

## *Achatina fulica* INFECTED BY *Angiostrongylus cantonensis* IN MANAUS, BRAZILIAN AMAZON REGION, AND THE RISK OF TRANSMISSION OF EOSINOPHILIC MENINGITIS

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### ABSTRACT

*Angiostrongylus cantonensis*, which causes the zoonosis Eosinophilic Meningitis (EM), has become increasingly widespread in Brazil. Terrestrial mollusks, especially the exotic giant African snail *Achatina fulica*, have been found naturally infected with this nematode besides some few freshwater species. In this study, we collected several terrestrial and one freshwater mollusks from five different localities in the city of Manaus, in the Brazilian Amazon Region. One hundred and eighteen specimens from nine different species were obtained. In all, 49 specimens were analyzed parasitologically, including *A. fulica* (42 specimens), *Bulimulus* sp. (one specimen) and the freshwater species *Pomacea dolioides* (five specimens). The other specimens died or were represented by shells. Specimens of *A. fulica* from one locality were found naturally infected with *A. cantonensis*, which was confirmed by phylogenetic analysis of mitochondrial MT-CO1 gene sequences. A recent case of EM reported in the Amazon region, in the city of Macapá, reinforces the need of effective surveillance of areas in which the parasite is known to occur, as well as raising the awareness of local populations at risk of infection. We highlight both the role of *A. fulica* in the transmission of *A. cantonensis* and the need for effective measures to control the spreading of EM in Brazil.

**KEY WORDS:** Mollusk; neural angiostrongyliasis; neurotropic parasite; intermediate host.

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## INTRODUCTION

The relationship between nematodes parasites and terrestrial mollusks has raised attention in public health because these mollusks may act as intermediate host in the life cycle and in the dispersion of zoonotic helminthiasis. In this context, there has been an increase in the risk of cases of zoonosis associated with the invasion and dispersal of the African snail *Achatina fulica* Bowdich, 1822 in Brazil and other countries where this mollusk was introduced (Chitwood & Chitwood, 1937; Thiengo et al., 2007, 2022; Silva et al., 2022).

Neural angiostrongyliasis or eosinophilic meningitis (EM) is caused by *Angiostrongylus cantonensis* (Chen, 1935), the rat lung worm, that over the past two decades have been recorded in Brazil (Caldeira et al., 2007; Morassutti et al., 2014; Thiengo et al., 2022). Humans are accidental hosts as the life cycle of this nematode occurs between rodents and mollusks. *A. cantonensis* has become increasingly widespread in Brazil (Ramos-de-Souza et al., 2018), which appears to be associated with the ongoing dispersal of *A. fulica*. This mollusk now occurs throughout the country (Arruda & Santos, 2022; Thiengo et al., 2022), after being introduced in the 1970s (Barçante et al., 2005). Although *A. fulica* is considered to be the principal intermediate host of *A. cantonensis* in Brazil (Thiengo et al., 2022), a number of other synanthropic terrestrial mollusks are also known to act as intermediate hosts (Carvalho et al., 2012; Ramos-de-Souza et al., 2018). Among aquatic mollusks, the freshwater snail *Pomacea lineata* Spix in Wagner, 1827 is the only aquatic species already found naturally infected in Brazil, in the State of Pernambuco, although other species of the genus were reported as natural hosts in other countries, as *P. canaliculata* (Lamarck, 1822) in Asia, that is an abundant species in Southern Brazil (Thiengo et al., 2010; Song et al., 2016).

EM was initially endemic in Southeast Asia, having emerged in the western Pacific after the Second World War, but later appearing in the Caribbean and more recently, emerging in South America (Robles & Dorta-Contreras, 2016). In Brazil, *A. cantonensis* is known to occur in 14 of the 26 states (Ramos-de-Souza et al., 2018; Almeida et al., 2023), including Amazonas, where the species has been reported from the town of Barcelos (Morassutti et al., 2014), 400 km northwest of the city of Manaus.

The present study is part of an ongoing project that is investigating the involvement of *A. fulica* and other terrestrial mollusks in the transmission of nematodes that affect human and animal health in Brazil. Surveys of synanthropic terrestrial mollusks, in particular *A. fulica*, have been performed with specimens being collected along different Brazilian States. In this paper we provide the rates of parasitism by nematodes in synanthropic mollusks collected in the city of Manaus, capital of the State of Amazonas, in the central Amazon region.

