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Semperula wallacei (Mollusca, Veronicellidae) um hospedeiro natural recém-descoberto de Angiostrongylus cantonensis (Nematoda, Angiostrongylidae) na Bacia do Pacífico

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Abstract

Semperula wallacei (Issel, 1874) is a species of terrestrial slug that occurs in southeast China and the Pacific Basin and is the only species of its genus that occurs beyond the Oriental region and to the east of Wallace's line in the Australian region, where it has probably been introduced. In this study, we report for the first time *S. wallacei* as an intermediate host for *Angiostrongylus cantonensis* (Chen, 1935) based on histological and molecular analyses of slugs from Tuamasaga, Samoa, deposited at the Medical Malacological Collection (Fiocruz-CMM). DNA was obtained from the deparafinized tissues scraped from specimen slides. Polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) targeted to the *internal transcribed spacer 2* (*ITS2*) region were carried out using the restriction enzyme *Cla* I. The RFLP profile observed for our larval specimen of *S. wallacei* was identical to the profile previously established for *A. cantonensis*, demonstrating that *S. wallacei* can be naturally infected with *A. cantonensis* and is likely to be an intermediate host for this parasitic nematode species in the field. The potential for geographical range expansion of *S. wallacei* in the Pacific Basin, its small size, and the general role of veronicellids as crop pests and hosts of nematodes, indicate the significance of *S. wallacei* as an invasive species in the Pacific Basin. Our work also highlights the importance of biological collections for investigating the environmental impact of invasive species on agriculture, public health, and biodiversity conservation.

Introduction

Angiostrongylus cantonensis (Chen, 1935) is one of the main etiological agents of eosinophilic meningitis in humans (Eamsobhana, 2014). This metastrongyloid is endemic in Asia and the Pacific Basin, where most cases of human infection have occurred, although now it is reported from most parts of the world (Cowie, 2013; Eamsobhana, 2014; Jarvi *et al.*, 2017). Currently, *A. cantonensis* has been reported from Taiwan and other parts of Southeast Asia (Thailand, Malaysia), numerous Pacific islands, including New Caledonia, Vanuatu, Fiji, Guam, Saipan, Chuuk, Pohnpei, Marshall Islands, Tahiti, Cook Islands, Hawaii, Papua New Guinea, Western Samoa, and American Samoa, as well as Okinawa and mainland Japan, Indonesia, the Philippines, Australia, Sri Lanka, India, Réunion, Mauritius, Ivory Coast, Egypt, South Africa, Madagascar, Cuba, Jamaica, Puerto Rico, Haiti, Dominican Republic, Ecuador, Brazil, the Canary Islands, and the southeastern United States (Gomes & Thomé, 2001; Hirano *et al.*, 2019; Thiengo *et al.*, 2022; Cowie *et al.*, 2022). Additionally, an increasing number of cases have been recorded in locations where *A. cantonensis* is not considered to be naturally present, including various European countries and the northern United States (Cowie, 2013; Nguyen *et al.*, 2017; Ansdell & Wattanagoon, 2018; Federspiel *et al.*, 2020; Cowie *et al.*, 2022), mostly in people returning from regions believed to be within its native biogeographical range.

The life cycle of *A. cantonensis* occurs mainly in molluscs and rodents. The increasing spread of these intermediate and definitive hosts, respectively, due to globalization, are among the reasons for the currently wide distribution of this species of nematode (Kim *et al.*, 2014). The molluscs that act as intermediate hosts have been transported around the world either intentionally or accidentally through various pathways, notably the agricultural and horticultural industries (Cowie *et al.*, 2008).

The parasite *A. cantonensis* can use numerous species of terrestrial, and some aquatic molluscs as intermediate hosts (Valente *et al.*, 2020). Species of Veronicellidae are well known for their public health importance as intermediate hosts for nematodes (Bonetti & Graeff-Teixeira, 1998; Laitano

et al., 2001; Ohlweiler *et al.*, 2010; Carvalho *et al.*, 2012; Valente *et al.*, 2020; Modrý *et al.*, 2021) and for being important agricultural pests (Robinson & Hollingsworth, 2005; Ramos *et al.*, 2021). These molluscs are a diverse group that includes endemic species as well as more widespread species that have recently expanded beyond their original native ranges (Gomes & Thomé, 2004).

