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The giant African snail *Achatina fulica* as natural intermediate host of *Angiostrongylus cantonensis* in Pernambuco, northeast Brazil

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ABSTRACT

The human cases of eosinophilic meningitis recently reported from Brazil have focused the attention of the public health agencies on the role the introduced snail *Achatina fulica* plays as hosts of the metastrongylid nematodes. Determining the potential of this snail to host and develop infective larval stages of metastrongylids in the wild and identify the species harbored by them is crucial for designing effective control measures. Here we assess if *A. fulica* may act as intermediate host of *A. cantonensis* at the peridomiciliary areas of a patient's house from state of Pernambuco (PE), who was diagnosed with eosinophilic meningitis and a history of ingesting raw molluscs. Larvae obtained from naturally infected *A. fulica* were orally administered to *Rattus norvegicus*. The worms were collected from the pulmonary artery and brain, and were morphologically characterized and compared to the Japan isolate of *A. cantonensis*. Adult worms and infective L_3 larvae (PE isolate) recovered from *A. fulica* specimens were also analyzed by polymerase chain reaction and restriction fragment length polymorphism of ITS2 region from rDNA and compared to *A. cantonensis* (ES isolate), *A. vasorum* (MG isolate) and *A. costaricensis* (RS isolate). The large size of the spicules (greater than those observed in other species of *Angiostrongylus*) and the pattern of the bursal rays agree with the original species description by Chen (1935). Furthermore, the morphology of the PE isolate was similar to that of Japan isolate. The PCR-RFLP profiles obtained were distinctive among species and no variation in patterns was detected among adult individuals from *A. cantonensis* isolates from PE and ES. The importance of *A. fulica* as an intermediate host of eosinophilic menigoencepahlitis in Brazil is emphasized.

1. Introduction

Achatina (*Lissachatina*) *fulica* Bowdich, 1822, the giant African snail, has been introduced throughout the tropics and subtropics and has been considered an important snail pest in these regions. Introduced to Brazil in the 1980s for commercial purposes ("escargot" farming) *A. fulica* is now widespread in at least 24 of 26 Brazilian states and the Federal District (Brasília), including the Amazonian region and natural reserves, where it is a pest in ornamental gardens, vegetable gardens, and small-scale agriculture (Thiengo et al., 2007).

Brazil is currently experiencing the explosive phase of the *A. fulica* invasion. This rapid spread throughout almost the entire country followed a pattern similar to the initial introduction in the state of Paraná, that is, distribution for commercial purposes followed by the release of the snails into the wild when people gave up the enterprise (Thiengo et al., 2007).

In addition to the impact on ecosystem health and potential competition with native terrestrial molluscs, *A. fulica* can also act as an intermediate host of nematodes of medical and veterinary importance (Thiengo et al., 2008; Carvalho et al., 2003). Among them are, *Angiostrongylus cantonensis* (Chen, 1935), a nematode that can cause eosinophillic meningoencephalitis and *Angiostrongylus costaricensis* Morera and Céspedes, 1971, which causes abdominal angiostrongylosis. The global dispersal of *A. cantonensis*, endemic to some Asian countries and Pacific Islands, is closely associated with the spread of *A. fulica* (Kliks and Palumbo, 1992; Lv et al., 2009b). In 2007, Brazil reported its first two cases of

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Table 1
Measurements (μm) of L_1 larvae of *Angiostrongylus cantonensis* (PE, Brazil isolate) recovered from *Rattus norvegicus* feces experimentally infected and L_3 larvae from *Achatina fulica* collected in Pernambuco state.

Characteristics	*n*	L_1		*n*	L_3	
		Mean ± SE	Range		Mean ± SE	Range
Body length	25	254.4 ± 23.2	304.3–206	34	460.4 ± 31.8	544.6–460.3
Width	25	10.9 ± 2.4	15.1–5.2	34	24.8 ± 3.8	31.9–18.5
Esophagus	24	108.1 ± 14.8	137.6–71.2	34	168.4 ± 11.2	189.0–129.6
Nerve ring	8	40.2 ± 12.3	66.4–27.2	30	90.9 ± 27.0	76.3–51.0
Excretory pore	25	70.8 ± 17.2	101.4–32.3	33	157.9 ± 28.8	200–79.6
Genital primordium	17	9.82 ± 4.1	18.1–5.2	14	40.8 ± 14.0	65.9–22.2
Anus–tail	15	26.1 ± 9.4	57.6–12.3	29	41.2 ± 10.4	89.3–27.7

this zoonosis in the municipality of Cariacica, state of Espírito Santo. Both instances were the result of ingestion of raw veronicellid slugs (Caldeira et al., 2007).

