REVISTA DO INSTITUTO DE MEDICINA TROPICAL DE SÃO PAULO

JOURNAL OF THE SÃO PAULO INSTITUTE OF TROPICAL MEDICINE

ORIGINAL ARTICLE

http://dx.doi.org/10.1590/S1678-9946201860051

First records of molluscs naturally infected with *Angiostrongylus cantonensis* (Nematoda: Metastrongyloidea) in Northeastern Brazil, including new global records of natural intermediate hosts

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ABSTRACT

Human neural angiostrongyliasis is an emerging infectious disease caused by nematode *Angiostrongylus cantonensis*. The present study investigated the presence of *Angiostrongylus* spp. in terrestrial molluscs collected from the following areas in the Metropolitan Region of Aracaju, Sergipe State, Brazil: Barra dos Coqueiros, Nossa Senhora do Socorro, Sao Cristovao and Aracaju. In total, 703 specimens representing 13 mollusc species were screened for *Angiostrongylus* spp. Larvae of *Angiostrongylus* spp. were found in three species. Larvae recovered from *Achatina fulica* were used for experimental infection in Wistar rats (*Rattus norvegicus*). For specific identification of nematodes, the mitochondrial cytochrome c oxidase subunit I (COI) was sequenced from both larvae and adults recovered from molluscs and rats, respectively. Infection with *A. cantonensis* was detected in all municipalities and in the following three host species: *Bulimulus tenuissimus*, *Cyclodontina fasciata* (Barra dos Coqueiros), and *A. fulica* (Aracaju, Nossa Senhora do Socorro and Sao Cristovao). Co-infections were also found with *Caenorhabditis* sp. and *Strongyluris* sp. larvae. This is the first study of the helminth fauna associated with the terrestrial malacofauna in Sergipe State, and confirms that these three snail species are involved in the transmission of *A. cantonensis* in the state. In addition, *B. tenuissimus* and *C. fasciata* are newly reported natural hosts of the parasite.

KEYWORDS: Eosinophilic meningitis. *Angiostrongylus. Achatina fulica. Bulimulus tenuissimus. Cyclodontina fasciata.* Land snails.

INTRODUCTION

Achatina fulica Bowdich, 1822 is listed among 100 of the worst invasive species in the world and is present in almost all Brazilian states[1,2]. This species' rapid proliferation is the main reason it is considered an agricultural pest. It can also act as an intermediate host of *Angiostrongylus cantonensis* (Chen, 1935) and *Angiostrongylus costaricensis* Morera and Céspedes, 1971. These two species are the etiological agents of human neural angiostrongyliasis and human abdominal angiostrongyliasis, respectively[2-4].

Eosinophilic meningitis is the principal clinical manifestation of human neural angiostrongyliasis, an emerging infectious disease that causes severe impairment of the central nervous system and can lead to death[5-7]. This nematode has low specificity

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Received: 16 April 2018

Accepted: 16 August 2018

for its intermediate hosts, which means that many mollusc species have already been found to be naturally infected globally[8,9]. However, *A. fulica* is an important intermediate host for *A. cantonensis*[4,8,10-12].

Human neural angiostrongyliasis occurs primarily by ingestion of snails that are infected with L_3 larvae[13]. These larvae reach the brain where they mature into L_4 and L_5 but are unable to migrate from the brain to the heart (as they do in the definitive rat hosts) but move around in the brain causing neurological damage and, especially when they die, cause intense inflammatory reactions that result in additional neurological damage and symptoms[5,7,14].

More than 2,800 human cases of eosinophilic meningitis caused by *A. cantonensis* have been reported in more than 30 countries[11] since it was first reported in Taiwan in 1945[15]. In Brazil, out of 84 suspected cases, there have been 34 confirmed cases of *A. cantonensis* infections in humans as well as the confirmation of definitive hosts and infected intermediate hosts in different regions of the country[10].