Semperula wallacei (Issel, 1874) is a small veronicellid, having a body length of approximately 40 mm and width of approximately 20 mm. This species can be identified based on both its reproductive system characteristics and molecular markers (Gomes & Thomé, 2001; Schilthuizen & Liew, 2008; Gomes *et al.*, 2010). *Semperula wallacei* occurs in Australia, China, Fiji, Sarawak, Sulawesi, Sumatra, Samoa, Vanuatu (Gomes & Thomé, 2001), American Samoa (Kim *et al.*, 2016), and Japan (Hirano *et al.*, 2019). This species is the only member of the genus *Semperula* found beyond the Oriental region and to the east of Wallace's line in the Australian region.

In the present study, we report the occurrence of *S. wallacei* from Tuamasaga, Samoa, infected with larvae of *A. cantonensis*, using molecular identification of DNA recovered from larval specimens found in histological slides of the snail host.

Material and methods

The results presented below are based on the analysis of four specimens originally from the collection of terrestrial molluscs of the United States of Department of Agriculture (USDA), which were donated to the Collection of the Medical Malacology Research Center René of the Instituto René Rachou/Fiocruz/Minas in the state of Minas Gerais, Brazil. The specimens were collected from the district of Tuamasaga on the island of Upolu, Samoa.

To identify the specimens, three specimens were dissected under a stereomicroscope, starting with a posterior to anterior longitudinal central incision, following the methodology of Thomé & Lopes-Pitoni (1973). The anatomical characteristics of the genus and species were compared with those described by Gomes & Thomé (2001, 2004).

For histological analysis, one specimen preserved in 70% ethanol was analyzed. A cross-sectional sample of the slug's body was clipped, dehydrated in an ethanol series, and then infiltrated with paraffin. Serial cross sections of the paraffin-embedded block of tissue were stained with hematoxylin-eosin (HE) and examined by bright field microscopy. The slides were photographed using a stereoscopic microscope coupled to a camera and captured using the LAS V4.9 software.

For the molecular identification of *A. cantonensis*, the same histological slides were placed in glass containers containing xylol for 48 h, followed by 10 consecutive washes in absolute alcohol and distilled water. Deparaffinized tissues were scraped from the slides and placed in 1.5 ml Eppendorf tubes containing 600 µl of nuclear lysis solution (Wizard Genomic DNA Purification, Promega). Five µl of proteinase K (125 mg/ml) were added to the lysate and then incubated for 24 h at 55°C. Afterwards, DNA extraction was undertaken using the DNA Wizard Genomic Purification kit (Promega, Madison, USA), according to the manufacturer's instructions. The resulting pellet was treated with 50 µl DNA dehydration solution for 30 min at 65°C and stored at –20°C (Magalhães et al., 2008).

For comparison, other nematode species were also included in the study: *A. cantonensis* and *A. costaricensis*, which had been stored at –70°C. Polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) directed to the *internal transcribed spacer 2* (*ITS2*) region were carried out using the restriction enzyme *Cla* I (Caldeira *et al.*, 2003).

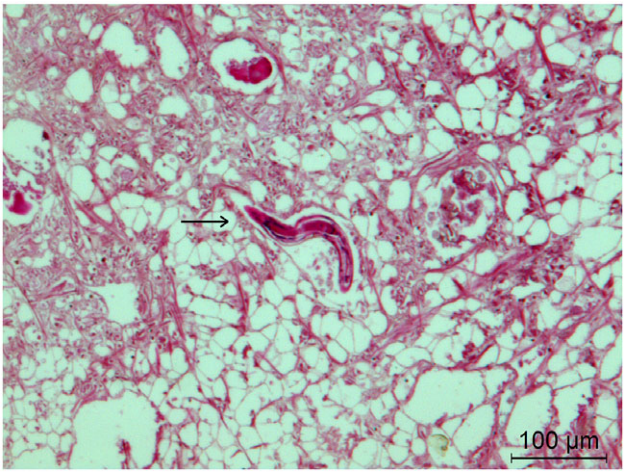

Figure 1. Hematoxylin-eosin-stained histological section showing a larva of the nematode *Angiostrongylus cantonensis* (arrow) in the fibromuscular layer of the integument of *Semperula wallacei.*

