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Research paper

Trypanosoma teixeirae: A new species belonging to the T. cruzi clade causing trypanosomiasis in an Australian little red flying fox (Pteropus scapulatus)

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ABSTRACT

Little is known about the genetic diversity and pathogenicity of trypanosomes in Australian bats. Recently a novel trypanosome species was identified in an adult female little red flying fox (Pteropus scapulatus) with clinical and pathological evidence of trypanosomiasis. The present study used morphology and molecular methods to demonstrate that this trypanosome is a distinct species and we propose the name Trypanosoma teixeirae sp. n. Morphological comparison showed that its circulating trypomastigotes were significantly different from those of Trypanosoma pteropi and Trypanosoma hippocideri, two species previously described from Australian bats. Genetic information was not available for T. pteropi and T. hippocideri but phylogenetic analyses at the 18S ribosomal RNA (rRNA) and glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) loci indicated that T. teixeirae sp. n. was genetically distinct and clustered with other bat-derived trypanosome species within the Trypanosoma cruzi clade.

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1. Introduction

Bats (order Chiroptera) are reservoirs of numerous zoonotic pathogens including rabies, Australian bat lyssavirus, severe acute respiratory syndrome (SARS), Hendra virus, Nipah virus and Ebola virus (Wood et al., 2012). Trypanosomes are blood-borne flagellate protozoan parasites that can infect a wide range of vertebrate hosts including humans. Numerous trypanosome species have been identified in bats in Asia, Africa, South America and Europe (Hoare, 1972; Baker, 1973; Marinkelle, 1976, 1979; Gardner and Molyneux, 1988a,b; Hamanaka and Pinto Ada, 1993; Steindel et al., 1998; Barnabe et al., 2003; Grisard et al., 2003; Lisboa et al., 2008; Cottontail et al., 2009; Maia da Silva et al., 2009; Cavazzana et al., 2010; Garcia et al., 2012; Hamilton et al., 2012; Lima et al., 2012, 2013; Marcili et al., 2013; Silva-Iiturriza et al., 2013; Cottontail et al., 2014; Ramirez et al., 2014).

In Australia, three Trypanosoma spp. have been described in bats to date: Trypanosoma pteropi from the black flying fox (Pteropus gouldii) (Breinl, 1913; Mackerras, 1959), Trypanosoma hippocideri from the dusky horseshoe bat (Hipposideros bicolor albanensis) and Trypanosoma vegrandis, in pteropid bats (Yangochiroptera) and microbats (Yinpterochiroptera) (Austen et al., 2015). None of these have been associated with clinical disease. In addition, Mackie et al. (2015) recently described the first case of trypanosomiasis in a little red flying fox (Pteropus scapulatus—suborder Yinpterochiroptera) from eastern Australia, caused by an apparently novel trypanosome species.

Molecular and phylogenetic studies have suggested that bat trypanosomes are implicated in the evolutionary history of the T. cruzi clade and may potentially be the precursor of trypanosomes from Australian marsupials and several African terrestrial mammals (Hamilton et al., 2012; Lima et al., 2013). There is however very limited knowledge about the genetic diversity of Australian bat trypanosomes, where only 9 of 76 indigenous bat species have been screened for this parasite (Thompson et al., 2014).

In the present study, we describe the morphological and genetic characterisation of the novel trypanosome in the little red flying fox.
Table 1
Genbank accession numbers and sources (where known) of trypanosome isolates included in the phylogenetic analyses.

| Trypanosome species | Host origin | Geographic origin | GenBank accession numbers |
|---------------------|-------------|-------------------|--------------------------|
| T. brucei rhodesiense | Human (Homo sapiens) | Europe | AY246138 |
| T. brucei gambiense | Human (Homo sapiens) | Australia | AJ013156 |
| T. evansi | Cavybara (Hydrochoerus) | Brazil | AJ013154 |
| T. congolense | Koala (Phascolarctos cinereus) | Australia | AJ010710 |
| T. rhodesiense | Human (Homo sapiens) | Uganda | AJ010712 |
| T. brucei | Human (Homo sapiens) | Nigeria | AJ013158 |
| T. cruzi | Human (Homo sapiens) | Brazil | AJ013156 |
| T. lewisi | Rat (Rattus rattus) | England | AJ013160 |
| T. vivax | Cattle | Australia | AJ013160 |
| T. brucei | Human (Homo sapiens) | Europe | AY246138 |
| T. brucei | Human (Homo sapiens) | Australia | AJ013156 |
| T. evansi | Cavybara (Hydrochoerus) | Brazil | AJ013154 |
| T. congolense | Koala (Phascolarctos cinereus) | Australia | AJ010710 |
| T. rhodesiense | Human (Homo sapiens) | Uganda | AJ010712 |
| T. brucei | Human (Homo sapiens) | Nigeria | AJ013158 |
| T. cruzi | Human (Homo sapiens) | Brazil | AJ013156 |
| T. lewisi | Rat (Rattus rattus) | England | AJ013160 |
| T. vivax | Cattle | Australia | AJ013160 |

(Mackie et al., 2015), for which we proposed the name Trypanosoma teixeirae sp. n.