## MATERIAL AND METHODS

Field expeditions received support from the Amazonas State Public Health Monitoring Foundation (Fundação de Vigilância em Saúde do Amazonas: FVS-AM), which surveyed five neighborhoods in the urban zone of city of Manaus. We collected mainly *A. fulica* and a few other land mollusks found in proximity to human dwellings. The neighborhoods were Shangrilá (3°04'14.2" S, 60°00'6.3" W), Novo Aleixo (3°03'2.86" S, 59°59'0.63" W), Redenção (3°02'59.9" S, 60°02'49.19" W), Tarumã (2°58'51.5" S, 60°03'7.19" W), and São Pedro (2°59'49.1" S, 60°02'16.7" W) – they were selected as survey sites due to previous reports of *A. fulica* infestation by FVS-AM. Additionally, we collected freshwater mollusks of the genus *Pomacea* due to their known role as an intermediate host of *A. cantonensis* (Thiengo et al., 2010; Song et al., 2016).

The specimens were collected in December 2021 and sent to the National Reference Laboratory for Schistosomiasis – Malacology (LRNEM) at the Oswaldo Cruz Institute/Oswaldo Cruz Foundation (IOC/Fiocruz), where parasitological analysis and taxonomic identifications were carried.

Mollusks were identified based on Simone (2006), Thomé et al. (2006) and Thiengo et al. (2017), as well as through comparisons with voucher specimens deposited in the Mollusk Collection of the Oswaldo Cruz Institute (CMIOC). The young specimens were identified at genus level. Specimens of terrestrial and freshwater mollusks were examined based on the artificial digestion of their soft tissue in 0.7% HCl (Graeff-Teixeira & Morera, 1995) to determine the possible occurrence of nematode larvae.

The nematode larvae with morphological traits of the family Angiostrongylidae were separated for molecular analysis, with a pool of 10 larvae being extracted from each positive sample, stored in 10 µL of PBS buffer (Thermo Fisher Scientific, Massachusetts, USA), and cryopreserved at -20 °C until the extraction of the DNA for molecular identification at the LRNEM. The partial genomic DNA was extracted by the thermal shock method.

The nematode species was identified by sequencing the mitochondrial cytochrome *c* oxidase subunit I (MT-CO1) for comparison with existing sequences of known nematode species on Genbank. For this, the Polymerase Chain Reaction (PCR) solution was prepared in a volume of 20 µL containing 9.25 µL of ultrapure water, 5.0 µL of 5× Green GoTaq Flexi Buffer, 2.0 µL of 2.5 mM dNTPs, 2.5 µL of 25 mM MgCl<sub>2</sub>, 0.5 µL of each forward and reverse primer (0.2 µM of Nem\_F3 and Nem\_R3, modified from Prosser et al. (2013), and 0.25 µL of GoTaq G2 Hot Start Polymerase (Promega). A 5 µL aliquot of the DNA sample was added to this mixture to a final volume of 25 µL. Ultrapure water was used as the negative control template for all the reactions, and the positive control was a template of the genomic DNA of *A. cantonensis* confirmed by Sanger sequencing.

The PCR amplification protocol was as follows: initial denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 40 s, 51 °C for 40 s, and 72 °C for 1 min, with a final extension at 72 °C for 1 min. The purified products were sequenced bidirectionally using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, California, USA) according to the manufacturer's instructions. The samples were sequenced in an ABI 3730 DNA Analyzer (Applied Biosystems) at the DNA Sequencing Platform of the Oswaldo Cruz Institute (PDTIS/FIOCRUZ) subunit RPT01A. Chromatograms of the sequences obtained from this amplification were edited in Geneious, version R9 (Kearse et al., 2012) (<http://www.geneious.com>), to produce a consensus sequence (contig). The BLAST Search tool was used to the sequence match the target organism and obtain sequences of similar size from GenBank for the phylogenetic analysis. All the sequences obtained in this study were deposited in GenBank.

To support the molecular identification of the species, the sequences obtained here were included in a phylogenetic analysis based on Bayesian Inference (BI), which included available *A. cantonensis* of similar size obtained from GenBank, with outgroups, *Angiostrongylus* spp. and *Aelurostrongylus abstrusus* (Railliet, 1898) (Angiostrongylidae). The MT-CO1 of 26 sequences in 596 bases pairs (bp) were aligned using the Muscle tool, which was implemented in Geneious R9 (Kearse et al., 2012), and the Bayesian Inference was run in MrBayes, version 3.2.7 (Ronquist et al., 2012) with the command blocks for the GTR + G evolutionary model. The MrBayes analysis was run in the CIPRES Science Gateway v.3.3 (Miller et al., 2010).