Populations of *A. fulica*, generally with many large individuals, occur in urban areas and are often a nuisance because of the high density of their populations, which often leads people to seek assistance in controlling them from local public health institutions. For the past seven years, the Laboratório de Malacologia do Instituto Oswaldo Cruz/Fiocruz (LRNM), National Reference for Medical Malacology, has examined samples of *A. fulica* sent by the Brazilian health and environmental agencies for larvae of *Angiostrongylus* spp.

We report the first finding of *A. fulica* naturally infected with *A. cantonensis* larvae in northeast Brazil and present the results of light microscopy morphology of adult and larvae nematodes obtained by experimental infection as well as the molecular diagnostic of both third-stage larvae (L_3) and adults. We also discuss the epidemiological aspects of eosinophilic meningitis in Brazil.

2. Material and methods

In March 2008 a 6-year-old female, with anamnesis of ingesting raw molluscs from her yard, in the municipality of Escada, state of Pernambuco (PE), was diagnosed with meningitis in the hospital Correto Picanço in Recife, PE, Brazil. Three months later, a 26-year-old female from the municipality of Olinda, PE, was diagnosed with the same condition. Similarly to the other patient, the anamnesis emphasized the ingestion of raw molluscs from her yard. Therefore, in June and September 2008 collection of terrestrial and freshwater molluscs in peridomiciliary areas of the two patients' houses was undertaken by us and health agents from the Secretaria Estadual de Saúde de Pernambuco. In all 1544 molluscs were collected randomly. The cephalopodal mass of each of the molluscs was individually minced and digested in a 0.7% HCl solution for 6 h. The digested samples were placed in a Baermann apparatus and allowed to sediment overnight. Infective *A. cantonensis* larvae obtained from *A. fulica* collected in Olinda were used to infect 30 three-month-old *Rattus norvegicus*, Wistar strain, at a concentration of 100 L_3/animal (Youssif and Ibrahim, 1978).

Nematodes collected from the brain and the pulmonary artery 30 and 120 days after infection, respectively, were washed in physiologic solution and fixed in hot AFA solution (2% glacial acetic acid, 3% formaldehyde, and 95% ethanol). Specimens (10 males and 10 females) from each of those sites were cleared in lactophenol, mounted on slides in lactophenol solution and examined under a light microscope. Drawings for the morphometric analyses were made using a light microscope with the aid of a camera lucida. Taxonomic identification of nematodes was based on morphological and morphometric parameters following Anderson (1978) and Ash (1970). Specimens of *A. cantonensis* isolate from Akita, Japan were used for comparison. Measurements are given in micrometers (unless otherwise stated).

Ten male and 18 female adult helminthes, and a pool of L_3 were fixed in >90% ethanol and washed three times in distilled water prior to use in molecular analysis. Genomic DNA (gDNA) extractions were undertaken using the Wizard Genomic Purification Kit (PROMEGA, Madison, USA) according to manufacturer's instructions. Comparative material was obtained from other nematode samples provided by the Laboratório de Helmintologia e Malacologia Médica do Centro de Pesquisas René Rachou, Fiocruz including: *A. cantonensis* (from Cariacica, state of Espírito Santo), *A. costaricensis* (from state of Rio Grande do Sul) and *A. vasorum* (from the

