Angiostrongylus cantonensis (Nematode: Metastrongyloidea) in molluscs from harbour areas in Brazil

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Angiostrongylus cantonensis *is the most common aetiological agent of human eosinophilic meningoencephalitis. Following a report indicating the presence of this parasite in Brazil in 2007, the present study was undertaken to investigate the presence of* A. cantonensis *in the surrounding Brazilian port areas. In total, 30 ports were investigated and the following molluscs were identified:* Achatina fulica, Belocaulus *sp.,* Bradybaena similaris *sp.,* Cyclodontina *sp.,* Helix *sp.,* Leptinaria *sp.,* Melampus *sp.,* Melanoides tuberculata, Phyllocaulis *sp.,* Pomacea *sp.,* Pseudoxychona *sp.,* Rhinus *sp.,* Sarasinula marginata, Streptaxis *sp.,* Subulina octona, Succinea *sp.,* Tomigerus *sp.,* Wayampia *sp. and specimens belonging to Limacidae and Orthalicinae. Digestion and sedimentation processes were performed and the sediments were examined. DNA was extracted from the obtained larvae and the internal transcribed spacer region 2 was analysed by polymerase chain reaction-restriction fragment length polymorphism after digestion with the endonuclease* Cla*I. Of the 30 ports investigated in this study, 11 contained molluscs infected with* A. cantonensis *larvae. The set of infected species consisted of* S. octona, S. marginata, A. fulica *and* B. similaris. *A total of 36.6% of the investigated ports were positive for* A. cantonensis, *indicating a wide distribution of this worm. It remains uncertain when and how* A. cantonensis *was introduced into South America.*

Key words: *Angiostrongylus cantonensis* - molluscs - eosinophilic meningoencephalitis - ports - Brazil

Two species of Metastrongylidae from the *Angiostrongylus* genus can affect humans: *Angiostrongylus costaricensis* (Morera & Céspedes 1971), which is the aetiological agent of abdominal angiostrongyliasis in the Americas (Morera & Céspedes 1971), and *Angiostrongylus cantonensis* (Chen 1935), which is the aetiological agent of eosinophilic meningoencephalitis (Alicata 1962) and occurs primarily in southeastern Asia and throughout the Pacific Basin (Alicata 1991).

A. cantonensis has been reported in Asia, Africa, Europe, Oceania, southeast Asia, North America and Central America (Wang et al. 2008). The first report of the occurrence of snails infected with *A. cantonensis* in South America was by Caldeira et al. (2007) and Pincay et al. (2009) later described the first human case in South America.

The cycle of *A. cantonensis* occurs mainly in molluscs and rodents. Molluscs become infected through the oral route or through percutaneous penetration by L_1-stage larvae eliminated in the host's faeces. The parasite has no specific intermediate host and infects numerous species of terrestrial and aquatic molluscs (Malek & Cheng 1974, Caldeira et al. 2007). Rodents can potentially acquire the parasite by eating infected molluscs and/or food contaminated with L_3-stage larvae. After migration through the central nervous system, where two moults (L_3 - L_4 - L_5) take place, the adult worms migrate to their final habitat, the terminal branches of the pulmonary arteries. Humans are considered an accidental host because the migration of juvenile forms of the parasite is interrupted in the brain (meninges, medullar parenchyma and cerebellum) or rarely in the lungs and the worms do not reach the adult form, instead causing local inflammatory reactions.

As a result of the discovery of *A. cantonensis* in Brazil (Caldeira et al. 2007) and the hypothesis that the introduction of this parasite occurred through the migration of infected rodents on ships coming mainly from Asia and Africa, a project was implemented to investigate the presence of *A. cantonensis* in the areas surrounding the main ports of Brazil (Fig. 1). This project received financial support from the Ministry of Health of Brazil, which was coordinated by one of the authors (OSC).

The recorded presence of *A. cantonensis* larvae in Brazil is important because the parasite can cause clinical manifestations that can be confused with infectious meningitis of different aetiologies due to the transit of larvae through the meninges.