## RESULTS

A total of 118 mollusks (live individuals and shells) were collected, including both terrestrial and freshwater specimens. They represented nine different taxa, *A. fulica* (n = 44 live specimens), *P. dolioides* (n = 22 live specimens), two live specimens of *Sarasinula linguaeformis* (Semper, 1885), *Subulina octona* (Brugüière, 1792) (n = 5 live specimens, n = 9 shells), *Leptinaria unilamellata* (d'Orbigny, 1835) (n = 3 live specimens; n = 3 shells), *Allopeas gracille* (Hutton, 1834) (n = 5 live specimens), *Bulimulus* sp. (n = 2 live specimens, n = 13 shells), *Solaropsis* sp. (n = 2 shells of young specimens) and one species of Risssooidea (n = 8 live specimens). Voucher specimens of each taxon were deposited in the CMIOC (CMIOC 13270-13282; CMIOC 13321-13322).

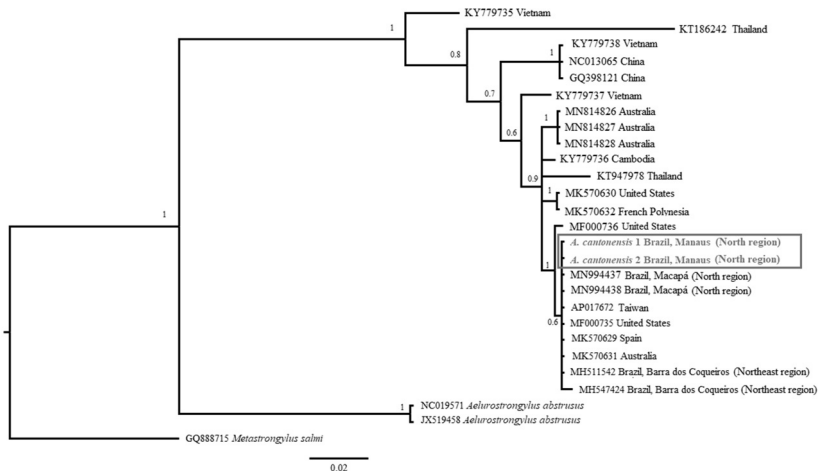
Angiostrongylid larvae were obtained from three *A. fulica* specimens collected from the Novo Aleixo neighborhood (12.5% of the 24 *A. fulica* specimens examined from this neighborhood). The results of the parasitological examination are shown in Table. Of these three positive specimens, larval samples from two mollusks were sequenced to confirm the parasite species.

The MT-CO1 sequences obtained from each sample of *Angiostrongylus* had 700 base pairs (bp). The sequences obtained here were deposited in GenBank (OQ592051- OQ592052).

*Table.* Live mollusks included in the parasitological analysis based on the artificial digestion of tissue in 0.7% HCl.

Neighborhood	Species	Specimens		Specimens (%)
		Collected	Analyzed	testing positive
Shangrilá	<i>Achatina fulica</i>	18	17	0 (0.0)
	<i>Bulimulus</i> sp.	2	1	0 (0.0)
Novo Aleixo	<i>A. fulica</i>	25	24	3 (12.5)
Tarumã	<i>A. fulica</i>	1	1	0 (0.0)
Redenção	<i>Pomacea dolioides</i>	3	1	0 (0.0)
São Pedro	<i>P. dolioides</i>	19	5	0 (0.0)

The BLAST search revealed that both *Angiostrongylus* sequences were 100% similar to the GenBank sequences of *A. cantonensis*. This BI phylogenetic analysis (Figure 1) confirmed the molecular diagnosis of *A. cantonensis* (Figure 2) from the two sequenced samples from Manaus.



*Figure 1.* Cladogram of the *Angiostrongylus cantonensis* MT-CO1 sequences analyzed by Bayesian Inference using the GTR + G evolutionary model. The outgroups are *Angiostrongylus* spp. and the angiostrongyloid *Aelurostrongylus abstrusus*. The samples from Manaus are highlighted.