2. Material and methods

2.1. Sample collection

A venous blood sample was collected from the cephalic vein of an adult female little red flying fox that presented to the Australia Zoo Wildlife Hospital (AZWH) in April, 2014. The flying fox had been rescued from the ground at Redcliffe in south-eastern Queensland, Australia and was moribund with anaemia and icterus. Clinical and pathological evidence of disease consistent with trypanosomiasis in this flying fox was described by Mackie et al. (2015).

2.2. Morphological analyses

Thin blood smears were made from a drop of fresh blood and stained with Diff Quick (Siemens, Germany). After air-drying, the slides were then cover-slipped using DePeX mounting medium Gurr (Merck Pty. Limited, Kilsyth, Victoria, Australia). Stained films were systematically examined using a BX50 microscope (Olympus, Japan) with screen views generated by a DP Controller (version 3.2.1.276, Olympus, Japan). Digital light micrograph images of any trypanostagotes observed were taken at ×1000 magnification.

Digital images of the organisms identified in the blood films were used to measure key morphological features such as total length (TL), width (W), posterior to kinetoplast (PK), kinetoplast to nucleus (KN), nucleus to anterior (NA) and free flagellum (FF), according to parameters described by Hoare (1972) and Mackerras (1959). Means and standard errors were calculated. The morphological measurements were taken using the software Image J (Abramoff et al., 2004).

As two trypanosome species have previously been described in Australian bats based on morphological analysis only (Breinl, 1913; Mackerras, 1959), morphometrics of the novel trypanosome was compared statistically with available measurements for T. piropli and T. hippodermis. Mean values for each morphological feature were calculated for T. teixeirae sp. n. whilst median values of reported ranges were used as input data for T. piropli and T. hippocraes, as means were not available in the bibliographical references. Statistical analyses were conducted using the same sample t-test, in the software PAST 1.43 (Hammer et al., 2001).
2.3. DNA extraction

Genomic DNA was extracted from 200 μl of whole blood, using the MasterPure Purification Kit (Epicentre Biotechnologies, USA). A DNA extraction blank (with sterile molecular-grade water instead of blood) was included in the extraction to exclude the contamination of reagents and consumables with DNA.

2.4. 18S rRNA and GAPDH amplification and sequencing

A nested PCR protocol using generic *Trypanosoma* sp. primers SLF, S762R, S823F and S662R (Maslov et al., 1996; Mclnnes et al., 2009) was performed to amplify an approximately 900 bp fragment of the 18S rRNA gene, as previously described by Mclnnes et al. (2009). The DNA sample was also amplified at the glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) gene using a heminested PCR protocol (Mclnnes et al., 2009).

PCR products were run on a 2% agarose gel containing SYBR Safe Gel Stain (Invitrogen, USA), and visualized with a dark reader trans-illuminator (Clare Chemical Research, USA). The gel bands were purified using an in-house filter tip method as previously described (Yang et al., 2013). All controls (positive, negative and DNA extraction blank) produced appropriate PCR results.

The purified PCR products were sequenced using the corresponding internal reverse primers diluted at 3.2 picomoles with an ABI PrismTM Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) on an Applied Biosystem 3730 DNA Analyzer.

2.5. Phylogenetic analysis

Nucleotide sequences obtained at both 18S rRNA and GAPDH loci were aligned with additional trypanosome sequences retrieved from GenBank (Table 1) by MUSCLE (Edgar, 2004) using the default settings. Ambiguous regions containing gaps or poorly aligned were removed by Gblocks (Castrasana, 2000), available on the Phylogeny.fr platform (Dereeper et al., 2008), using low stringency parameters. The curated alignments were imported into MEGA 6 (Tamura et al., 2013) and the most appropriate nucleotide substitution model was selected using the dedicated function.

The evolutionary histories at both 18S rRNA and GAPDH genes were inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The gamma shape parameter was estimated directly from the data. Reliability for internal branch was assessed using the bootstrapping method (500 bootstrap replicates) and support values (>60%) indicated at the left of each node. The phylogenetic trees were drawn to scale, with branch lengths measured in the number of substitutions per site.

Estimates of genetic divergence between sequences were generated in MEGA 6 based on the Tamura-Nei algorithm, using uniform rates and a partial deletion of 95%.