In Sergipe State, 19 municipalities have reported infestation of *A. fulica* (Comissao de Combate ao Caramujo Africano, personal communication) in addition to Barra dos Coqueiros municipality[8]. The objective of this study was to verify the infection rate by nematode larvae in *A. fulica* and other terrestrial molluscs collected in the Aracaju Metropolitan Region, Sergipe State, Northeastern Brazil.

MATERIALS AND METHODS

This study was undertaken in urban environments of four municipalities that constitute the Metropolitan Region of Aracaju, Sergipe State (Figure 1): Aracaju, Barra dos Coqueiros, Nossa Senhora do Socorro and Sao Cristovao (Table 1).

Each point of collection was georeferenced, followed by manual random sampling of terrestrial molluscs using forceps and gloves. The collections were carried out in the following three seasons: April (autumn), July (winter) and October (spring) of 2016, at three locations in each municipality. The samples were sent to the National Reference Laboratory for Schistosomiasis - Malacology (LRNEM) of the Instituto Oswaldo Cruz, Fundação Oswaldo Cruz (IOC/Fiocruz, Rio de Janeiro), where they were analyzed. The identification of the molluscs species was done based on conchological characteristics compared to photos and descriptions from catalogues[16-19]. We also compared our material with material deposited in the Instituto Oswaldo Cruz. Samples of each species and from each collection locality were deposited in the Mollusca Collection of the Instituto Oswaldo Cruz (CMIOC 10103 - 10134, 11206).

To collect the nematode larvae, the molluscs were artificially digested by a modified Wallace and Rosen technique[20]. The recovered material was observed under a stereoscopic microscope following the LRNEM Identification Guide and based on the literature[4,21]. The *Angiostrongylus* sp. (Nematoda: Metastrongylidae) larvae were identified under a compound microscope according to Thiengo *et al.*[4]. Ten *Angiostrongylus* sp. larvae from each sample were collected and cryopreserved at -20 °C until DNA extraction for subsequent molecular analysis. Some rhabditiform larvae were observed and prepared for DNA extraction. In addition, *Strongyluris* sp. was morphologically identified, based on Thiengo[21].

To assess the viability of the parasites, *Angiostrongylus* sp. larvae recovered from *A. fulica* from the municipality of Aracaju were used to infect two specimens of *Rattus norvegicus* through an orogastric tube at a concentration of 50 L_3 larvae per rodent. Fifty days after infection, the animals were killed and necropsied to confirm the presence of adult *A. cantonensis*[22]. This procedure was carried out following the Ethics Commission on Animal Use of the Oswaldo Cruz Foundation (LW-47/14).

Figure 1 - Study area: Aracaju Metropolitan Region, including the capital of Sergipe State, the municipalities of Barra dos Coqueiros, Nossa Senhora do Socorro and Sao Cristovao. The black spots represent the collection localities

Table 1 - Geographical coordinates by location of collection of terrestrial molluscs

Municipalities	Description of locality	Geographical coordinates
Barra dos Coqueiros	Square / open area	10°49'30.1"S 36°56'46.7"W
	Outer clothing area	10°49'42.6"S 36°56'08.2"W
	School backyard / Rubble	10°49'12.2"S 36°56'58.9"W
Sao Cristovao	Street / close to houses	10°55'57.8"S 37°06'26.6"W
	Street / close to houses	10°55'27.7"S 37°06'43.2"W
	Street and house backyard	10°55'43.1"S 37°07'16.2"W
Aracaju	Street / close to houses	10°58'29.7"S 37°06'16.7"W
	Ground / adjacent to a commercial establishment	11°00'11.9"S 37°05'03.2"W
	Close to sewer-line	10°54'24.1"S 37°05'23.3"W
Nossa Senhora do Socorro	Garden and houses backyard	10°50'19.0"S 37°03'15.0"W
	Houses backyard	10°51'02.2"S 37°05'38.1"W
	Street and wasteland	10°53'23.0"S 37°08'50.9"W