MATERIALS AND METHODS

Thirty Brazilian ports were investigated in 28 municipalities in 16 states (Table). All of the collection points were georeferenced. Molluscs were collected, packed and sent to the Laboratory of Medical Helminthology and Malacology at the René Rachou Institute-Oswaldo Cruz Foundation (Fiocruz) for examination. To verify the presence of *A. cantonensis* larvae, the molluscs were digested and the products were sedimented according to the protocol proposed by Wallace and Rosen (1969) and the Baermann-Moraes method (Moraes 1948). Molluscs

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of the same species and from the same location were pooled and then digested due to the large number of samples that were collected. After sedimentation, the material was analysed under a stereomicroscope for nematode larvae. The larvae from each collection point (pool) were submitted to molecular studies. DNA extraction was performed using the DNA Wizard Genomic Purification kit (Promega, Madison, USA) according to the manufacturer's instructions. The DNA was amplified to detect polymorphisms in internal transcribed spacer region 2 of the rRNA gene after restriction with *Cla*I endonuclease (polymerase chain reaction-restriction fragment length polymorphism) (Caldeira et al. 2003). *A. cantonensis* (Department of Parasitology, Medical School, Akita University, Japan) and *A. costaricensis* [Laboratory of Pathology, Instituto Oswaldo Cruz-Fiocruz, Rio de Janeiro (RJ), Brazil] were used as controls. Data management, visualisation and mapping was carried out in ArGIS version 9.3 (ESRI, Redlands, CA, USA).

RESULTS

A total of 6,095 mollusc specimens were collected: *Achatina fulica* (Bowdich, 1822) (Achatinidae), *Belocaulus* sp. Hoffmann, 1925 (Veronicellidae), *Bradybaena similaris* (Férussac, 1821) (Bradybaenidae), *Bulimulus* sp. Leach, 1814 (Bulimulidae), *Cyclodontina* sp. Beck, 1837 (Bulimulidae), *Helix* sp. Linné, 1758 (Helicidae), *Leptinaria* sp. Beck, 1837 (Subulinidae), *Melampus coffeus* (Linnaeus, 1758) (Ellobiidae), *Melanoides tuberculatus* (Muller, 1774) (Thiaridae), *Phyllocaulis* sp. Colosi, 1922 (Veronicellidae), *Pomacea* sp. Perry, 1811 (Ampulariidae), *Pseudoxychona* sp. Pilsbry, 1931 (Bulimulidae), *Rhinus* sp. Martens in Albers, 1860 (Bulimulidae), *Sarasinula marginata* (Semper, 1885) (Veronicellidae), *Streptaxis* sp. Gray, 1837 (Streptaxidae), *Subulina octona* (Bruguière, 1792) (Subulinidae), *Succinea* sp. Draparnaud, 1801 (Succineidae), *Tomigerus* sp. Spix, 1827 (Bulimulidae), *Wayampia* sp. Tillier, 1980 (Systrophiidae) and specimens from the Orthalicinae (Bulimulidae) subfamily and the Limacidae family.