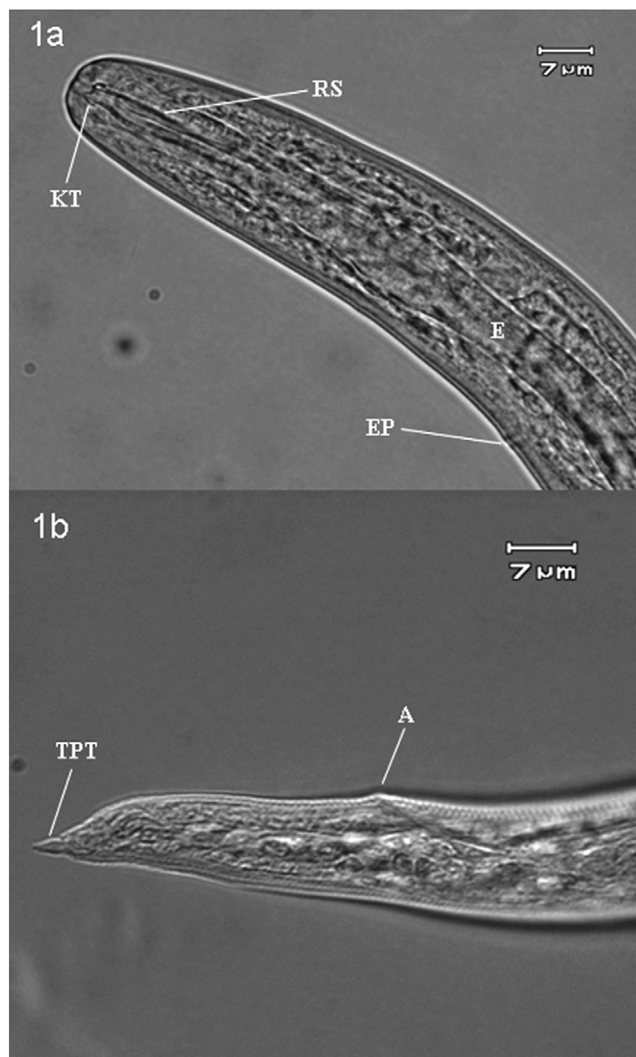
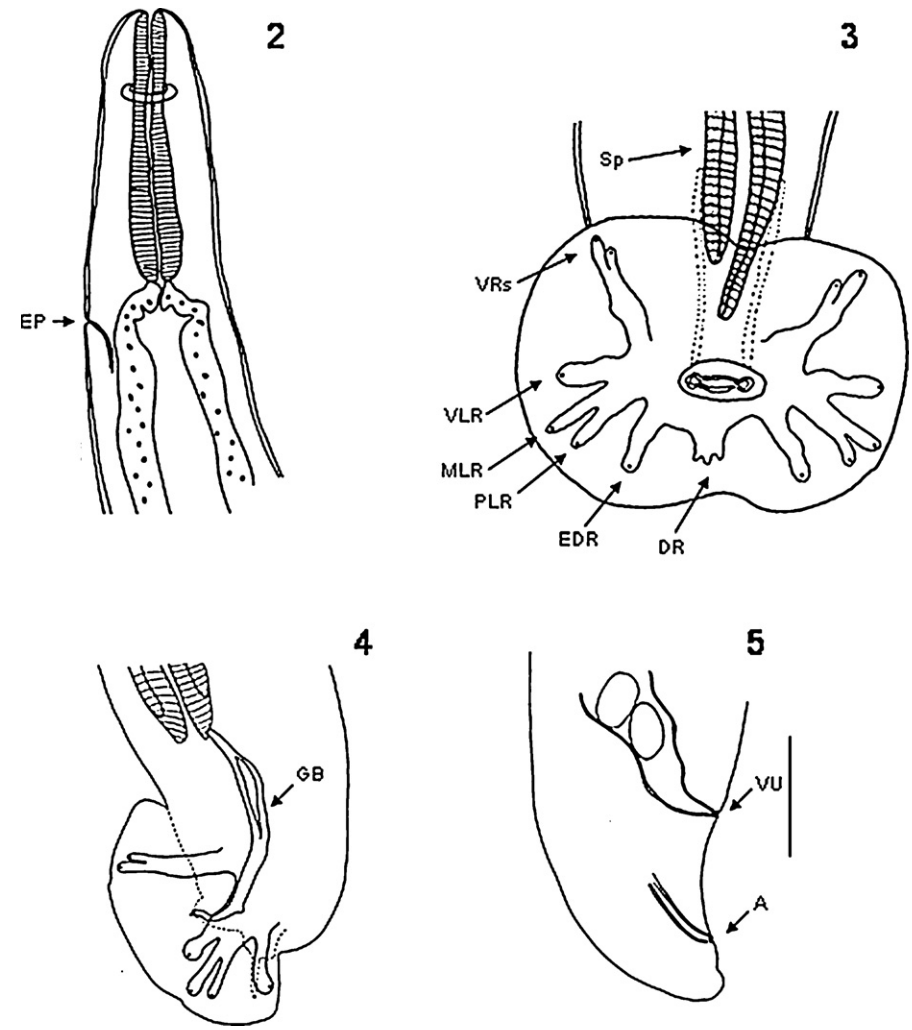