Molecular diagnosis of nematode larvae

DNA was extracted from 10 *Angiostrongylus* sp. larvae obtained from each mollusc in which larvae had been found and resuspended in 30 µL of PCR Buffer solution (Thermo Fisher Scientific, Massachusetts, USA). The polymerase chain reaction (PCR) mixtures were prepared in a volume of 20 µL containing 8 µL of ultrapure water, 5 µL of 10% trehalose, 2.5 µL of 10x PCR Reaction Buffer, 2 µL of 2.5 mM dNTPs, 1.25 µL 50 mM $MgCl_2$, 0.5 µL each of 5 µM forward and reverse primer (Nem 3 from Prosser *et al.*[23]), and 0.25 µL of recombinant Taq DNA polymerase (Thermo Fisher Scientific). A total of 5 µL of the DNA sample was added to the mixture, producing a final volume of 25 µL for each reaction. For all the reactions, ultrapure water was used as a negative control template, and the positive control was performed with genomic DNA of *A. cantonensis* as template. The 700 bp fragment of the *Angiostrongylus* sp. larvae mitochondrial cytochrome *c* oxidase subunit I (COI) gene was amplified using the following PCR conditions: initial denaturation at 94 °C for 1 min, five cycles at 94 °C for 40 s, 45 °C for 40 s, and 72 °C for 1 min, followed by 30 cycles at 94 °C for 40 s, 51 °C for 40 s, and 72 °C for 1 min and a final extension at 72 °C for 1 min.

For rhabditiform larvae, individual DNA from some isolates was used to amplify the 480 bp ribosomal 18S region[24].

The PCR products were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK) following the manufacturer's protocol. Purified products after amplification were bidirectionally sequenced using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, California, USA) according to the manufacturer's instructions. Chromatograms of the sequences obtained were analyzed and edited using Geneious version R9 software (http://www.geneious.com), resulting in a consensus sequence (contig). Then, a comparative similarity search was performed on GenBank (www.ncbi.nlm.nih.gov/genbank/)[25] using BLAST (Basic Local Alignment Search Tool) to identify the closest match. The obtained sequences are available in GenBank under the access Nº MH511539 - MH511542, and MH547424.

RESULTS

In total, 703 terrestrial gastropods were analyzed (190 in Aracaju, 250 in Barra dos Coqueiros, 159 in Nossa Senhora do Socorro, and 104 in São Cristovão) and identified as the following 13 species: *Achatina fulica* (Bowdich, 1822), *Allopeas gracile* (Hutton, 1834), *Subulina octona* (Bruguière, 1789), *Leptinaria unilamellata* (d'Orbigny, 1835), *Bulimulus tenuissimus* (d'Orbigny, 1835), *Cyclodontina fasciata* (Potiez & Michaud, 1838), *Latipes erinaceus* (Colosi, 1921), *Sarasinula linguaeformis* (Semper, 1885), *Streptartemon cookeanus* (Baker, 1914), *Streptartemon quixadensis* (Baker, 1914), *Tamayoa banghaasi* (Thiele, 1927), *Helicina* sp. and *Omalonyx* sp.

Angiostrongylus sp. larvae were found in specimens of *A. fulica*, *B. tenuissimus* and *C. fasciata* and were later confirmed by molecular analysis as *A. cantonensis* (Table 2).

Achatina fulica was found in all four municipalities, with snails harboring infection by *A. cantonensis* in three of them (Aracaju, Sao Cristovao and Nossa Senhora do Socorro). *Bulimulus tenuissimus* occurred in all municipalities, while specimens of *C. fasciata* were found only in Aracaju and Barra dos Coqueiros. Both *B. tenuissimus* and *C. fasciata*

Table 2 - Prevalence of nematodes in terrestrial molluscs from the Metropolitan Region of Aracaju, Sergipe State, Northeast Brazil, from April to October 2016