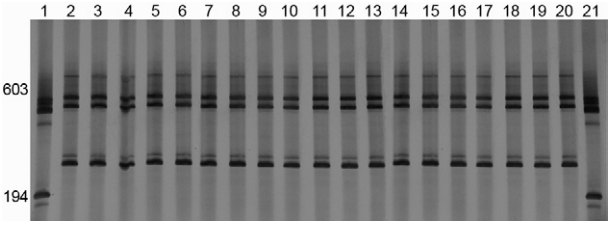

Fig. 1: silver-stained polyacrylamide gel (6%) showing polymerase chain reaction-restriction fragment length polymorphism profiles for the internal transcribed spacer region 2 of rDNA digested with the *Cla*I enzyme. Lane 1: adult *Angiostrongylus costaricensis* worm [Rio de Janeiro, state of Rio de Janeiro (RJ)]; adult *Angiostrongylus cantonensis* worm (Akita/Japan); 3-7: L3 pool obtained from *Subulina octona* [Belém, state of Pará (PA), Recife, state of Pernambuco, Ilhéus, state of Bahia (BA), São Sebastião, state of São Paulo (SP), Santos (SP)]; 8-12: L3 pool obtained from *Sarasinula marginata* [Belém, Salvador (BA), Vila Velha, state of Espírito Santo, Niterói (RJ), Santos]; 13-16: L3 pool obtained from *Achatina fulica* [Ilhéus, Angra dos Reis (RJ), Paranaguá, state of Paraná, Navegantes, state of Santa Catarina (SC)]; 17-19: L3 pool obtained from *Bradybaena similaris* (Ilhéus, Navegantes, Santos); 20: adult *A. cantonensis* worm (Akita/Japan); 21: *A. costaricensis* worm (Rio de Janeiro). Molecular size markers are shown on the left.

At 11 (36.6%) of the 30 ports investigated, molluscs infected with larvae of *A. cantonensis* were found (Table). Fig. 1 shows the molecular profiles of these larvae (Lanes 3-19) compared with the profiles of *A. cantonensis* from Japan (Lanes 2, 20) and of *A. costaricensis* samples from Rio de Janeiro, RJ (Lanes 1, 21). *A. cantonensis* larvae were present in the following mollusc species: (i) *S. octona* (Belém, state of Pará, Recife, state of Pernambuco (PE), Ilhéus, state of Bahia (BA), São Sebastião and Santos, state of São Paulo (SP), (ii) *S. marginata* [Belém, Salvador (BA), Vila Velha, state of Espírito Santo (ES), Niterói (RJ), Santos], (iii) *A. fulica* [Ilhéus, Angra dos Reis (RJ), Paranaguá, state of Paraná, Navegantes, state of Santa Catariana (SC)] and (iv) *B. similaris* (Ilhéus, Navegantes, Santos). Fig. 2 shows the map with the locations of the 30 ports investigated.

DISCUSSION

The presence of *A. cantonensis* in Brazil was first suspected by Moll et al. (2006) at the V Congress of Infectology, at which a clinical case of eosinophilic meningoencephalitis in RJ that resulted in death 10 days after the ingestion of *A. fulica* was reported.

The results of the study that first identified *A. cantonensis* in Brazil (Caldeira et al. 2007) demonstrated the need for further studies. The parasite was found in two states in the Southeast Region, ES and SP. In ES, the parasite was found in the municipality of Cariacica, approximately 20 km towards the interior of the country on the margins of one of the main routes linking the coast to the centre of Brazil. The infected molluscs were *B. similaris* (100% infection) *S. octona* (76%), *S. marginata* (84%) and *A. fulica* (66%). Concurrently, *A. cantonensis* larvae were isolated and identified from the faeces of *Rattus norvegicus* collected in the peridomicile of patients from Cariacica, demonstrating the importance of this rodent in the lifecycle of the parasite. Experimental infection of *R. norvegicus* revealed that after 25 days of infection, several young adult worms were present in the meninges and after 57 days, male and female adult worms were found in the pulmonary arteries. In the city of São Vicente (SP), *A. cantonensis* larvae were found in *A. fulica* (Caldeira et al. 2007).

In Ecuador, Pincay et al. (2009) reported 26 cases of eosinophilic meningoencephalitis and several of these patients were reported to have consumed raw molluscs prepared as a *ceviche*. One of the patients died and the autopsy results revealed the presence of *A. cantonensis* larvae in the subarachnoid space. This is the first report of a human infection proven by parasitology in South America. Molluscs and two rodents (*R. norvegicus* and *Rattus rattus*) harbouring adult *A. cantonensis* worms were collected. After the first report of *A. cantonensis* in Brazil by Caldeira et al. (2007), several other authors described the presence of *A. cantonensis* in this country. In Olinda

TABLE

Occurrence of *Angiostrongylus cantonensis* in molluscs from harbour areas in Brazil

