ANGISTRONGYLUS CANTONENSIS AND ANCYLOSTOMA CANINUM DETECTION IN SNAILS OF SÃO PAULO CITY (2016-2017), BRAZIL

DETECÇÃO DE ANGISTRONGYLUS CANTONENSIS AND ANCYLOSTOMA CANINUM EM CARAMUJOS DA CIDADE DE SÃO PAULO (2016-2017), BRASIL

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SUMMARY

Achatina fulica snails cause environmental problems and represent a public health hazard since it is a host in the life cycles of various parasites, among them, *Angiostrongylus cantonensis* and, less frequently, *Ancylostoma caninum.* We report the occurrence of *Angistrongylus cantonensis*, as well as the unexpected finding of *Ancylostoma caninum*, in a total of 936 specimens of *Achatina fulica* snails from different regions of São Paulo city, Brazil. Samples were divided into 492 pools which were screened for nematodes. If present, larvae were submitted to DNA extraction and PCR protocol targeting, the ITS-2 gene junction. From the 183 positive pools for larvae presence, 97 showed specific 650 bp band at electrophoresis and 21 presented bands nearly 300 bp. Two amplicons from each size were and sequenced. A BLAST/n of 650 bp sequences presented identity with *Angistrongylus cantonensis*, while the two of 300 bp, showed identity with *Ancylostoma caninum*, also supported by phylogenetic analysis. This is the second report of *Ancylostoma caninum* found in these snails in the world, therefore, this study allows a better understanding about these diseases and highlights the need of continue systematically mapping sites that can be infested with the mollusc.

KEY-WORDS: Brazil. Snail. Nematode. Zoonosis.

RESUMO

Os caramujos *Achatina fulica* causam problemas ambientais e representam um perigo em Saúde Pública uma vez que são hospedeiros de vários parasitas, entre eles o *Angiostrongylus cantonensis* e menos frequentemente o *Ancylostoma caninum.* Nós relatamos a ocorrência de *Angistrongylus cantonensis*, bem como o achado de *Ancylostoma caninum*, a partir de 936 espécimens de caramujos *Achatina fulica* de diferentes regiões da cidade de São Paulo, Brasil. Amostras foram divididas em 492 pools os quais foram triados para nematóides. Se presentes, larvas foram submetidas a extração de DNA e umprotocolo de PCR tendo como alvo a junção do gene ITS-2. De 183 pools contendo larvas, 97 apresentaram bandas específicas de 650 pb e na eletroforese 21 apresentaram bandas próximas aos 300 pb. Dois amplicons de cada tamanho foram sequenciados. A submissão ao BLAST/n das sequências de 650 pb apresentaram identidade das sequências com *Angistrongylus cantonensis*, enquanto que as duas de 300 pb apresentaram identidade com *Ancylostoma caninum*, também corroboradas por análises filogenéticas. Este é o segundo relato do encontro de *Ancylostoma caninum* nestes caramujos no mundo, sendo assim, este estudo permite um melhor entendimento destas doenças e denota a necessidade de contínuo monitoramento de regiões que estejam infestadas pelo molusco.

PALAVRAS-CHAVE: Brasil. Caramujo. Nematóide. Zoonose.

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INTRODUCTION

Listed among 100 of the worst invasive species by the Global Invasive Species Database (2019) (GISD, 2019), the giant african snail, *Achatina fulica* (Bowdich, 1893) is known for promoting environmental imbalance and substantial biodiversity and economic losses in the areas it has been introduced (Ohlweiler et al., 2010; Pavanelli et al., 2017).

Originated from east Africa, these molluscs were driven to the worldwide distribution since the 1800, reaching Asia in the middle of 1920s, the USA in the 60s and Guadeloupe in 1988 (Thiengo et al., 2007; Roda et al., 2016). They were illegally introduced in Brazil between 1988 and 1989, as a commercial alternative to the *Helix aspersa* (Müller) snail to be consumed as escargot (Thiengo et al., 2007; Pavanelli et al., 2017). The commercial collapse of breeders led to the inappropriate release of specimens and consequent dissemination in most of Brazilian territory. Currently, *Achatina fulica* is found in 23 of the 26 Brazilian states, including Federal District and the Amazon region (Thiengo et al., 2008; Moreira et al., 2013).

