani* was observed by microscopy in all human blood cultures from day 5 to day 19 post inoculation (PI). The mechanism for normal human serum (NHS) resistance in *T. copemani* is not known. The results of this study show that at least one native Australian trypanosome species may have the potential to be infective for humans.

Keywords: Trypanosome, BIIT, quokka, zoonosis, marsupial.

1.0 Introduction

Only a few trypanosome species are known to cause disease in mammals. These include the etiological agents of animal trypanosomiasis (*T. congolense, T. vivax, T. brucei brucei,* and *T. evansi*) and human African trypanosomiasis (*T. b. rhodesiense* and *T. b. gambiense)* as well as *T. cruzi*, which causes Chagas's disease in the Americas (Hoare 1972). *Trypanosoma rangeli* has also been detected in humans but it is not considered to be pathogenic (Stevens and Brisse 2004). *Trypanosoma rangeli* and *T. cruzi* share a large number of vertebrate reservoirs including animals in five orders: Edentata, Marsupialia, Carnivora, Rodentia and Primates (Guhl and Vallejo 2003).

Trypanosoma brucei rhodesiense and *T. b. gambiense* are resistant to the cytotoxic action of normal human serum because they are resistant to the trypanosome lytic factors (TLFs) that are naturally present in the blood of all humans and primates (Milner and Hajduk 1999, Molina-Portela Mdel, Lugli et al. 2005). Resistance to TLFs are conferred by the presence of the serum resistance associated gene (SRA) in *T. b. rhodesiense* (Xong et al., 1998; De Greef and Hamers, 1994). In *T. b. gambiense,* an SRA-like protein that is specific to *T. b. gambiense* (TgsGP), has been shown to be essential for human serum resistance (Capewell et al., 2013). The resistance of *T. cruzi* to human serum is based on resistance to the alternative complement pathway (ACP) (Cestari and Ramirez, 2010). However, not all of the life-cycle stages of *T. cruzi* are resistant to direct serum lysis. For example, the epimastigote life-cycle stage derived from the vector's gut is efficiently lysed in human serum but the trypomastigote and amastigote stages from the vertebrate host are not (Tomlinson and Raper 1998). Trypomastigotes of *T. cruzi* resist lysis because they are able to prevent the initiation of the complement cascade by expression of complement system inhibitors (Joiner, daSilva et al. 1988).

The Blood Incubation Infectivity Test (BIIT) developed by Rickman and Robson, (1970) was originally designed to distinguish the human-infective *T. b. rhodesiense* from the non human-infective *T. b. brucei*. This test is based on the observation that non human-infective trypanosomes are lysed by human serum while human-infective trypanosomes resist lysis. Therefore, this test can provide a method to identify new species of trypanosome that are potentially human-infective (Turner, McLellan et al. 2004). *Trypanosoma copemani* infects a wide variety of Australian marsupials including the critically endangered Gilbert's potoroo *(Potorous gilbertii)*, the common wombat (*Vombatus ursinus*), the koala (*Phascolarctos cinereus*), the brush-tailed bettong (*Bettongia penicillata*), southern brown bandicoot (*Isoodon obesulus*), tiger quoll (*Dasyurus maculatus*) and brush tail possum (*Trichosurus vulpecula*) (Noyes et al., 1999; Austen et al., 2009; McInnes et al., 2010; Paparini et al., 2011; Botero et al., 2013; Thompson et al., 2013). Molecular and phylogenetic characterisation of *T. copemani* has shown that it is similar to *T. cruzi* (Noyes et al., 1999; Austen et al., 2009; Botero et al., 2013). Recently, the vector for *T. copemani* has been identified as a tick, (*Ixodes* sp.) (Austen et al., 2011). The present study was performed to determine the relative susceptibility of *T. copemani* to human serum.

2.0 Materials and Methods

2.1 Study site and sample collection

A quokka (*Setonix brachyurus*) (Q2088), previously identified as positive for *T. copemani* by PCR was trapped at Two Peoples Bay (34° 58'S, 118°11'E) near Albany, Western Australia under Murdoch University animal ethics permit W2204/09 and Department of Parks and Wildlife permit number SC000767. The quokka was anaesthetised with isoflurane and

approximately 5 millilitres (mL) of blood was collected by venepuncture of the lateral caudal vein. Fifty microliters (µL) of blood were used for the BIIT test. The remainder of the blood was added to commercial blood storage tubes containing ethylene diamine tetra-acetic acid (EDTA) (Sarstedt, Australia) and stored at 4°C for a maximum of 14 days. Thin-blood smears were prepared from 10µL of blood and stained with Modified Wright's stain using an automated slide stainer (Hematek, Bayer). The blood smears were examined at 200× magnification and the number of trypanosomes counted using a haemocytometer to determine the numbers of trypanosomes / mL of blood.

