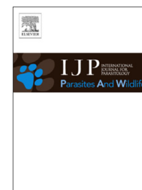
International Journal for Parasitology: Parasites and Wildlife 2 (2013) 77–89

Contents lists available at SciVerse ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw



Trypanosomes genetic diversity, polyparasitism and the population decline of the critically endangered Australian marsupial, the brush tailed bettong or woylie (*Bettongia penicillata*)

Adriana Botero [a,*], Craig K. Thompson [a], Christopher S. Peacock [b,c], Peta L. Clode [d], Philip K. Nicholls [a], Adrian F. Wayne [e], Alan J. Lymbery [f], R.C. Andrew Thompson [a]

[a] School of Veterinary and Life Sciences, Murdoch University, 90 South Street, Murdoch, WA 6150, Australia
[b] School of Pathology and Laboratory Medicine, University of Western Australia, Crawley, WA 6009, Australia
[c] Telethon Institute for Child Health Research, 100 Roberts Road, Subiaco, WA 6008, Australia
[d] Centre for Microscopy, Characterisation and Analysis, University of Western Australia, Stirling HWY, Crawley, WA 6009, Australia
[e] Department of Environment and Conservation, Science Division, Manjimup, WA, Australia
[f] Fish Health Unit, School of Veterinary and Life Sciences, Murdoch University, South Street, Murdoch, WA 6150, Australia

ARTICLE INFO

Article history:
Received 28 January 2013
Revised 13 March 2013
Accepted 14 March 2013

Keywords:
Australian trypanosomes
Marsupials
Tissue tropism
Intracellular
Pathogenicity
Population decline

ABSTRACT

While much is known of the impact of trypanosomes on human and livestock health, trypanosomes in wildlife, although ubiquitous, have largely been considered to be non-pathogenic. We describe the genetic diversity, tissue tropism and potential pathogenicity of trypanosomes naturally infecting Western Australian marsupials. Blood samples collected from 554 live-animals and 250 tissue samples extracted from 50 carcasses of sick-euthanized or road-killed animals, belonging to 10 species of marsupials, were screened for the presence of trypanosomes using a PCR of the 18S rDNA gene. PCR results revealed a rate of infection of 67% in blood and 60% in tissues. Inferred phylogenetic trees using 18S rDNA and glycosomal glyceraldehyde phosphate dehydrogenase (*gGAPDH*) sequences showed the presence of eight genotypes that clustered into three clades: a clade including *Trypanosoma copemani*, a new clade closely related to *Trypanosoma gilletti*, and a clade including *Trypanosoma* H25 from an Australian kangaroo. Trypanosome infections were compared in a declining and in a stable population of the endangered Australian marsupial, the brush tailed bettong or woylie (*Bettongia penicillata*). This marsupial showed high rates of infection with Clade A genotypes (96%) in the declining population, whereas in the stable population, Clade B genotypes were predominant (89%). Mixed infections were common in woylies from the declining but not from the stable population. Histopathological findings associated with either mixed or single infections involving Clade A genotypes, showed a strong inflammatory process and tissue degeneration predominantly in heart, oesophagus and tongue. Trypanosomes were successfully grown in culture and for the first time we demonstrate that a genotype within Clade A has the capacity to not only colonize different tissues in the host but also to invade cells *in vitro*. These results provide evidence for the potential role of trypanosomes in the decline of a formerly abundant marsupial that is now critically endangered.