Besides causing environmental problems, this snail represents a public health hazard (Roda et al., 2016) as the intermediate host in the life cycles of various parasites, among them, *Angiostrongylus cantonensis* (Oliveira et al., 2015). First described in Canton, China, these nematodes are found in lung arteries of rodents (*Rattus norvegicus* and *Rattus rattus*) (Moreira et al., 2013), definitive hosts of this parasite. Since then, they were already reported in Asia, Europe, Oceania, and Americas, including Brazil, Caribbean Islands and the USA (Lu et al., 2018).

First stage larvae (L1) are released in rodents' feces and can be ingested by snails, in the tissues of which L1 turns to third stage larvae (L3) (Thiengo et al., 2013) the life cycle is completed when rats eat infected snails or slugs and the larvae further mature to become adult worms (Wang et al., 2008). Due to their low host specificity, L1 may also infect other paratenic hosts as crabs, freshwater shrimp, amphibians, flatworms, and fish, infected by ingesting intermediate or other paratenic hosts, although the parasite does not complete the life cycle as it does in rats (Lu et al., 2018). Humans may also get accidentally infected by eating raw or undercooked snails or any of the paratenic hosts. Although some people are asymptomatic or have mild symptoms that don't last very long, in some rare cases, the angiostrongyliasis may lead to neurological implications such as eosinophilic meningitis (Lima et al., 2009).

Naturally infected terrestrial snails were already found in Rio de Janeiro (Acuña et al., 2009; Maldonado et al., 2010; Oliveira et al., 2015), São Paulo (Guerino et al., 2017), Santa Catarina (Maldonado et al., 2010) and in northeast (Albuquerque et al., 2008; Thiengo et al., 2010) same places where human cases of eosinophilic meningitis were also reported (Lima et al., 2009; Moreira et al., 2013; Morassutti et al., 2014).

Ancylostoma caninum is a common nematode of the canine gastrointestinal associated with cutaneous *Larva migrans* in humans (Shepherd et al., 2018). The parasitic cycle in the definitive hosts (dogs and cats) occurs when the L3 larvae in the soil, penetrate the organism of these animals through the skin, followed by migration to the lungs and then the trachea, being swallowed. The adult worms reproduce in the intestine and the eggs are eliminated in the faeces (Velho et al., 2003). This nematode is widespread in Brazil (Lima et al., 1984; Velho et al., 2003), being sandboxes and playgrounds risk areas, due to presence of favorable environmental conditions for egg hatching and larvae development, as well as access of the definitive hosts (Nunes et al., 2000).

In this study we report the occurrence of *Angistrongylus cantonensis*, as well as the unexpected finding of *Ancylostoma caninum*, in *Achatina fulica* snails, found in different regions of São Paulo city, Brazil, confirmed through partial genetic nucleotide sequencing.

MÉTHODS

Samples **-** A total of 936 specimens of snail species were collected in different regions of São Paulo City, between the years of 2016 and 2017, and forwarded to Zoonosis Surveillance Division, São Paulo Municipality (DVZ-SP), in order to proceed to morphological identification. Snails confirmed as belonging to the species of *Achatina fulica* were further prepared. According to its size, they were tested individually or pooled (from 2 to 10 specimens), summing up 492 pools. The shells were broken and the cephalopodium was cut into thin slices that were mixed with 4% pepsin solution and 0.7% of HCl and heated to 37ºC for 2 h using a water bath. Finally, they were filtered and the solid content was immersed in a sedimentation flask with distilled water at 42ºC for 2 h. The sediment was screened for the larvae presence by visual observation using a magnifier (5X). If present, larvae were recovered in microtubes by pipetting and centrifuged at 12,000 x g for 1 min, discarding the supernatant.

Larvae DNA extraction **-** Glass beads were added to the larvae tube and vortexed together with 180µL of ATL Buffer (DNeasy® Blood and Tissue Kit - QIAgen®, Hilden, Germany) and 20µL of Proteinase K. The samples were submitted to water bath at 56°C for 150 min, followed by the DNA extraction according to manufacturer's protocol.