Fig. 1. Light microscopy of third-stage larvae (L_3) of *Angiostrongylus cantonensis*. (a) Anterior end showing knob-like tips (KT), rod-like structure (RS), esophagus (E), excretory pore (EP); (b) posterior end showing tail pointed tip (TPT) and anus (A).



Figs. 2–5. *Angiostrongylus cantonensis* adult worm. Scale bar: 100 μm. (2) Anterior extremity, right lateral view, female, showing excretory pore (EP). (3) Male, caudal bursa, ventral view, showing spicules (Sp), ventral rays (VRs), ventrolateral ray (VL), mediolateral ray (MLR), posterolateral ray (PLR), externodorsal ray (EDR) and dorsal ray (DR). (4) Lateral view, caudal bursa, showing gubernaculum (GB). (5) Female, posterior extremity, lateral view, showing vulva (VU) and anus (A).

municipality of Caratinga, state of Minas Gerais). Polymerase chain reaction associated with restriction fragment length polymorphism (PCR-RFLP) analysis was performed on all adult and L_3 directed to the internal transcribed spacer 2 (ITS2) region and using the restriction enzyme *Cla*I (Caldeira et al., 2003).

R. norvegicus and *Biomphalaria glabrata* (Say, 1818) (Belo Horizonte strain) have been used for the maintenance of *A. cantonensis* life cycle. Snails were individually put into culture wells with filtrated–dechlorinated water, and a solution containing first stage larvae (L_1) was added. The parasitic load varied from 368 to 3400 L_1/mollusc.

Representative adult specimens of *A. cantonensis* were deposited in the Helminthological Collection of the Oswaldo Cruz Institute, numbers 37.225 (slide) and 35.661a-b (wet material).

3. Results

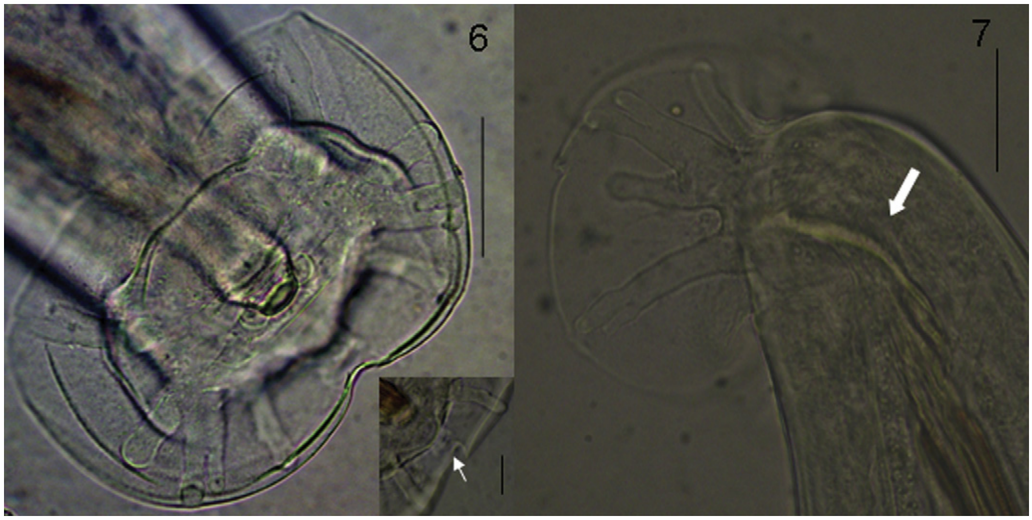
In the municipality of Escada 1511 molluscs were collected, 272 in June and 1239 in September. The majority (1310) were freshwater snails: 1215 *Biomphalaria straminea* (Dunker, 1848) and 95 *Pomacea lineata* (Spix and Wagner, 1827). As for the terrestrial molluscs, 15 *A. fulica*, 158 *Leptinaria unilamellata* (d'Orbigny, 1835), 20 *Sarasinula marginata* (Semper, 1885) and 8 *Subulina octona* (Bruguière, 1792) were collected. In addition to other larvae, a few *A. cantonensis* larvae were recovered from 5 specimens of *P. lineata* and larvae of *Aelurostrongylus abstrusus* (Railliet, 1898) were identified from 7 *A. fulica*.