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Introduction

Trypanosomes are flagellated blood parasites that are capable of infecting virtually all classes of vertebrates. They range from non-pathogenic species to those that are highly pathogenic and are the causative agents of many diseases of medical and veterinary importance, including Chagas disease in South America and sleeping sickness and Nagana in Africa (Hoare, 1972). Some trypanosomes, normally considered as non-pathogenic, are capable of inducing detrimental effects in the host, particularly when the parasite encounters new or naïve host species following their introduction into a new habitat (Maraghi and Molyneux, 1989) or when an infected host is exposed to additional or increased levels of stress (Brown et al., 2000; Wyatt et al., 2008). This may have been the case with the introduction of *Trypanosoma lewisi* to immunologically naïve rodent hosts on Christmas Island, which caused a collapse in the population of the endemic rat *Rattus macleari* to the point of complete extinction (Pickering and Norris, 1996; Wyatt et al., 2008). Studies have also shown that *Trypanosoma theileri*, a non-pathogenic trypanosome of bovids that infects most cattle worldwide, may be considered potentially pathogenic in the presence of stress, gestation, poor nutritional status, and concurrent infections (Hussain et al., 1985; Doherty et al., 1993; Seifi, 1995).

* Corresponding author. Tel.: +61 8 93602690; fax: +61 8 93602466.
E-mail address: L.Boterogomez@murdoch.edu.au (A. Botero).

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http://dx.doi.org/10.1016/j.ijppaw.2013.03.001

Within Australia, numerous trypanosome species and genotypes have been described naturally infecting a wide range of native marsupials. These include *Trypanosoma thylacis* in northern brown bandicoots (*Isoodon macrourus*) (Mackerras, 1959), *Trypanosoma irwini* and *Trypanosoma gilletti* in koalas (*Phascolarctos cinereus*) (McInnes et al., 2009, 2011a), *Trypanosoma binneyi* in platypus (*Ornithorhynchus anatinus*) (Noyes et al., 1999) and *Trypanosoma copemani* in quokkas (*Setonix brachyurus*), Gilbert's potoroo (*Potorous gilbertii*) (Austen et al., 2009) and koalas (McInnes et al., 2011a,b). Different genotypes are also seen in the woylie (*Bettongia penicillata*), common wombat (*Vombatus ursinus*), eastern grey kangaroo (*Macropus giganteus*), swamp wallaby (*Wallabia bicolor*) brush tailed possum (*Trichosurus vulpecula*), chuditch (*Dasyurus geoffroii*), golden bandicoot (*Isoodon auratus*), southern brown bandicoot (*Isoodon obesulus*) and burrowing bettong (*Bettongia lesueur*) (Bettiol et al., 1998; Noyes et al., 1999; Hamilton et al., 2005; Thompson et al., 2008; Smith et al., 2009; Paparini et al., 2011). Phylogenetic analysis has shown that most of these trypanosomes fall into several distinct clades. Some of them are closely related to trypanosomes from outside Australia, but the majority cluster with species/genotypes within Australia (Stevens et al., 2001; Hamilton et al., 2005; Thompson et al., 2008; Smith et al., 2009; Austen et al., 2009; McInnes et al., 2011a; Paparini et al., 2011). Despite the identification of this large list of trypanosomes and their marsupial hosts, there are many unanswered questions related to host–parasite interactions and their pathogenic potential during single and mixed natural infections.

A commonly infected Australian native marsupial is the woylie (Thompson et al., 2008; Smith et al., 2009), which occupied most of the southern half of the Australian mainland before European settlement in 1788. However by the 1970s, woylies were listed as critically endangered due to a drastic reduction in abundance from habitat destruction and introduced predators (Start et al., 1995; Orell, 2004; de Tores et al., 2008). The geographical distribution of the woylie became confined to three locations in Western Australia (WA), the Upper Warren Region, Tutanning Nature Reserve and Dryandra Woodland (Fig. 1) (DEC, 2008; de Tores et al., 2008). Significant conservation efforts, which included predator control and captive breeding and release, led to an increase in abundance from the mid 1970s into the early 2000s. As a consequence of these efforts, woylies were removed from the endangered species list in 1996 (Start et al., 1998). However, since 1999, remaining populations have undergone a dramatic 90% reduction in abundance despite no apparent increase in the number or type of predators in the region and no apparent decrease in natural resources (Wayne et al., in press a,b). As a consequence of these population declines, woylies were included once again on the endangered species list (Wayne et al., 2008; Groom, 2010). Karakamia wildlife sanctuary, a predator-proof fenced reserve located 50 km north-east of Perth, is the only substantial translocated population of woylies that has remained stable in Western Australia. Disease, possibly caused by *Trypanosoma* parasites shown to be at high prevalence levels, has been under consideration as an important factor associated with the decline (Thompson et al., 2008).