PCR - Helminths DNA larvae were identified by PCR targeting primarily the ITS-2 gene junction, using the primers NC1 (5'-ACGTCTGGTTCAGGGTTGTT-3') and NC2 (5'-TTAGTTTCTTTTCCTCCGCT-3') (Gasser et al., 1993). A volume of 5µL of DNA was added to the PCR mix, which consisted of 1X PCR buffer (Invitrogen™, Carlsbad , California), 0.2mM of each DNTP, 0.5mM of each primer, 1.5mM $MgCl_2$, 1.25U Taq DNA polymerase (Invitrogen™, Carlsbad, California), completed with ultrapure water up to 25µL. The thermocycling conditions were: initial

denaturation at 94ºC for 5 min; 30 amplification cycles (94ºC for 1 min, 58ºC for 1 min, and 72ºC for 1 min); and a final extension at 72ºC for 10 min. Products were resolved on a 1.5% agarose gel stained with 3µL of ethidium bromide.

DNA sequencing **-** PCR amplicons were purified with EXOSAP-it (USB®, Waltham, MA, USA) reagent, submitted to bidirectional DNA sequencing using BigDye 3.1 (Applied Biosystems™ Waltham, MA, USA), and resolved in an ABI-3500 Genetic Analyzer (Applied Biosystems™ Waltham, MA, USA), according to the manufacturer's instructions.

Phylogenetic analysis **-** The nucleotide sequences were submitted to BLAST/n in order to define the most probable larvae family found. Nucleotide sequences were retrieved from Genbank, aligned and trimmed with Bioedit software v. 7.0.5.3 (© 1997-2005 Tom Hall). The nucleotide substitution Hasegawa-Kishino-Yano and Kimura-2-parameter models were determined for each gene separately, according to the lowest Bayesian Information Criterion (BIC), for *Angiostrongylidae* and *Ancylostomatinae* sequence datasets, respectively, using MEGA software v. 7.0.26 (© 1993-2019 Kumar, Tamura & Stecher). The phylogenetic trees were defined using this same software, supported by 1,000 bootstrap replicates.

Accession numbers **-** Nucleotide sequences from this study were deposited in GenBank under the accession numbers: MF371331 and MF371332 (*Angiostrongylus cantonensis*); MF371321 and MF371322 (*Ancylostoma caninum*).

RESULTS

Nematode larvae were found by visual observation in 183 out of 492 sample pools and were tested by PCR. From those, 97 pools showed specific 650 bp band at agarose gel electrophoresis, compatible with *Angiostrongylus cantonensis* positive control and 21 presented bands nearly 300 bp.

The amplicon sequencing and BLAST/n analysis of two different 650 bp positive samples chosen at random confirmed PCR results, presenting identity with *Angistrongylus cantonensis*, while two amplicons of nearly 300 bp, showed identity with *Ancylostoma caninum*. Maximum Likelihood phylogenetic trees using partial nucleotide sequences were generated using either *Angiostrongylidae* ITS2 gene and *Ancylostomatinae* rRNA5.8s/ITS-2 gene junction. The phylogenetic trees depicted that nucleotide sequences defined in this study clustered together with *Angiostrongylus cantonensis*, while the topology maintained the remaining species apart from each other and supported by high bootstrap values (Figura 1). Regarding to the sequences generated from ~300 bp amplicons, both clustered together with *Ancylostoma caninum* species, also supported by high bootstrap value (Figura 2).