3. Results

3.1. Microscopy and morphometric analysis of *T. teixeirae* sp. n.

A total of nine organisms morphologically consistent with a trypanosome were detected by light microscopy in blood films from the little red flying fox. The extracellular organisms were slender with tapered ends, with long free flagellums and either an undeveloped or absent undulating membrane. A nearly central nucleus and a terminal small round deeply staining internal structure consistent with a kinetoplast were also observed (Fig. 1a–c). The trypomastigotes varied in length from 20.4 to 30.8 μm (average 25.9 μm) and in width from 1.3 to 2.3 μm (average 1.9 μm) (Table 2).

Among the nine long slender organisms observed, two were not true trypomastigotes as their kinetoplast was located at the very end of the posterior, what made it impossible to calculate the PK distance. In another instance, the trypomastigote’s free flagellum was apparently under a red blood cell, hence any measurements taken of PF or TL would have been inaccurate. We have therefore only measured what was feasible, which explains the divergence in the number of organisms measured for each morphological feature (Table 2).

Three flagellate round forms with a flagellum running round the organism about 90 degrees were also observed (Fig. 1d). Their body shape was consistent with a sphaeromastigote or a round epimastigote if their flagellar position was considered.

Morphometric analysis revealed that although the reported length and width ranges for the *T. teixeirae* sp. n. and *T. pteropi* overlap, the former was significantly longer and thinner than the latter (p < 0.01) (Table 3). There was no significant difference between KN, NA and FF dimensions between *T. teixeirae* sp. n. and *T. pteropi*. In addition, *T. hippodideri* was significantly smaller than *T. teixeirae* sp. n. for TL and FF dimensions (p < 0.01) (Table 3). No significant difference was observed for B, PK and KN between *T. teixeirae* sp. n. and *T. hippodideri*.

3.2. Sequence and phylogenetic analysis

Maximum Likelihood analysis at both the 18S rDNA and GAPDH loci (Figs. 2 and 3, respectively) produced concordant tree topologies and revealed that *T. teixeirae* sp. n. grouped with other trypanosomes belonging to the *T. cruzi* clade, including 7 bat-derived isolates (*T. cruzi* Tcbat, *T. cruzi* marinkellei, *T. ernyai*, *T. dionisi*, *T. rangeli*, *T. vespertilionis* and *T. sp. AJ012418/GQ140365) and three isolates from Australian marsupials (T. sp. H25 from a kangaroo- AJ099168/A620276; T. sp. AB-2003-G8 from a woylie- KC753537/KC812988; and T. sp. AP-2011-64 from a brush-tailed possum – JN315383/A620276). The phylogenetic trees also corroborated the evolutionary relationships among all major trypanosome clades described in previous broader analyses. Nucleotide sequences were obtained at both loci for *T. teixeirae* sp. n. were submitted to GenBank under the following accession numbers: KT907061 and KT907062.

Estimates of evolutionary divergence between nucleotide sequences revealed that *T. teixeirae* sp. n. was genetically distinct but most closely related to *T. minasense* and *T. rangeli* (genetic distances of 1% at the 18S rDNA locus and 14%–15% at the gGAPDH, respectively) (Table 4).

4. Species description

Species Name: *Trypanosoma teixeirae* sp. n. (Fig. 1)

Type host: Little red flying fox (*Pteropus scapulatus*).

Other hosts: Unknown

Type Locality: Redcliffe peninsula, Queensland, Australia.

Prevalence: Unknown

4.1. Morphology

*T. teixeirae* sp. n. trypomastigotes are on average 25.9 μm long and 1.9 μm wide, exhibiting a slender shape with tapered ends, a nearly central nucleus and a small terminal kinetoplast. Undulating membrane either absent or under-developed.

4.2. Etymology

The species is named *T. teixeirae* sp. n. in honour of Prof. Marta Maria Geraldes Teixeira, from the University of Sao Paulo, who has
greatly contributed to the biology and phylogeny of trypanosome species.
Fig. 2. Phylogenetic relationships of *Trypanosoma teixeirae* sp. n. with other trypanosomes, based on 18S rDNA partial sequences (∼730 bp). Evolutionary relationships were determined by Maximum Likelihood, based on the Tamura-Nei model (Tamura et al., 2013). Bootstrap values (>60%) based on 500 replicates are indicated at the left of each supported node. The scale bar is the proportion of base substitutions per site. Trypanosome species from bats are shown with an asterisk.

Unfortunately no genetic data was available for two of the previously reported bat-derived trypanosomes in Australia (*T. pteropi* and *T. hipposideri*). *Trypanosoma pteropi* was described as having a slender body (total length 18–22 μm; width 2–4 μm), an underdeveloped undulating membrane and a long free flagellum whereas *T. hipposideri* is very small and slender (total length 10.5–13 μm; width 1.5–2 μm), with a delicate short free flagellum at the anterior end (Breinl, 1913; Mackerras, 1959). Statistical analysis however, revealed that *T. teixeirae* sp. n. was significantly larger than both *T. pteropi* and *T. hipposideri*, even though they had several other overlapping morphometric features. However, morphology alone is not a reliable tool to delimit trypanosome species due to the interspecific similarities and intraspecific variability (Dunn et al., 1963; Marinkelle, 1966; Dunn, 1968).