Locality	Species	Nº specimens (n)	Positive molluscs (n)		
			A. cantonensis	*Caenorhabditis* sp.	*Strongyluris* sp.
Barra dos Coqueiros	*A. fulica*	110	-	48	22
	B. tenuissimus	23	1	3	-
	C. fasciata	112	2	48	23
	Helicina sp.	-	-	-	-
	S. cookeanus	1	-	-	-
	S. linguaeformis	2	-	-	1
	S. octona	2	-	-	-
	S. quixadensis	-	-	-	-
	T. banghaasi	-	-	-	-
Sao Cristovao	*A. fulica*	63	22	25	1
	B. tenuissimus	17	-	1	-
	S. linguaeformis	10	-	1	-
	S. octona	14	-	1	-
Aracaju	*A. fulica*	97	36	46	12
	A. gracille	-	-	-	-
	B. tenuissimus	19	-	2	-
	C. fasciata	43	-	4	1
	S. cookeanus	2	-	-	-
	S. linguaeformis	5	-	-	-
	S. octona	24	-	2	-
	T. banghaasi	-	-	-	-
Nossa Senhora do Socorro	*A. fulica*	85	5	34	-
	B. tenuissimus	13	-	3	-
	L. erinaceus	-	-	-	-
	L. unilamellata	47	-	5	-
	Omalonyx sp.	2	-	1	-
	S. linguaeformis	2	-	-	-
	S. octona	10	-	4	-
	T. banghaasi	-	-	-	-

were infected by *A. cantonensis* in the municipality of Barra dos Coqueiros, near the port area of Sergipe State.

Co-infections were observed between *A. cantonensis* and other nematodes, such as *Strongyluris* sp. and rhabditiform larvae (Table 3). The rhabditiform larvae were analyzed molecularly and were found to be 99% similar to a sequence from GenBank identified as *Caenorhabditis* sp.

Infections of *Caenorhabditis* sp. were observed in *A. fulica*, *B. tenuissimus*, *C. fasciata*, *S. octona*, *L. unilamellata*, *S. linguaeformis* and *Omalonyx* sp. Infections by *Strongyluris* sp. were observed in *A. fulica*, *C. fasciata* and *S. linguaeformis*.

Two rats that were experimentally infected with *Angiostrongylus* sp. isolates displayed symptoms 50 days after infection. Forty *A. cantonensis* adult worms were recovered in the first rat (37.5% males and 62.5% females), and 42 adult worms were recovered in the second (40.4% males and 59.5% females).

DISCUSSION

The snail *A. fulica* has already been reported as being parasitized with *A. cantonensis* by several authors[2,4,8,10,19,26]. In Brazil, the species is considered one of the main

Table 3 - Prevalence of co-infection between *Angiostrongylus cantonensis*, *Caenorhabditis* sp. and *Strongyluris* sp. in land molluscs from the Aracaju Metropolitan Region, Sergipe State, Northeast Brazil, from April to October 2016

Locality	Species	Specimens analyzed (n)	Positive specimens for *A. cantonensis*		Co-infection (n)		
			(n)	(%)	*A. cantonensis* + *Strongyluris* sp.	*A. cantonensis* + *Caenorhabditis* sp.	*A. cantonensis* + *Caenorhabditis* sp. + *Strongyluris* sp.
Barra dos Coqueiros	*B. tenuissimus*	23	1	4.3	-	1	-
	C. fasciata	109	2	1.8	-	-	-
São Cristovao	A. fulica	63	22	34.9	-	6	-
Aracaju	*A. fulica*	99	36	36.4	3	10	7
Nossa Senhora do Socorro	*A. fulica*	85	5	5.9	-	-	-

potential transmitters of human neural angiostrongyliasis, considering their widespread distribution, high population densities and proximity to humans[10,26].

Despite knowledge of the presence of the giant African snail in Sergipe State since 2006[27,28], only one previous investigative study has been carried out in this region[8]. Ten years after the first detection of *A. cantonensis* in Brazil, this study records its presence in Sergipe State for the first time; it is also the first report of *C. fasciata* and *B. tenuissimus* as natural intermediate hosts of the parasite. In addition, we observed co-infection with other nematodes, which is consistent with other studies[12,26,28].