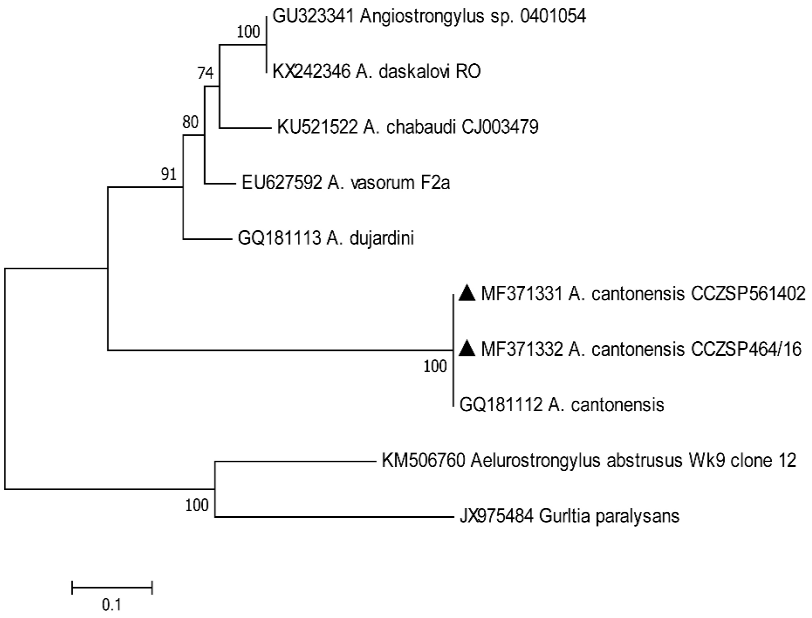

Figure 1 - Maximum likelihood tree (HKY substitution model) of the partial ITS2 gene (493-nt), according to *Angiostrongylidae* family representatives. Strains detected in this study are preceded by black triangles. The numbers at each node are bootstrap values greater than 70% from 1,000 bootstrap replicates, and the scale bar represents the number of substitutions per site.

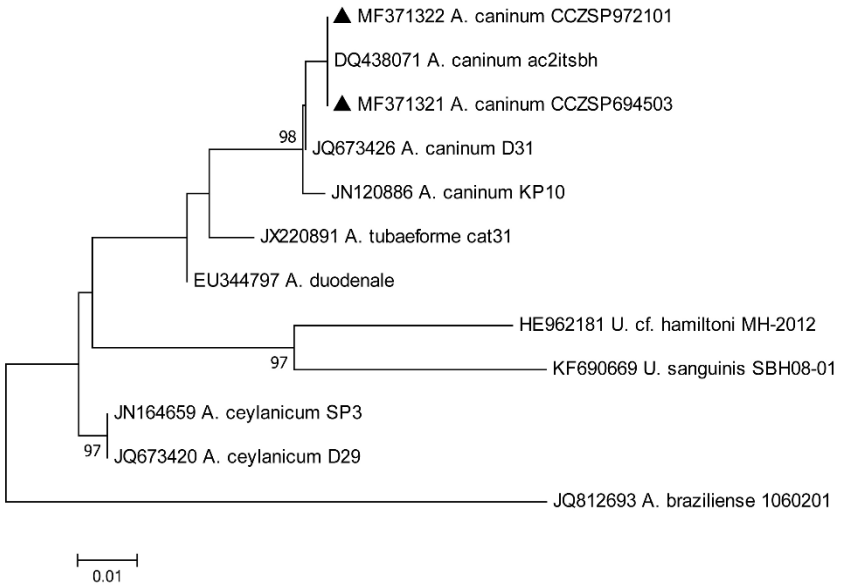

Figure 2 - Maximum likelihood tree (K2P substitution model) of the partial rRNA5.8s/ITS-2 gene junction gene (293-nt), according to *Ancylostomatinae* family representatives. Strains detected in this study are preceded by black triangles. The numbers at each node are bootstrap values greater than 70% from 1,000 bootstrap replicates, and the scale bar represents the number of substitutions per site.

DISCUSSION

Since 2016, Zoonosis Surveillance Division, São Paulo Municipality (DVZ-SP) has been carrying out a routine diagnosis for *Angiostrongylus* spp. larvae in *Achatina fulica* specimens collected all over the city, in order to implement epidemiological zoonosis surveillance and to control the dissemination of the giant snail. *Achatina fulica* species is widely spread throughout several Brazilian cities, among them some that border the city of São Paulo (Ohlweiler et al., 2010).

Pools were composed of specimens collected on the same site in order to give an idea of their circulation, maximizing the sensibility and reducing costs of the monitoring activity, once many snails present small dimensions. The L3 larvae were visualized in stereoscopic microscopes, allowing a fast and cheap screening of the infection state in those molluscs. When the larvae were observed, the pool was submitted to PCR reaction aiming to detect *Angiostrongylus cantonensis* or *Angiostrongylus costaricensis*.

Remarkably, 37.2% (183 out of 492 pools) contained the L3, demonstrating a high frequency, highlighting the importance of *Achatina fulica* as vector for nematodes dissemination. Snail infection rates and parasite load are highly variable as they depend on a myriad of factors but, when compared to a study carried out in 12 different sites from Metro Manila urban area, in Philippines, where 61 out of 365 individuals (16.7%) were parasitized by nematodes (Santos et al., 2014), the rates we have obtained in Brazil are much higher.