In Olinda only terrestrial molluscs were collected, including: *A. fulica* (4 specimens in June and 19 in September), *L. unilamellata* (6 specimens in September) and *S. marginata* (1 in June and 3 in September). Out of the 33 snails collected in total, 14 *A. fulica* (42%) were found heavily infected with *A. cantonensis*. All *A. fulica* infected were collected in September, corresponding to 73.7% of the sample.

In order to maintain the life cycle of *A. cantonensis* in laboratory for further studies rats and snails have been infected with L_3 and L_1, respectively. Lower mortality rate was observed in infected *B. glabrata* with <2000 L_1/snail.

The measurements of first- and third-stage larvae are given in Table 1. First stage larvae have a slender tail, while the third-stage larvae are characterized by a sharp pointed tail, with a marked indentation on dorsal surface and the cuticle with only faint transverse striations. The genital primordium of the larvae was situated along the posterior third of the intestine making it impossible to differentiate sex (Fig. 1).

Adult *A. cantonensis* (Figs. 2–7) are characterized by a filiform body in both sexes, tapering at the anterior end. Females are larger and more robust than males. The cephalic vesicle is absent, oral aperture simple, circular, surrounded by 6 papillae (2 dorsal, 2 lateral and 2 ventral) and 2 lateral amphids. The esophagus is claviform and the excretory pore posterior to esophagus (Fig. 2). Nerve



Figs. 6 and 7. Light microscopy of *Angiostrongylus cantonensis*. Scale bar: 25 μm. (6) Male, ventral view of caudal bursa. Detail showing dorsal ray thick, with 3 short branches (→). (7) Male, lateral view of caudal bursa, showing gubernaculum (⇨).

Table 2
Measurements (mm) of larvae L_5 and adults of *Angiostrongylus cantonensis* from Brazil (*) and Japan (**).

	Larvae L_5				Adult			
	Pulmonary artery*		Brain*		Pulmonary artery*		Pulmonary artery**	
	♂ (*n* = 10)	♀ (*n* = 10)	♂ (*n* = 10)	♀ (*n* = 10)	♂ (*n* = 9)	♀ (*n* = 10)	♂ (*n* = 10)	♀ (*n* = 10)
Body length	12.91 ± 1.65	14.54 ± 1.23	11.88 ± 0.71	14.51 ± 1.23	22.82 ± 1.76	32.84 ± 2.16	18.35 ± 1.83	26.42 ± 5.69
Width	0.24 ± 0.05	0.28 ± 0.02	0.23 ± 0.02	0.26 ± 0.03	0.35 ± 0.05	0.48 ± 0.03	0.30 ± 0.03	0.36 ± 0.05
Width at the base of esophagus	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.0.1	0.04 ± 0.01
Esophagus	0.28 ± 0.02	0.34 ± 0.08	0.30 ± 0.03	0.30 ± 0.03	0.31 ± 0.01	0.34 ± 0.03	0.30 ± 0.02	0.31 ± 0.06
Nerve ring	0.08 ± 0.01	0.12 ± 0.04	0.08 ± 0.02	0.10 ± 0.01	0.09 ± 0.01	0.10 ± 0.02	0.08 ± 0.02	0.09 ± 0.02
Excretory pore	0.33 ± 0.03	0.29 ± 0	0.28 ± 0.05	0.29 ± 0.02	0.43 ± 0.03	0.40 ± 0.04	0.40 ± 0.05	0.42 ± 0.01
Spicules	1.26 ± 0.05	–	1.23 ± 0.05	–	1.29 ± 0.06	–	1.29 ± 0.07	–
Gubernaculum	0.10 × 0.02	–	0.11 × 0.02	–	0.08 × 0.02		0.11 × 0.02	–
Vulva–tail	–	0.16 ± 0.02	–	0.16 ± 0.02	–	0.19 ± 0.02	–	0.25 ± 0.07
Anus–tail	–	0.05 ± 0.01	–	0.05 ± 0.01	–	0.06 ± 0.01	–	0.07 ± 0.02
Eggs	–	–	–	–	–	0.06 × 0.04	–	0.06 × 0.03