Regions States Municipalities/ports	Collection date (month/year)	Geographical coordinates	Collected molluscs		
			Identification	n	PCR-RFLP assay (*A. cantonensis*)
North					
Amazonas					
Manaus/Manaus	Oct 2008	3°08'08''S 60°0'41''W	*Subulina octona*	206	-
			Sarasinula marginata	NI	-
Pará					
Barcarena/Porto de Vila do Conde	Feb 2010	1°33'41''S 48°45'30''W	*S. marginata*	20	-
			S. octona	179	-
			Bulimulus sp.	4	-
Belém/Belém	Feb 2010	1°26'30''S 48°29'41''W	*S. octona*	200	+
			S. marginata	21	+
			Bulimulus sp.	4	-
Northeast					
Maranhão					
São Luiz/Itaqui	Apr 2009	2°34'33''S 44°22'05''W	*S. octona*	585	-
			Bradybaena similaris	34	-
			Succinea sp.	66	-
			Pomacea sp.	03	-
			S. marginata	28	-
			Tomigerus sp.	4	-
Ceará					
Fortaleza/Mucuripe	Mar 2009	3°43'11''S 38°28'30''W	*S. octona*	NI	-
			Achatina fulica	64	-
			B. similaris	29	-
São Gonçalo do Amarante/Pecém	Mar 2009	3°32'53''S 38°48'53''W	*A. fulica*	120	-
Rio Grande do Norte					
Natal/Natal	Apr 2009	5°46'06''S 35°12'13''W	*B. similaris*	164	-
Paraíba					
Cabedelo/Cabedelo	Dec 2009	6°58'12''S 34°50'17''W	*S. octona*	63	-
			A. fulica	18	-
			Bulimulus sp.	24	-
Pernambuco					
Recife/Suape	Dec 2009	8°23'47''S 34°58'54''W	*A. fulica*	18	-
Recife/Recife	Dec 2009	8°35'87''S 35°00'65''W	Orthalicinae	47	-
		8°03'12''S 34°52'16''W	*Melampus coffeus*	31	-
			S. octona	26	+

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Regions States Municipalities/ports	Collection date (month/year)	Geographical coordinates	Collected molluscs		
			Identification	n	PCR-RFLP assay (*A. cantonensis*)
Alagoas					
Maceió/Jaraguá	Feb 2010	9°40'39''S 35°43'19''W	*S. marginata*	5	-
			Bulimulus sp.	86	-
			Rhinus sp.	7	-
			S. octona	117	-
Sergipe					
Barra dos Coqueiros/Terminal Marítimo Inácio Barbosa	Dec 2009	10°49'33''S 36°56'21''W	*A. fulica*	87	-
			S. octona	79	-
			Cyclodontina sp.	54	-
			Pseudoxychona sp.	259	-
			Bulimulus sp.	20	-
			Melanoides tuberculatus	1	-
Bahia					
Salvador/Salvador	Dec 2009	12°57'51''S 38°30'20''W	*S. marginata*	66	+
			B. similaris	5	-
			S. octona	13	-
			Bulimulus sp.	37	-
			A. fulica	16	-
Ilhéus/ Ilhéus	Nov 2009	14°47'02''S 39°01'58''W	*A. fulica*	127	+
			S. octona	373	+
			Wayampia sp.	28	-
			B. similaris	16	+
			Bulimulus sp.	03	-
Southeast					
Espírito Santo					
Vitória/Tubarão	Nov 2010	20°17'25''S 40°14'26''W	*S. octona*	93	-
			B. similaris	56	-
			S. marginata	18	-
			Bulimulus sp.	187	-
			Succinea sp.	35	-
			Leptinaria sp.	24	-
Vitória/Vitória	Nov 2010	20°19'26''S 40°21'02''W	*S. marginata*	02	-
			Bulimulus sp.	30	-
Vila Velha/Porto de Capuaba	Nov 2010	20°19'31''S 40°20'13''W	*B. similaris*	180	-
			S. marginata	19	+
			Bulimulus sp.	16	-
			Succinea sp.	9	-
Anchieta/Porto de Ubú	Nov 2010	20°46'27''S 40°35'04''W	*B. similaris*	05	-
			S. marginata	04	-
			Bulimulus sp.	15	-