When submitted to PCR reaction, 53.0% (97/183) of the tests were positives for *Angiostrongylus cantonensis*. Even though they presented amplicons with the expected sizes, two of the positive pools for *Angiostrongylus cantonensis* were further sequenced to confirm the findings. These sequences, were submitted to phylogenetic analysis where could be observed they group with GQ181112 *Angiostrongylus cantonensis* sample with high bootstrap values. Despite the previously proven susceptibility of *Achatina fulica* to *Angiostrongylus costaricensis* (Carvalho et al., 2003), this nematode was not detected in tested samples. This was expected, once there are no reports of snails naturally infected by *Angiostrongylus costaricensis* in the Americas (Pavanelli et al., 2017).

Besides the samples with the expected amplicons, in 11.5% (21/183) of PCRs gel bands of approximately 300 bp could be observed. Those samples were sequenced and confirmed to be closely related to a fragment of the genome from *Ancylostoma caninum* ac2itsbh (DQ438071). With the purpose of explaining these findings, the used primers were submitted to BLAST (data not shown). *In silico* analysis demonstrated that these primers theoretically can produce a ~312 bp gel band, consistent with these results.

A study in the city of São Paulo using 278 samples of dogs, presented 56.6% prevalence of hookworm infections, of which 61.4% were positives for *Ancylostoma caninum*, 12.5% for *A. braziliense* and 26.1% had mixed infections (Arbex et al., 2017). Likewise, the occurrence of gastrointestinal parasites was assessed in fecal samples from 3,099 dogs in the metropolitan region of São Paulo, SP and indicated *Ancylostoma* spp. as the most common nematode in these samples (7.1%), found both in adult and young dogs (Ferreira et al., 2016).

Infections of *Achatina fulica* snails with *Ancylostoma caninum* are unusual findings and were first reported in 2014, from 4 different sites in the

metropolitan area of Manila (Santos et al., 2014). It is known that this parasite is able to complete its migration in humans, with occasional worms reaching the human gut, and it was described the possibility of eosinophilic gastroenteritis, in addition to pruritus and skin irritation (Croese et al., 1994; Walker et al., 1995; Shepherd et al., 2018) therefore, skin contact and ingestion of this nematode should be avoided.

Our data does not allow to confirm whether the parasite is developing part of its cycle in the host or is only a mechanical vector, but there is certainly contact of the snail with animal feces.

Despite showing larvae in the visual inspection, 35.52% (65/183) of the pools resulted negative in the test. Thus, at least two hypotheses may be raised: the first of these could be failures during extraction and/or DNA amplification processes, which can be weakened by the presence of positive controls in every reaction. Besides that, procedures were strictly followed and negative samples were tested twice, in order to confirm the results. The second, more likely, is that snails were infected with other nematodes which were not detectable by the used PCR technique.

In fact, other agents have already been detected in *Achatina Fulica*, including *Aelurostrongylus abstrusus larvae* (Railliet, 1898) (Nematoda: *Metastrongylidae*), that was previously described in the cities of Guaratinguetá (Ohlweiler et al., 2010) and Jundiaí (Thiengo et al., 2008), approximately 190 km and 55 km distant from the city of São Paulo, respectively. Parallelly, in Colombia was detected *Aelurostrongylus abstrusus*, *Angiostrongylus vasorum*, *Troglostrongylus brevior*, and *Crenosoma vulpis* infecting *Achatina fulica* snails, individually or in co-infection (Tabares et al., 2019). Similarly, in Philippines, *Ancylostoma caninum* and *Rhabditis sp* were found, besides the already mentioned *Angiostrongylus cantonensis* (Santos et al., 2014).

Therefore, additional studies are required on the characterization of the larvae found to allow assessing whether they represent a risk to public and animal health as well as on the improvement of the screening and diagnostic procedures.

As very little is known about the occurrence of these parasites in the city of São Paulo, data shown in this study allows a better understanding about this disease and highlights the need of continue systematically mapping sites that can be infested with the mollusc. Thus, as a key to prevent these diseases, bigger malacological and parasitological surveillance actions should be taken, combined with a continuous and integrated snails, rats, dogs and cats control.

Public Health education is also an essential component for prevention discouraging consumption (accidental or not) of this mollusc by animals or people, once these snails can be unintentionally eaten together with raw vegetables poorly washed and sanitized (Santos et al., 2014).

CONCLUSIONS

We report the occurrence of *Angistrongylus cantonensis* (n=97 pools), as well as the unexpected finding of *Ancylostoma caninum* (n=21 pools), in a total of 936 specimens (n=492 pools) of *Achatina fulica* snails from different regions of São Paulo city, Brazil.

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