2.2 DNA extraction

Whole genomic DNA was extracted from both fresh blood samples and cultured trypanosomes using a MasterPure™ DNA Purification Kit (Epicentre® Biotechnologies, Madison, Wisconsin, U.S.A.) following the manufacturer's instructions and the DNA stored at -20 ºC until required.

2.3 Molecular characterisation of T. copemani

A 1,439 bp fragment of the 18S ribosomal RNA (rRNA) gene and a 841bp fragment of the GAPDH gene was amplified and sequenced as previously described (Austen et al., 2009; McInnes et al., 2010), to confirm that the trypanosomes isolated from quokka 2088 were *T. copemani*. Amplified products were purified using a QIAquick® PCR Purification kit (Qiagen, USA) and sequenced directly. Sequencing reactions were performed using an ABI Prism Dye Terminator Cycle Sequencing Core kit (Applied Biosystems, USA).

2.4 In vitro human serum resistance: Blood Incubation Infectivity Test

Serum prepared from fresh whole blood from healthy human volunteers was used during this experiment. The use of human subjects for this study was approved by Murdoch University human ethics committee (project number 2010/053).

The BIIT assay was performed in triplicate using serum freshly prepared from the blood of five healthy human volunteers as the test samples (labelled HA, HB, HC, HD and HE) and from horse blood as the control samples (labelled H0A, H0B and H0C). Five sets of Modified Sloppy Evans Medium (MSEM) (Noyes, Stevens et al. 1999), used for the growth of the trypanosomes following the BIIT challenge were made up with each sample of the homologous human blood (HuMSEM) along with three sets of horse blood MSEM (HoMSEM). The BIIT was performed by adding a 50 µL aliquot of fresh quokka blood (containing ~five trypanosomes) into separate tubes, each containing 250 µL of one of the five samples of fresh undiluted human serum, or horse serum. These tubes were then incubated in a water bath at 37°C for 5 hours. The entire contents of each incubated tube were then added to individual tubes containing 1 mL of HuMSEM or HoMSEM, and incubated at room temperature in the dark for 24 hours before examination. In addition, a 50 µL control sample of fresh quokka blood was directly transferred into a HuMSEM and a HoMSEM without the initial incubation in serum, and incubated at room temperature in the dark. Microscopic examination of wet-smear preparations of the medium from each culture was performed every day at 200× and 400× magnification to detect the presence of motile trypanosomes. If trypanosomes were detected, Giemsa-stained thin blood smears were prepared for further microscopic examination.

On day 14 when high numbers of motile trypanosomes were observed in HuMSEM, 100 µL volumes of the culture (approx. 7.5 x 10^7 organisms) were transferred into 1 mL of HuMSEM with a 0.5 mL overlay of RPMI 1640 supplemented with 10% horse serum, 1000IU /

mL of ICN penicillin and 10000MCG / mL of streptomycin. Cultures were incubated at 37ºC with 5% CO^2 to mimic mammalian conditions and determine survival of *T. copemani* in liquid culture.

2.5 Statistical analysis

The relative rate of replication of *T. copemani* in HuMSEM and HoMSEM after the BIIT was assessed by performing triplicate counts on three human cultures (HA, HB, HC) and 1 horse culture (H0C) at several time points after inoculation using a hemocytometer counting chamber. Half of the minimum level of detection (300) was added to each of the triplicate counts of *T. copemani* in HuMSEM and HoMSEM after the BIIT and Log_{10} transformed. The mean log-transformed count was plotted against time for each of the 4 cultures (HA, HB, HC and H0C). Non-linear regression was performed on the log-transformed counts from each culture using the *plateau followed by one phase association* function in GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, USA). The counts recorded for the human serum culture HA on days 18 and 19 were omitted from the analysis to improve the goodness of fit. Outputs of the regression analysis include: day of initiation of exponential growth, maximum trypanosome density and replication rate and their 95% confidence intervals. Values were considered significantly different if the 95% CI's for each output variable from the regression analysis did not overlap.

3.0 Results

3.1 Microscopy

Blood from quokka 2088 contained approximately 100 trypanosomes per mL. The morphology of the trypanosomes detected in the blood smear was consistent with the trypomastigote life-cycle stage (Figure 1) (Hoare 1972).