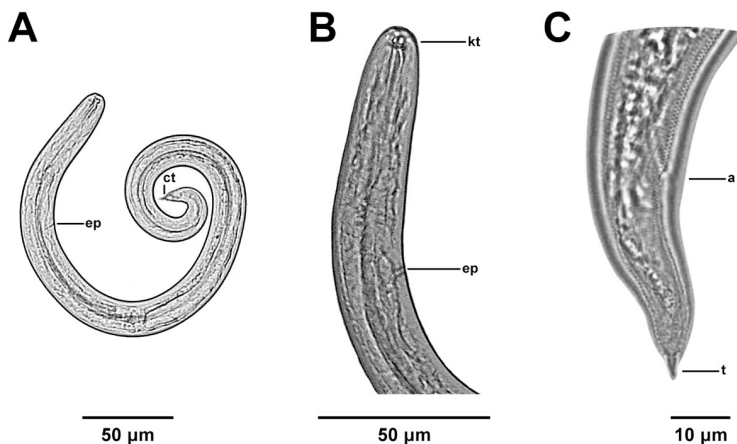


Figure 2. *Angiostrongylus cantonensis* of third stage (L<sub>3</sub>) larvae, obtained *Achatina fulica* naturally infected: A – General view, highlighting the excretory pore (ep) and coiled tail (ct); B – anterior end with expanded knob-like tips (kt) and excretory pore (ep); and C – posterior end presenting the anus (a) and lanceolate tail (t).

## DISCUSSION

We highlight that the occurrence of *A. cantonensis* in Manaus may indicate a risk of the parasite spreading, since the Manaus Free Trade Zone is a major economic hub in the central Amazon region, which was established to support the local industrial, commercial, and agricultural sectors. This zone is supported logistically by international shipping, which may facilitate the entry and exit of *A. cantonensis* in cargos (Carvalho et al., 2012). Even though only *A. fulica* was naturally found infected, we consider epidemiological surveillance regarding this, and other species previously reported as natural intermediate hosts for this parasite to be important.

In Brazil, the distribution of *A. cantonensis* is closely associated with the dispersal of *A. fulica* (Thiengo et al., 2022), even though this nematode has been recorded infecting naturally several other mollusks in this country, including *P. lineata*, *Bradybaena similaris* (Férussac, 1821), *Subulina octona*, *Sarasimula linguaeformis*, and more recently, *Bulimulus tenuissimus* (d' Orbigny, 1835), *Cyclodontina fasciata* (Potiez & Michaud, 1838), and *Belocaulus willibaldoi* Ohlweiler, Mota & Gomes (Thiengo et al., 2010; Carvalho et al., 2012; Ramos-de-Souza et al., 2018; Mota et al., 2020). The positive samples of *A. fulica* in this study have confirmed diagnosis to *A. cantonensis* in the city of Manaus

by BI phylogenetic analysis. Interestingly, in addition to other samples, from northern and northeastern Brazil, the sequences closest to the Manaus samples were from geographically diverse countries, including the United States, Spain, Australia, and Taiwan. These samples were obtained in an urban area, in the garden of a church, where people from different places often gather at religious events, which demonstrates the proximity that can occur between people and infected mollusks.

Carvalho et al. (2012) investigated several major ports in Brazil, including Manaus, and examined both *S. octona* and *S. marginata* (syn. *S. linguaeformis*) for parasites, but recorded no infections by *A. cantonensis*. Given this, this study represents the first record of mollusks being parasitized by *A. cantonensis* in Manaus, which further reinforces the role of *A. fulica* as an important intermediate host of this nematode. In fact, *A. fulica* was the only mollusk parasitized by *A. cantonensis* in the city of Manaus, which implies a close relationship between the two species (Thiengo et al., 2022). Considering that Manaus is an important area of commercial import and export and that *A. fulica* is an invasive species of easy displacement by transporting various products and rapid dispersion (Cowie & Robinson, 2003; Andrade-Porto et al., 2012), it represents an important disperser of *A. cantonensis*.

This first record of *A. cantonensis* from Manaus is also an important alert on the dispersal of this nematode in northern Brazil, given that its only previous record from the State of Amazonas was from the municipality of Barcelos, approximately 400 km northeast from Manaus (Morassutti et al., 2014). In 2020, the first human case of EM was reported from the Brazilian Amazon region, being recorded in the city of Macapá, the capital of Amapá State (Barbosa et al., 2020). This reinforces the need to increase the monitoring of its intermediate hosts, and in particular control the invasive populations of *A. fulica*. Another angiostrongylid, *A. abstrusus*, had previously been found infecting *A. fulica* in Manaus (Andrade-Porto et al., 2012), although this nematode is only of veterinary concern.

Given the data here highlighted, surveillance of land mollusks, in particular *A. fulica*, will be essential for the understanding of epidemiological patterns and for the control of the diseases transmitted by these gastropods. This study also reinforces the need for social programs to increase the awareness of local human populations, to minimize human infection.

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## CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest to disclose.

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