Results and discussion

Our results demonstrate that the larva present in the fibromuscular layer of the integument of *S. wallacei* (Figure 1) belongs to *A. cantonensis* because the molecular profile of the samples analyzed were identical to the profile previously established for this metastrongylid species (Figure 2). This observation was possible because of the efficiency of the method of DNA extraction of *A. cantonensis* from formalin-fixed, paraffin-embedded, HE-stained histological sections. Using this same methodology, our group was also previously able to detect the liver fluke *Fasciola hepatica* (Plagiorchiida: Fasciolidae) in the freshwater snail *Lymnaea viatrix* d'Orbigny, 1835 (Gastropoda: Lymnaeidae) in histological sections using multiplex-PCR (Magalhães *et al.*, 2008). The wide geographical distribution of *S. wallacei* in the Pacific Basin and its known proximity to human habitations suggest that it may be a potentially important intermediate host of *A. cantonensis*. Schilthuizen & Liew (2008) mentioned that, although the holotype of *S. wallacei* is from Sarawak and was collected during a period when alien species introductions resulting from anthropogenic activity were still relatively rare, this

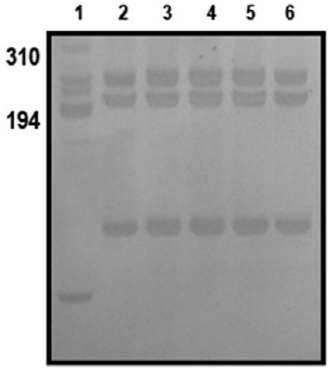

Figure 2. Silver-stained 6% polyacrylamide gel showing the PCR-RFLP profiles of the *ITS2* rDNA digested with the enzyme *Cla* I. Lane 1: L3 pool of *Angiostrongylus costaricensis* recovered from *Sarasinula linguaeformis* (Instituto Oswaldo Cruz, Fiocruz RJ); lanes 2–3: adult worm of *Angiostrongylus cantonensis* Department of Parasitology, Medical School, Akita University, Japan; lanes 4–6: larvae of *A. cantonensis* from a histological section of *S. wallacei* from Tuamasaga, Samoa (this study). Molecular size in base pairs is indicated on the left side of the figure.

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species appears not to be native to Borneo because it is only found in disturbed vegetation near human habitation, being present in and around most towns and cities of Sabah, as well as more widely in Southeast Asia and the Pacific islands.

The nonnative snail/slug faunas of many of the islands and archipelagos in the Pacific are often composed of cosmopolitan invasive species and contain a subset of this suite species. In many disturbed areas, for example in Hawaii, only nonnative species are present, and these include large species, such as *Achatina fulica* and other veronicellid slugs (Cowie, 2001).

In recent years, cases of human angiostrongyliasis have increased significantly in China due to increase of living standards and income and modern food consumption trends (Eamsobhana, 2014). By the end of 2009, angiostrongyliasis cases/outbreaks had been reported from at least nine provinces in China, where 457 cases had been identified (Wang *et al*., 2012).

Several species of gastropods, including terrestrial and freshwater species, can act to varying degrees as intermediate hosts for *A. cantonensis* (Valente *et al*., 2020). Of 37 mollusc species analyzed from the Hawaiian Islands, 16 tested positive for *A. cantonensis* (Kim *et al*., 2014). In Brazil, among 21 species of gastropods collected in 30 ports, four were found positive for *A. cantonesis* (Carvalho *et al*., 2012).

This finding reinforces the importance of health surveillance for eosinophilic meningitis in Samoa because there are already recorded cases of this zoonosis in the Pacific region and possibly in Samoa (Cowie *et al*., 2022). Also, there are other alien species of veronicellids reported in Samoa (Gomes & Thomé, 2004) that has potential to act as intermediate hosts of nematodes of medical and veterinary importance, considering the historic of this family of slugs, such as *Sarasinula plebeia* (Fischer, 1868), *Veronicella cubensis* (Pfeiffer, 1840), and *Laevicaulis alte* (Férussac, 1822). The potential expanding geographical range of *S. wallacei* in the Pacific Basin through agriculture trade, its small size, and the generally known role of veronicellids as crop pests and hosts of nematodes, indicate the potential importance of *S. wallacei* as an invasive species in the Pacific Basin. Our work also highlights the importance of biological collections for investigating the environmental impact of invasive species on agriculture, public health and biodiversity conservation.

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Competing interest. The author(s) declare none.

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