3.2 Molecular characterisation of T. copemani

The identity of the trypanosome infecting quokka 2088 was confirmed as *T. copemani* (genotype B) using sequence analysis of both the 18S rRNA and GAPDH genes before and after the BIIT test. The 18S rRNA (1439bp) and GAPDH (841bp) gene sequences of the quokka trypanosome isolate were found to be 100% identical to reference *T. copemani* sequences both before and after the BIIT challenge, and therefore were deposited in the GenBank database under the accession numbers (18S rRNA) HQ267094 and (GAPDH) HQ267095.

3.3 Blood Incubation Infectivity Test (BIIT)

Trypanosoma copemani isolated from quokka 2088 multiplied successfully in MSEM containing either human or horse blood, after undergoing the BIIT. There was no significant difference in the time to initiation of exponential growth in cultures containing human blood or horse blood and trypanosomes were detected in all cultures by day 5. Plots of the log-transformed counts of *T. copemani* in cultures containing serum from 3 humans and 1 horse are presented (Figure 2). Motile *T. copemani* was also observed in both the control HuMSEM and HoMSEM inoculated with blood taken directly from the quokka. Unfortunately complete growth curves for *T. copemani* directly inoculated into HuMSEM and HoMSEM from infected blood were not possible due to the delayed detection of trypanosomes in the first 9 days. Numbers of trypanosomes / mL were however measured at several time points from day 10 (Figure 3) with *T.*

copemani shown to multiply at a slightly faster rate in HuMSEM at days 10, 12 and 14 compared to the growth rate of *T. copemani* in HoMSEM. On day 17, the numbers of trypanosomes in HuMSEM dropped compared to that detected in HoMSEM (Figure 3).

The first trypanosome life-cycle stages to be detected in both HuMSEM and HoMSEM post-BIIT were slender, rapidly moving trypomastigotes and thin epimastigotes (Figure 4a), with the former life-cycle stage being the most abundant. Larger epimastigotes and sphaeromastigotes (Figure 4b), were detected by day 10, but in fewer numbers than the trypomastigotes and epimastigotes. There were no obvious morphological differences between the epimastigote and trypomastigotes life-cycle stages seen following culture in either human or horse blood. Motile *T. copemani* was observed in the subcultures grown in human MSEM overlayed with RPMI that were incubated at 37°C. Trypomastigotes were the most abundant life-cycle stage detected with similar morphology to the trypomastigotes observed in quokka blood (Figure 4c).

4.0 Discussion

This study is the first time that the susceptibility of an Australian mammalian trypanosome to human serum has been assessed. Results from comparing the growth curves of *T. copemani* in cultures containing human and horse serum showed that there was no consistent effect on the trypanosome, attributable to either host species. *Trypanosoma copemani* was detected as early as day 5 after the BIIT challenge, compared to direct inoculation into either HuMSEM or HoMSEM, in which trypanosomes were first detected on day 10. This suggests a faster rate of multiplication with the addition of liquid serum allowing the trypanosomes to readily gain access to the required nutrients compared to the solidified blood agar medium. The use of solidified MSEM by Noyes et al. (1999) to detect novel trypanosome species isolated from the blood of a wombat and kangaroo shows similar findings with trypanosomes first

209 detected on days 13 and 20 respectively. Overall the growth of *T. copemani* inoculated directly
210 into HuMSEM and HoMSEM appeared to be similar with only a slight increase in multiplication
211 observed initially in the HuMSEM. Molecular analysis of the 18S rRNA and GAPDH genes was
212 conducted to confirm that the trypanosomes isolated from the blood of quokka 2088 were *T.*
213 *copemani*.

214 The BIIT is designed to enable the identification of potentially human infective
215 trypanosomes. In previous studies (Rickman and Robson 1970, Hawking 1978), the BIIT
216 protocol included inoculation of human serum-exposed trypanosomes into rats and mice to
217 determine viability. Inoculation of rodents was not possible in the present study because early
218 attempts to infect rodents with *T. copemani* were unsuccessful (Austen, unpublished data). The
219 failure of rodents to demonstrate infectivity with *T. copemani* may have resulted in the lack or
220 low prevalence of metacyclic trypanosomes within the singular inoculum at the time of
221 infection. For example, for natural infections to occur, multiple bites are needed from *Glossina*
222 *spp.* (Hoare, 1972). This may also be the case for *T. copemani,* which may require multiple
223 inocula to establish infection within the host. The immune response of the host may have also
224 overcome the singular inoculum of *T. copemani,* however future studies are required to confirm
225 this and to also understand the infective stages of *T. copemani* and how they are transmitted.

