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Confirmation of a unique species of *Giardia*, parasitic in the quenda (*Isoodon obesulus*)

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ABSTRACT

The 'quenda genotype' of *Giardia* was first identified in quenda (syn. southern brown bandicoots, *Isoodon obesulus*) in Western Australia in 2004. We aimed to formally describe this genotype as a species of *Giardia*, *Giardia peramelis*. Seventy five faecal samples positive for *G. peramelis* were obtained from quenda within the Statistical Division of Perth, Western Australia. These samples were used in morphological and molecular characterisation of *G. peramelis*. PCR amplification and sequencing was most successful at the 18S rRNA and ITS1-5.8s-ITS2 loci. Phylogenetic analyses placed *G. peramelis* external to the '*Giardia duodenalis* species complex' and *Giardia microti*. This confirmed the uniqueness of *G. peramelis*, warranting classification as a separate species of *Giardia*. Study findings suggest quenda are a natural host for *G. peramelis*.

1. Introduction

A novel genotype of *Giardia* was isolated and described from a quenda (syn. southern brown bandicoot, *Isoodon obesulus*) in southwest Western Australia (Adams et al., 2004). This was believed to be a distinct species of *Giardia*, but the lack of samples from additional infected animals precluded formal description of this genotype as a separate species at that time.

Since this initial description, the 'quenda genotype' of *Giardia* has been documented in quenda in several other locations in Western Australia (Thompson et al., 2010). It has also been identified using PCR from a calf in Western Australia, though it is unclear whether this reflected infection or cysts passing through the calf gut after ingestion of contaminated pasture (Ng et al., 2011a). The 'quenda genotype' of *Giardia* has not been isolated from other Australian marsupial species surveyed for *Giardia* spp. (McCarthy et al., 2008; Thompson et al., 2008, 2010; Ng et al., 2011b; Thomasz, 2014; Vermeulen et al., 2015).

We undertook a parasitological survey of quenda in the greater Perth region, investigating the epidemiology of *Giardia* spp. infections in this species, and found infection with the 'quenda genotype' of *Giardia* to be common. We aimed to formally describe the 'quenda genotype' of *Giardia* as a separate species, *Giardia peramelis*, by describing the morphology of cysts and trophozoites and genetically characterising the parasite. It is recognised that for any parasite, once adequate data are available the names should be formalised at the species level (Brooks and Hoberg, 2000). This provides stability and is essential for effective communication. More specifically, we considered formal description of this parasite important to expand knowledge of the phylogenetic range of the genus *Giardia*; and in consideration of the public health significance of *Giardia* spp. in Australian marsupials, in differentiating zoonotic and non-zoonotic 'strains' of the parasite.

2. Materials and methods

2.1. Obtaining *G. peramelis* specimens

Faecal samples were collected from quenda trapped across 51 locations in the Statistical Division of Perth, Western Australia. Faecal material was also collected from the large intestine of quenda carcasses, obtained opportunistically from the same area. All samples were obtained under Murdoch University Animal Ethics Permit (R2530/12), and Department of Parks Wildlife Regulation 17 (SF009640) and Regulation 4 (CE004287) permits.

From each quenda, 2 mL faeces were thoroughly mixed in to

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8 mL 10% buffered formalin, and 1 mL faeces were thoroughly mixed in to 8 mL 70% ethanol. Preserved faecal samples were stored at 4 °C until analysis.

The formalin-preserved faecal samples were screened for *Giardia* spp. cysts using immunofluorescence microscopy. Merifluor Cryptosporidium/Giardia kits (Meridian Bioscience, Inc. USA) were used according to manufacturer's directions for unconcentrated faecal samples. Slides were examined at 200X magnification. Samples positive for *Giardia* spp. were differentiated to a species level via PCR and sequencing (methodology in section 2.4). In addition, ten immunofluorescence negative samples from trapped quenda were randomly selected and subject to the same PCR and sequencing protocols as the immunofluorescence positive samples.

2.2. G. peramelis morphological description-trophozoites

Wet mounts were prepared from the small intestinal mucosa of two quenda carcasses, which were positive for *G. peramelis* on faecal testing (and were not positive for any other species of *Giardia*), and were considered sufficiently fresh, with minimal mucosal autolysis, for detection of trophozoites. The first third of the small intestine was gently scraped and the scrapings mounted on a microscope slide. The slides were examined for trophozoites using an Olympus BX50 microscope. A sample of mucosal scrapings from each quenda was also used to seed flasks containing *Giardia* media (section 2.2, below), and cultures were monitored regularly for the appearance of trophozoites.

Excystation of *G. peramelis* cysts was attempted three times, using faecal samples from three quenda that were positive for *G. peramelis* by immunofluorescence microscopy and PCR (and not positive for any other species of *Giardia*).

To purify *G. peramelis* cysts, 1 g of fresh faeces containing *G. peramelis* was homogenised in 1X PBS, passed through layers of gauze, and centrifuged at 0.6 G. Two wash steps were carried out, where the supernatant was removed, the pellet was resuspended in 1X PBS, and the sample was centrifuged at 0.6 G. Two sucrose samples were made-one to a specific gravity of 1.18, and another made up to 0.8 M. The 0.8 M solution was layered on top of the SG 1.18 solution, and the purified sample was layered on top. The sample was centrifuged at 0.2 G, and cysts were collected at the water/sucrose interphase. 1X PBS was added to this isolation and it was centrifuged at 0.6 G, with the supernatant removed subsequently. The cysts were resuspended in a final volume of 1 ml 1X PBS, and examined under the microscope.