Considering the pathogenic potential of trypanosomes when encountering new host species or when an infected host is exposed to increased levels of stress, it is becoming increasingly important to establish their presence, rate of infection, genetic diversity and phylogenetic status within Australian wildlife. To help clarify these issues, this study aims to: (i) determine the genetic variability and phylogenetic relationships of trypanosomes circulating in Western Australia marsupials; (ii) investigate the life cycle of trypanosomes in the marsupial host; (iii) characterize trypanosome behavior in axenic culture; and (iv) evaluate the pathogenicity of trypanosomes and their influence in the decline of the woylie. Together these data will help clarify the impact of trypanosomes on the health of native Australian marsupials.

Materials and methods

Sample collection

Tissue and blood samples were collected from 10 species of marsupials at different locations throughout Western Australia (WA) as part of the WA Department of Environment and Conservation (DEC) fauna research and monitoring program (Fig. 1, Table 1). A total of 554 heparinized peripheral blood samples were collected from trapped and released marsupials during ongoing trapping sessions by DEC. 237 of these samples were collected from woylies at the stable population in Karakamia Sanctuary and 257 from woylies at the declining population in the Upper Warren Region. Tissue samples were collected from sick-euthanized animals that were presented to Perth Zoo Veterinary Department for treatment and from dead (accidently killed on roads) animals sent to Murdoch University for necropsy. Sick animals were euthanized due to very poor body condition, marked lethargy and poor prognosis for return to the wild. A total of 250 tissue samples were collected from 50 carcasses and at least two of the following tissues were collected from each animal: spleen, liver, lung, heart, kidney, brain, oesophagus, tongue, skeletal muscle and bone marrow. Wildlife sampling was carried out under Murdoch University animal ethics approval permit numbers NS1182-06, W2172-08 and W2350-10, and DEC animal ethics approval permit number DECAEC/52/2009. All tissue samples were extensively washed with phosphate buffered saline (PBS) and stored in 100% ethanol and 10% formalin for DNA isolation and histopathological analysis respectively.

Isolation, growth behavior and morphology of trypanosomes

Cultures were established by inoculation of approximately 50 μl of peripheral blood in biphasic media containing Brain-Heart Infusion (BHI) medium with agar and 10% defibrinated horse blood as a solid phase, and either RPMI or HMI9 medium supplemented with 10% heat-inactivated Fetal Calf Serum (FCS) and 50 μg/ml Penicillin–Streptomycin as a liquid phase. All isolates were expanded in liquid media until they reached a density of approximately 1×10^6 parasites/ml and were subsequently maintained with successive passages every week at 27 °C in the dark. Thin blood smears taken from naturally infected marsupials and smears of logarithmic and stationary phase cultures were fixed in methanol and stained with the commercial Romanowsky-type stain 'Diff-Quik' for examination by light microscopy. For scanning electron microscopy (SEM), culture forms were fixed in a 1:1 mixture of 5% glutaraldehyde in 0.01 M PBS: cell culture media (pH 7.2), before being mounted on poly-L-lysine coated coverslips, progressively dehydrated through a series of ethanols and critical point dried as previously described (Edwards et al., 2011). Coverslips were mounted on stubs with adhesive carbon, coated with 2 nm Pt and imaged at 3 kV using the in-lens secondary electron detector on a Zeiss 55VP field emission SEM.

Cell infection

Metacyclic trypomastigotes (1.4×10^4/well) from one week old cultures growing in liquid media were used to infect monolayers of L6 (skeletal muscle) and Vero (kidney epithelial) cells grown to 50% confluency in RPMI media supplemented with 10% FCS, on 16 well glass coverslips. In parallel, cells were infected with *Trypanosoma cruzi* Tulahuen strain for comparison. All cells were maintained at 37 °C and 5% CO_2. Two days post-infection, cells

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