226 The *in vitro* methodology used in this study was adapted from a previous method
227 (Tomlinson, Jansen et al. 1995), where an *in vitro* assay was used to analyse the human serum
228 resistance of various *T. brucei* genotypes. To reduce the likelihood of false positive results, only
229 fresh whole human blood was used in this study to eliminate the potential effect an anticoagulant
230 may have had on either the complement system or the human serum high density lipoprotein
231 (HDL) trypanolytic factor. This is important because it has been shown that lipoprotein lipase
232 activity and the level of HDL in rats infected with *T. b. gambiense* were increased if heparin was
233 administered parenterally, which caused a reduction in the number of trypanosomes in the rat

(Nishimura et al., 2005). Furthermore, *in vitro* culture of *T. b. gambiense* was inhibited by the addition of plasma from infected rats treated with heparin (Nishimura et al., 2005). In contrast, the commonly used anticoagulant EDTA was found to inhibit the trypanolytic reaction of normal human serum on *T. congolense* TC35U (Ferrante and Allison, 1985). This uncoated trypanosome is normally lysed by human serum but is resistant to lysis by human serum containing EDTA. In 2006, *T. evansi* was identified in a human patient that lacked Apolipoprotein L-1 (APOL1), a human specific protein that binds to HDL and together cause the osmotic swelling of *T. brucei* and eventual death (Vanhollebeke et al., 2006). The potential role of APOL1 in the results of this study were not investigated because trypanolytic factors (TLFs), of which APOL1 is a major component, have been shown to have no effect on the replication of *T. cruzi* in an *in vivo* model (Samanovic et al., 2009). This is important because *T. copemani* is closely related to *T. cruzi* both biologically and phylogenetically (Austen et al., 2009; McInnes et al., 2011).

The low numbers of trypomastigotes (100 trypanosomes/mL of blood) detected in the quokka's blood at the time of sampling resulted in exposure of only about five trypomastigotes to human serum in the BIIT and subsequent culturing in HuMSEM. The low inoculum is both representative of a natural challenge, and also ensures that any potential defence mechanisms present in the blood samples being used in the test are not overwhelmed by a large inoculum, giving a spurious apparent survival of trypanosomes. The low number of trypanosomes used in the present study is in contrast to both the large inocula of 2 $x10^6$ *T. congolense* exposed to 50% NHS by Xong et al. (2002), and of $1x10^7$ *T. b. brucei* exposed to 25% NHS by Turner et al. (2004). These studies also used diluted serum in contrast to the undiluted normal human serum used in the present study, which shows that *T. copemani* blood life-cycle stages have a high level of resistance to human serum. The low inoculum used in the present study may have accounted for the initial lag phase in growth, which prevented the detection of viable parasites in the first

four days of the BIIT. Detection of *T. copemani* was first possible on day five, a time lag which may have allowed the parasites to efficiently multiply and adapt to the vector life cycle stages, given that their maintenance *in vitro* is a methodology used to mimic the conditions of the vector (Hoare 1972).

The observation that *T. copemani* is able to survive in the presence of human serum is interesting but must be interpreted cautiously. Survival of trypanosomes when subjected to the BIIT test is strongly correlated to pathogenicity, in studies of *T. b. brucei* (Rickman and Robson 1970). In addition, *T. lewisi* for instance, which is considered a rodent trypanosome (Hawking 1978) has been reported to infect humans on eight occasions (Lun, Reid et al. 2009). Similarly, *T. evansi* which is responsible for a widely distributed disease called ‘‘surra’’ in domestic and wild animals found in Asia, Africa, South America, and even Europe has been identified in humans on four occasions (Lun, Reid et al. 2009). However, survival in the BIIT test does not necessarily correlate with an ability to infect humans and cause disease. Furthermore, the risk of human infection with *T. copemani* would require interaction between the marsupial hosts, their tick vector and susceptible humans. *Trypanosoma copemani* does however have a broad marsupial host range, and increasing human encroachment on marsupial habitats, where ticks coexist with their natural marsupial hosts, may increase the risk of humans becoming infected with *T. copemani*.