↳

Regions States Municipalities/ports	Collection date (month/year)	Geographical coordinates	Collected molluscs		
			Identification	n	PCR-RFLP assay (*A. cantonensis*)
Rio de Janeiro					
Rio de Janeiro/Rio de Janeiro	Oct 2009	22°53'15''S 43°13'08''W	*A. fulica*	126	-
Niterói/Estaleiro ENAVI e RENAVE	Oct 2009	22°51'42''S 43°06'25''W	*S. marginata*	22	+
			S. octona	97	-
			B. similaris	9	-
			Wayampia sp.	14	-
			Bulimulus sp.	11	
Angra dos Reis/Bras Fels	Oct 2009	22°59'59''S 44°14'27''W	*B. similaris*	25	-
			S. octona	07	-
			A. fulica	55	+
Macaé/Imbetiba	Oct 2009	22°23'11''S 41°46'16''W	*A. fulica*	11	-
			S. octona	32	-
			Wayampia sp.	3	-
			S. marginata	2	-
			B. similaris	1	-
São Paulo					
São Sebastião/São Sebastião	Oct 2009	23°48'37''S 45°23'57''W	*A. fulica*	37	-
			S. octona	120	+
			B. similaris	61	-
			Bulimulus sp.	01	-
			Streptaxis sp.	03	-
			Rhinus sp.	08	-
Santos/Santos	Mar 2011	23°93'17''S 46°32'12''W	*B. similaris*	184	+
			A. fulica	73	-
			S. octona	88	+
			S. marginata	14	+
South					
Paraná					
Paranaguá/Paranaguá	Nov 2008	25°30'22''S 48°31'39''W	*B. similaris*	NI	-
			A. fulica	NI	+
			S. marginata	NI	-
			S. octona	NI	-
Santa Catarina					
Itajaí/Itajaí	May 2009	26°53'01''S 48°40'11''W	*B. similaris*	78	-
			Belocaulus sp.	1	-
			A. fulica	17	-
			Sarasinula sp.	2	-

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Regions States Municipalities/ports	Collection date (month/year)	Geographical coordinates	Collected molluscs: Identification	n	PCR-RFLP assay (*A. cantonensis*)
São Francisco do Sul/São Francisco do Sul	May 2009	26°14'27''S 48°38'01''W	*Belocaulus* sp.	5	-
			B. similaris	150	-
			S. octona	130	-
			Leptinaria sp.	133	-
			Succinea sp.	108	-
			Cyclodontina sp.	15	-
Navegantes/Navegantes	May 2009	26°53'28''S 48°39'40''W	*B. similaris*	88	+
			A. fulica	176	+
			Phyllocaulus sp.	1	-
Rio Grande do Sul					
Rio Grande/Rio Grande	May 2009	32°02'40''S 52°04'51''W	*Helix* sp.	104	-
			Phyllocaulis sp.	6	-
			Limacidae	12	-
			Bulimulus sp.	2	-
Porto Alegre/Porto Alegre	Dec 2009	30°01'23''S 51°13'22''W	*Bulimulus* sp.	3	-
			Leptinaria sp.	30	-

NI: not informed; PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism.

(PE), Lima et al. (2009) described a clinically identified case with neurological impairment due to *A. cantonensis*. Thiengo et al. (2010) reported *A. cantonensis* larvae parasitizing *A. fulica* that had originated from a municipality in Escada (PE). Maldonado Junior et al. (2010) reported the presence of this nematode in the municipalities of Barra do Pirai, São Gonçalo (RJ) and Joinville (SC).