Besides the typical trypomastigotes, two round forms (sphaeromastigotes or round epimastigotes) were also observed. The term ‘sphaeromastigote’ (Brack, 1968) refers to the parasite body shape.
Fig. 3. Phylogenetic relationships of Trypanosoma teixeirae sp. n. with other trypanosomes, based on gGAPDH partial sequences (~775 bp). Evolutionary relationships were determined by Maximum Likelihood, based on the Tamura-Nei model (Tamura et al., 2013). Bootstrap values (>60%) based on 500 replicates are indicated at the left of each supported node. The scale bar is the proportion of base substitutions per site. Trypanosome species from bats are shown with an asterisk.

only and has been applied without reference to the flagellar development. However, as these forms may occur within different stages of the parasite’s development, it is more appropriate to characterise the round organisms observed in the present study as round epimastigotes, considering both their body form and flagellar features (Elliott et al., 1974). This stage normally occurs in the interior of the cell, in vessels or in the insect gut.

Evolutionary reconstructions at both 18S rDNA and gGAPDH loci revealed that T. teixeirae sp. n. was genetically distinct from all known trypanosomes. The use of these two genes is recommended for taxonomic analysis of trypanosomatids and validation of new trypanosome species (Hamilton et al., 2004; Viola et al., 2009; Teixeira et al., 2011; Lima et al., 2012, 2013; Borghesan et al., 2013).

Phylogenetic analyses at both 18S rDNA and gGAPDH loci revealed that T. teixeirae sp. n. clustered within the T. cruzi clade together with all other bat-derived trypanosome species described to date, except T. livingstonei (which was positioned basal to the T. cruzi clade), T. evansi (which belongs to the T. brucei clade) and T. ve grandi s (which forms a separate group associated with other marsupial-derived trypanosomes found in Australia) (Hamilton et al., 2007; Botero et al., 2013; Lima et al., 2013; Austen et al., 2015; Carnes et al., 2015). At the gGAPDH locus, T. teixeirae sp. n. was closest to T. minasense and T. rangel and exhibited 14% and 15% genetic
distance from these two species respectively. T. minasense has been found in neotropical non-human primates from South America (Zuccarelli and Loureno-de-Oliveira, 1998) whilst T. rangeli has been reported in a range of mammalian hosts including Brazilian bats (Maia da Silva et al. 2009). Although T. teixeirae sp. n. exhibited a relatively low (1%) genetic distance from its closest related species at the 18S rRNA locus, a similar pattern was observed when comparing other previously described species among each other. For instance, genetic distances between T. minasense and T. vespertilio were 1% and 12% at the 18S rRNA and gGAPDH loci respectively. Trypanosomes have few morphological features detectable using light microscopy which can adequately delimit species (Gibson, 2009). Previous studies have reported that a genetic distance of 3.75% at the GAPDH gene is sufficient to delimit a new trypanosome species (McInnes et al., 2011). By this criterion, T. teixeirae sp. n. is clearly a separate species.

Bat trypanosomes have been implicated in the evolutionary origin of T. cruzi, the causative agent of Chagas disease, one of the most important public health issues in South America (Hamilton et al., 2012; Bonney, 2014). The ‘bat-seeding’ theory suggests that T. cruzi evolved from within a broad clade of bat-derived species, which have made the switch into terrestrial mammals (Hamilton et al., 2012; Lima et al., 2013). The theory also implies that these arboreal trypanosome species could potentially be evolutionary precursors for the terrestrial trypanosome lineage within Australian mammals (Hamilton et al., 2012; Lima et al., 2013; Thompson et al., 2014). It is therefore possible that T. teixeirae sp. n. could be the precursor of three marsupial-derived trypanosomes belonging to the T. cruzi clade: T. sp. H25 (Averis et al., 2009), T. sp. AP-2011-64 (Paparini et al., 2011) and T. sp. AB-2013-G8 (Botero et al., 2013). As most native bat species remain unsampled (Thompson et al., 2014), future studies are required to provide more evidence to support the ‘bat-seeding’ theory in Australia and elucidate evolutionary relationships between trypanosomes.

Similarly to most bat trypanosomes described worldwide, the prevalence, distribution, vectors, life cycle and zoonotic potential of T. teixeirae sp. n. remain unclear. Therefore, more studies comprising a larger sample size are required to better understand the prevalence and clinical impacts of T. teixeirae sp. n. on bat populations, taking into account ecological and stress factors that could play a role in the expression of clinical disease.

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