The mechanism of resistance to NHS by *T. copemani* is unclear. If the mechanism is similar to *T. cruzi,* then the trypomastigotes may be able to inhibit the assembly, or accelerate the decay of C3 convertase, the central enzyme of the complement cascade (Tomlinson and Raper 1998). Chronic, non-pathogenic infection in the quokka may be maintained by the production of antibodies, which render the trypomastigotes sensitive to lysis via the alternative complement cascade, as occurs in mammalian hosts infected with *T. cruzi* (Krautz, Kissinger et al. 2000). There are significant morphological similarities between the life-cycle stages

observed in *T. copemani* cultures and blood smears compared to *T. cruzi*. Therefore, it should not be surprising that blood-stream trypomastigote stages of *T. copemani* are resistant to human serum. This is because trypomastigotes and amastigotes of *T. cruzi* from the vertebrate host are resistant to direct serum lysis from the complement system and epimastigotes from the gut of the vector are resistant (Tomlinson and Raper 1998, Krautz, Kissinger et al. 2000). It has been shown that cultured trypomastigotes of *T. cruzi* can form into both extracellular and intracellular amastigotes, both of which are infective to human monocytes *in vitro,* and may help to maintain the *T. cruzi* mammalian life-cycle (Ley, Andrews et al. 1988). Amastigote stages of *T. copemani* have been observed in culture and in quokka blood (Austen et al., 2014), and may contribute to the resistance of *T. copemani* to human serum lysis.

It is tempting to draw bold conclusions from the results of this study that *T. copemani*, and possibly other Australian trypanosomes, represent an extensive and latent pool that could give rise to new emerging infectious diseases (EIDs). Indeed, approximately 75% of EIDs that have affected human populations in the past 30 years have been zoonotic (Daszak, Epstein et al. 2007). In addition to *T. copemani*, Australian trypanosomes from kangaroos (H25), possums (*Pseudocheirus peregrinus*), woylies (*Bettongia penicillata*), a banded hare wallaby (*Lagostrophus fasciatus*) and boodies (*Bettongia lesueur*) have closer phylogenetic relationships with *T. cruzi* than *T. copemani* (Noyes et al., 1999; Paparini et al., 2011; Botero et al., 2013). In addition, there are a small but significant number of atypical human infections with "animal" trypanosomes that raises the possibility that many human infections remain undiagnosed (Lun, Reid et al. 2009); Truc et al., 2013). *Trypanosoma cruzi* is principally a parasite of sylvatic animals and it did not undergo the prolonged period of co-evolution with humans experienced by the *T. brucei* group. Humans became a host only when they became 'available' in the sylvatic life-cycle of *T. cruzi* ~9,000 years ago, which corresponds with the period when humans developed settled (rather than nomadic) populations. The presence of these sedentary

populations and their dwelling places altered the life-cycle of the vector, creating a new non-sylvatic cycle and a new 'human' disease (Aufderheide, Salo et al. 2004). Therefore, the observation that *T. copemani* is able to resist NHS is insufficient to conclude that it has significant zoonotic potential *per se*. Rather, a more complex chain of events would have to occur to significantly alter the life-cycle of the parasite and its mammalian hosts. It may also be possible that *T. cruzi* is unique in the trypanosome world in that the vector and sylvatic cycle contained the correct ingredients for establishment in the "human" domestic environment. Whilst the human population of Australia is unlikely to undergo a significant societal change equivalent to South America 9,000 years ago, Australians live in relatively close proximity to native marsupials and the level of exposure to marsupial-derived tick vectors is unknown. It is, therefore possible that isolated cases of human trypanosomiasis may occur in the Australian population. If this does occur, then information on the susceptibility of native trypanosomes to currently available trypanocidal drugs would be of enormous value.

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Figure 1. Light photomicrograph of *T. copemani* in a Modified Wrights stained blood smear from a quokka. Scale bar represents 10µm.

Figure 2. Mean $\log_{10}$-transformed count (plus half minimum detection level) of *T. copemani* grown in cultures containing serum from 3 humans (HA, HB, HC) and one horse (H0C). The trend line represents the mean log-transformed count and markers (×) represent individual counts.

Figure 3. Numbers of trypanosomes / mL measured at several time points after direct inoculation of infected quokka blood into HuMSEM and HoMSEM.

Figure 4. Light photomicrographs of *T. copemani* grown *in vitro*, in a culture of blood from the quokka, which had undergone the blood incubation infectivity test. (A) Epimastigote (e) and trypomastigote (t) forms at day 10, in a Modified Wrights stained smear from HuMSEM. (B) Sphaeromastigotes (s), epimastigotes and trypomastigote forms at day 10, in a Modified Wrights stained smear from HuMSEM. (C) Trypomastigote form of *T. copemani* at day 18, grown in HuMSEM overlayed with RPMI at 37ºC. Scale bar represents 10µm.