* *A. cantonensis* specimens from Pernambuco, northeast Brazil.
** *A. cantonensis* specimens from Akita, Japan.

ring anterior to the middle of esophagus, male caudal bursa small and slightly asymmetric, ventroventral rays smaller than ventrolateral ones with a common origin, bifurcated at distal third and do not reach the bursal margins. The lateral rays arising in a common trunk with ventrolateral ray cleft and smaller than the other lateral rays (Figs. 3 and 6). The externodorsal rays are separated at the base. The right mediolateral is thinner than the left one with the right mediolateral and posterolateral bifurcating at the middle of the trunk and the left mediolateral and laterolateral at the distal third. The dorsal ray is short and thick, bifurcating in 3 branches. The gubernaculum is conspicuous and curved (Figs. 4 and 7). Uterine tubules spiral around blood-filled intestine easily seen through the transparent cuticle. Tail long and rounded without cuticle expansion and papillae, slightly ventrally curved (Fig. 5). Table 2 shows the comparison of *A. cantonensis* adults and L_5 measurements with those of the Japan isolates.

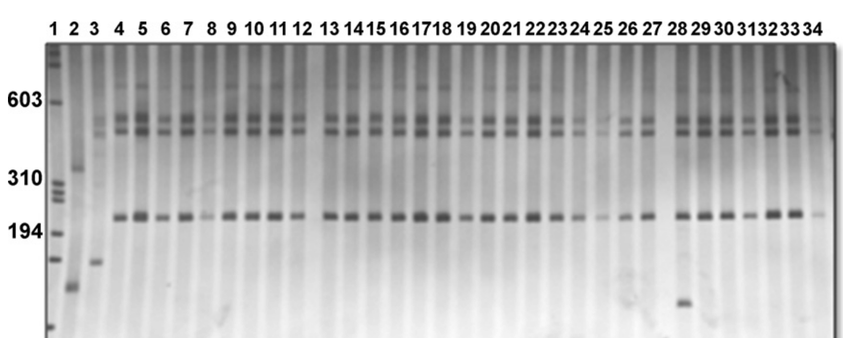


Fig. 8. Silver stained polyacrylamide gel (6%) shows PCR-RFLP profiles of the ITS2 of rDNA with the enzyme *Cla*I. Lane 1: molecular marker phi X 174. Lane 2: L_1 pool of *Angiostrongylus vasorum* (Caratinga/MG, Brazil); lane 3: adult worm of *A. costaricensis* (Rio Grande do Sul, Brazil); Lane 4: L_3 pool of *A. cantonensis* (Cariacica/ES, Brazil); lanes 5–14: adult worm males obtained from *Rattus norvegicus*; lane 15: L_3 pool obtained from *Achatina fulica* at Olinda/PE, Brazil; lanes 16–22: adult worm females obtained from the of lung *Rattus novergicus*. Lanes 23–33: adult worm females obtained from brain *Rattus novergicus* and lane 34: L_3 pool of *A. cantonensis* (Cariacica/ES, Brazil).

PCR-RFLP analysis of the ITS2 rDNA confirmed the identity of all adult worms and L_5 obtained from the brain and lungs of *R. norvegicus* and infective larvae recovered from *A. fulica* as *A. cantonensis* (Fig. 8, lanes 5–33). The band from the adult worm in lane 28 exhibited polymorphism when compared with the other specimens due to an additional band.