Since 2007, when the first Brazilian case of eosinophilic meningitis caused by *A. cantonensis* was recorded, in Espirito Santo State[29], reports of the disease have increased. Currently, the parasite has been found in 12 states (Figure 2)[2,7,10,29-31].

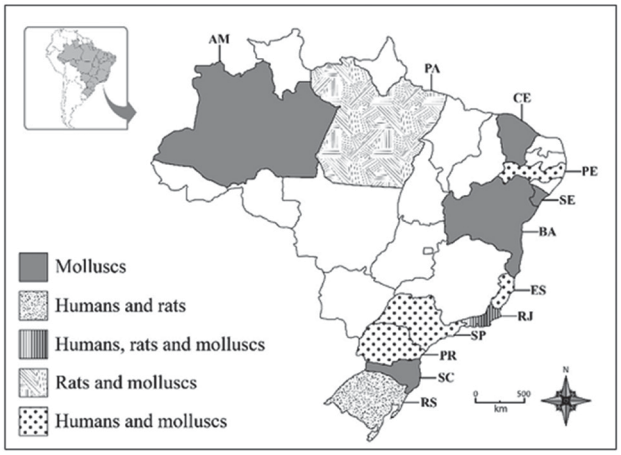

Figure 2 - Distribution in Brazil of *Angiostrongylus cantonensis* in natural vertebrate and invertebrate hosts, and in humans. Adapted from: Thiengo *et al.*[5], Carvalho *et al.*[8], Morassutti *et al.*[10], Espirito Santo *et al.*[30], Moreira *et al.*[31]. AM: Amazonas; PA: Para; CE: Ceara; PE: Pernambuco; SE: Sergipe; BA: Bahia; ES: Espirito Santo; RJ: Rio de Janeiro; SP: Sao Paulo; PR: Parana; SC: Santa Catarina; RS: Rio Grande do Sul

The first known occurrence of *A. fulica* naturally infected by *A. cantonensis* in the northeast region of Brazil was reported in 2008, with human cases of eosinophilic meningitis and naturally infected intermediate hosts in the municipalities of Escada and Olinda, Pernambuco State[4]. A study carried out in Brazilian port areas, including the port of Sergipe State, reported the presence of terrestrial and aquatic molluscs that, although previously negative for *A. cantonensis*, tested positive in the bordering Bahia State[8] and in Ceara State[10].

Several other terrestrial molluscs have also been found to be naturally infected with this nematode in Brazil[2,3,8,19] and in other countries[9,13,14]. Among these species are *S. linguaeformis* and *S. octona*[2,3,8,9], which, however, were not found to be infected in the present study. A possible reason for this is the lower abundance of these species in the sampled localities. The three species found infected by *A. cantonensis* were also those that were more abundant in the environment.

The present study identifies, for the first time, the following two species as natural hosts of the parasite: *C. fasciata*, which had not yet been described in the literature as an intermediate host for *A. cantonensis* or for other nematodes of human and veterinary interest, and *B. tenuissimus*, described as a host of metacercariae of the genus *Postharmostomum*[32]. This interaction between exotic and native species has become a new concern in the ecology of the disease and may result in increased parasitic spread as well as pose a threat to native species[9].

Studies indicate the need to implement control measures against molluscs associated with public health, particularly the exotic species *A. fulica*. However, passive geographic dispersal is the main form of spread of the giant African snail, which hinders its control[33].

The present study reinforces the need to investigate the current situation of *A. cantonensis* dispersal in the Northeastern region of Brazil, since there are reports of

its presence in four of its nine states, i.e., Bahia, Ceara, Pernambuco and Sergipe[7,8], as well as the evident dispersal of *A. fulica* in several municipalities in the Sergipe State.

ACKNOWLEDGMENTS

The authors are grateful to the Genomic Platform DNA Sequencing - RPT01A (Rede de Plataformas Tecnologicas FIOCRUZ) and for the technical support of the LRNEM/IOC/Fiocruz team, Eduardo Cinilha for help with map preparation, and the CAPES for the scholarship to Jucicleide R. Souza.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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