For excystment, cysts were resuspended in 10 mL of acidified Hanks Balanced Salt Solution (HBSS) (30 g/L biosate peptone (BD, Annapolis, USA), 10 g/L glucose (Sigma–Aldrich, St. Louis, USA), 2 g/L sodium chloride (Chem-Supply, Adelaide, Australia), 2 g/L cysteine (Sigma–Aldrich, St. Louis, USA), 1 g/L K_2HPO_4 (Chem-Supply, Adelaide, Australia), 0.6 g/L KH_2PO_4 (Merck, Melbourne, Australia), 0.01 g/L ferric ammonium citrate (Sigma–Aldrich, St. Louis, USA), 0.2 g/L ascorbic acid (Sigma–Aldrich, St. Louis, USA), 0.5 g/L bovine bile (Sigma–Aldrich, St. Louis, USA), 100 ml/L newborn calf serum (SAFC Biosciences, Lenexa, USA) and 10 ml/L penicillin/streptomycin (Sigma–Aldrich, St. Louis, USA), pH 2) and incubated at 37 °C for 30 min. Cysts were then centrifuged at 0.2 g and washed twice in HBSS (pH7.2), and finally resuspended in HBSS (pH 7.2) medium and incubated at 37 °C. Cysts were monitored daily for excystment, for one week.

2.3. G. peramelis morphological description-cysts

To describe the morphology of *G. peramelis* cysts, faecal smears were prepared using formalin-preserved faecal samples from eight quenda that were positive for *G. peramelis* by PCR with sequencing (and not positive for any other species of *Giardia*). Smears were examined by bright field and Nomarski differential interference microscopy, using an Olympus BX50 microscope. *G. peramelis* cysts were photographed at 1000X magnification. ImageJ software (US National Institute of Health, Bethesda, Maryland), was used to measure cyst length and width.

2.4. G. peramelis molecular characterisation

2.4.1. DNA extraction and PCR amplification

Amplification by PCR was attempted on all immunofluorescence microscopy positive faecal samples and the ten randomly selected immunofluorescence negative samples. Ethanol-preserved faecal samples were centrifuged to separate ethanol from faeces, with the ethanol supernatant discarded. Samples were then twice re-homogenised in distilled water, centrifuged and supernatant discarded. DNA extraction was then conducted using the Maxwell® 16 Instrument (Promega, Madison, USA) as per manufacturer's instruction.

Amplification by PCR was attempted at three loci: 18S rRNA, ITS1-5.8s-ITS2 and glutamate dehydrogenase (*gdh*). Initially, a semi-nested PCR protocol was employed to amplify a 130 bp product of the 18s rRNA, with primers RH11/RH4 and RH11/GiarR (Hopkins et al., 1997; Read et al., 2002). The PCR reaction was performed in 25 μl volumes, consisting of 1–2 μl of extracted DNA, 2.0 mM $MgCl_2$, 1 × reaction buffer (20 mM Tris-HCL, pH 8.5 at 25 °C, 50 mM KCl), 400 μM of each dNTP, 0.4 μM of each primer, 0.5 units of Taq DNA polymerase (Fisher Biotec, Perth, Australia), and DMSO 5%. Amplification conditions were modified from Hopkins et al. (1997), and involved a denaturing step of 95 °C for 6 min, then 40 cycles of 95 °C for 30 s, 53 °C for 30 s (56 °C in the secondary round) and 72 °C for 30 s, followed by a final extension of 72 °C for 7 min.

A nested PCR protocol was conducted to amplify a 330 bp product of the ITS1-5.8S-ITS2 region of the ribosomal gene, with primers developed by Caccio et al. (2010). The PCR reactions were the same as those used for 18s rRNA, but performed in 50 μl volumes. Conditions for amplifications were modified from Caccio et al. (2010), and involved an initial denaturing step of 95 °C for 5 min, then 40 cycles of 95 °C for 45 s, 59 °C for 30 s and 72 °C for 30 s, followed by a final extension of 72 °C for 7 min.

Finally, for *gdh*, a nested PCR protocol was used to amplify a 530 bp product, using the primer pairs Gdh1/Gdh2 and Gdh3/Gdh4 for the primary and secondary rounds respectively, as per Caccio and Ryan (2008). The PCR reaction was performed in 25 μl volumes, consisting of 2 μl of extracted DNA, 1.5 mM $MgCl_2$, 1 × reaction buffer, 200 μM of each dNTP, 0.4 μM of each primer, 1 unit of Taq DNA polymerase (Fisher Biotec, Perth, Australia) and DMSO 5%. Conditions for amplifications were the same for both rounds, and involved an initial denaturing step of 94 °C for 2 min, then 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s, followed by a final extension of 72 °C for 7 min.

2.4.2. Sequencing of amplified product

PCR products were purified using an Agencourt AMPure XP system (Beckman coulter, Beverly, USA). Sequence reactions were performed using the Big Dye Terminator Version 3.1 cycle sequencing kit (Applied Biosystems), according to the manufacturer's instructions. Reactions were electrophoresed on an ABI 3730 48 capillary machine. Amplicons were sequenced in both directions, with resultant nucleotide sequences compared with published sequences on NCBI GenBank® using the basic alignment search tool (BLAST). Further sequence analysis was conducted using the sequence alignment program Sequencher™ 4.8 (Gene Codes, Ann Arbour, MI, USA).