4. Discussion

The comparison of *A. cantonensis* adults and L_5 measurements with those of the Japan isolates showed a high degree of morphometric similarity (Table 2), confirming morphological and molecular analyses. Similarly to the L_3 larvae well compared to those described by Ash (1970). The only variation observed in adult worms was an additional band (Fig. 8, lane 28). As there are many copies of the rDNA cistrons in the genome, we believe that the variation in adult worm profile could be due to heterogeneity, i.e., the presence of two or more sequence types of the ITS in a single organism. This variation has been also described in other nematode parasites (Stevenson et al., 1995; Gasser et al., 1996) such as *Haemonchus contortus* (Rudolphi, 1803) Cobb, 1898 and *Ancylostoma caninum* (Ercolani, 1859).

A. cantonensis and other congeneric species such as *A. costaricensis* have low specificity for their intermediate hosts and many different freshwater and terrestrial mollusc species had been found naturally infected (Wallace and Rosen, 1969; Graeff-Teixeira et al., 1993; Caldeira et al., 2007; Lv et al., 2009a,b). Among them, *A. fulica* plays an important role in the worldwide transmission of *A. cantonensis* (Kliks and Palumbo, 1992; Thiengo, 2007; Lv et al., 2009b). The finding of infected specimens of *A. fulica* in the neighborhood of the patients in both the southeast (Caldeira et al., 2007) and northeast regions of Brazil reinforces the emerging public health threat presented by this species and closely related congenerics in Neotropical regions. In addition, *A. fulica* was found infected with other nematodes of veterinary importance such as *A. abstrusus* in seven Brazilian States from the southeast, northeast and midwest regions (Thiengo et al., 2008).

Similar to *A. fulica*, ampullariid species, including some from the Neotropics, mainly *Pomacea canaliculata* (Lamarck, 1822) introduced in Hawaii and Asia for commercial proposes, are implicated in numerous cases and outbreaks of eosinophilic meningitis in those regions, especially China (Tsai et al., 2001; Lv et al., 2009a,b). In the present study, although the parasitic index of *P. lineata* from municipality of Escada was low (5.26%) comparing with that found in the *A. fulica* from Olinda (42%), the patient probably got infected from the consumption of undercooked *P. lineata*, according to its clinical history.

It is noteworthy to mention that although there have been 4 cases of eosinophilic meningitis resulting from the ingestion of undercooked, or raw molluscs in Brazil, it is not common practice for Brazilians to consume molluscs prepared in this manner. This is in contrast to the common practice of eating raw or undercooked snails in many parts of Asia (Lv et al., 2009a,b). This points to the need for a different focus for preventing infections in Brazil, as most infections will probably result from inadvertent consumption of the larvae via unwashed vegetables and other produce. This is also the known mechanism of transmission in several cases of the zoonosis in Hawaii (Hollingsworth et al., 2007).

In spite of its global spread and the potential public health implications, the morphology of *A. cantonensis* has never been reviewed. This morphological study of this species isolated from Pernambuco confirms a new geographical occurrence and adds taxonomic characters for the accurate taxonomic identification.

Both nematodes from the brain and pulmonary artery present similar morphometry. However, the adults from pulmonary artery at 120 days post infection are longer and wider than L_5 and the excretory pore is situated posterior to the junction of esophagus whereas it is at the level of the esophagus base in L_5. The morphology of the caudal bursa and the size of the spicules were not variable among worms of differing ages, which permits the use of these parameters for species identification. The large size of the spicules [larger than those observed in any other *Angiostrongylus* spp (Souza et al., 2009)] and the patterns of the bursal rays agree with the species described by Chen (1935). Shorter morphological variability between Japan and Pernambuco population may be attributed to intraspecific variation.

A. cantonensis isolated from Brazil and Japan presented gubernaculums and Alicata (1968) reported the same structure in specimens from Hawaii. Although the gubernaculum had been represented in the original figure given by Chen (1935) there is no mention of this structure in his paper.

In Brazil there have been few studies of the impact of the introduction of *A. fulica* on the natural environment as well as its role in public health. Control measures, health education and surveillance in vulnerable areas for *A. cantonensis* introduction such as shipping ports are needed to prevent the further spread and outbreaks of eosinophilic meningitis.

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