The results presented in this study confirm the non-specificity of *A. cantonensis* in relation to the intermedi-

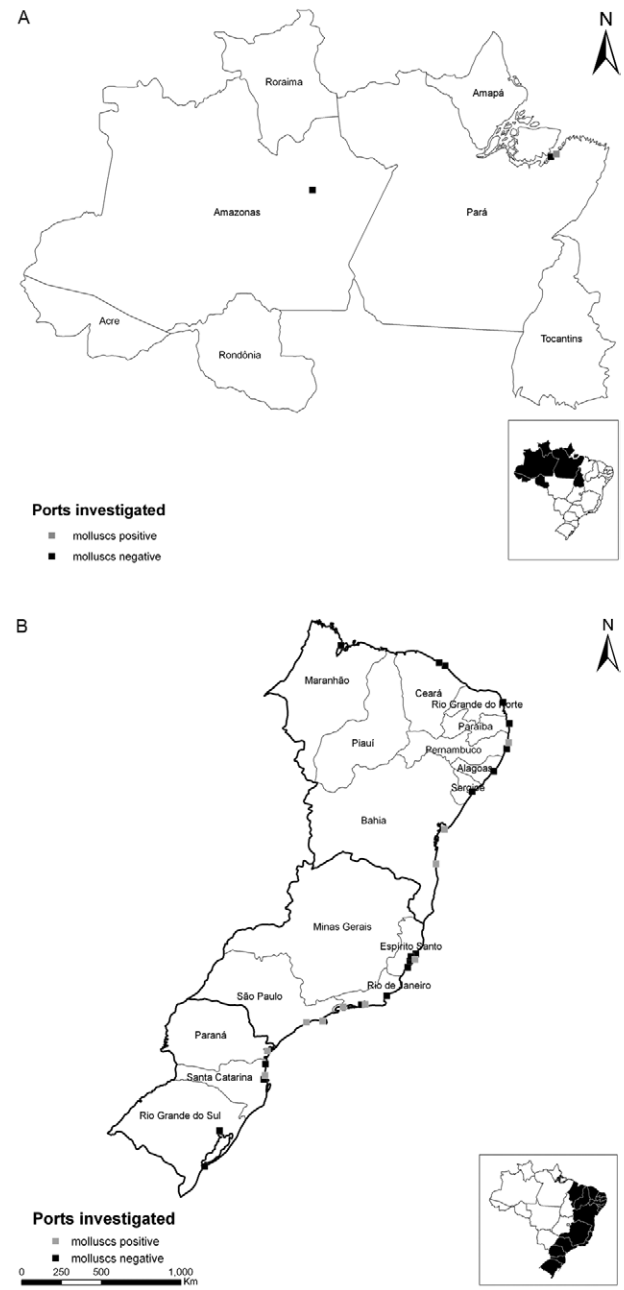

Fig. 2: geographical location of 30 ports of Brazil where molluscs were found not infected and infected with *Angiostrongylus cantonensis*. A: North Region of Brazil showing the location of three ports investigated; B: Northeast, Southeast and South Regions of Brazil showing the location of 27 ports investigated.

ate host because the parasite was found in a variety of gastropods, including *S. octona*, *S. marginata*, *A. fulica* and *B. similaris*. These molluscs have been shown to be hosts of *A. cantonensis* in South America in studies by Caldeira et al. (2007), Pincay et al. (2009), Maldonado Junior et al. (2010) and Thiengo et al. (2010).

The molecular diagnostic method utilised in this study was shown to be accurate, cost-effective and quick and therefore the use of this method would enable immediate responses to be obtained during outbreaks. In contrast to the molecular diagnostic method, the parasitological method requires approximately 30 days to obtain the adult forms for morphological identification and must be performed by an expert taxonomist, making this method cumbersome.

When and how the introduction of *A. cantonensis* in South America occurred remains unknown. There is, however, a concordance that the introduction occurred through the migration of infected rodents on ships travelling from other continents, especially Africa and Asia (Caldeira et al. 2007, Pincay et al. 2009, Maldonado Junior et al. 2010).

Future studies are needed to investigate the prevalence of molluscs parasitized by *A. cantonensis* in the interior regions to map the distribution and migration route of this parasite.

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