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Original Article

First record of *Bulimulus tenuissimus* (Mollusca) as potential experimental intermediate host of *Angiostrongylus cantonensis* (Nematoda)

*F. G. Martins[a], J. S. Garcia[b], E. J. L. Torres[c], M. A. J. Santos[d], C. L. Massard[a,e] and J. Pinheiro[a,f]**

[a]Programa de Pós-graduação em Ciências Veterinárias, Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro – UFRRJ, BR 465, Km 7, CEP 23897-000, Seropédica, RJ, Brasil

[b]Laboratório de Biologia e Parasitologia de Mamíferos Silvestres Reservatórios, Fundação Oswaldo Cruz – FIOCRUZ, Avenida Brasil, 4365, Manguinhos, CEP 21040-360, Rio de Janeiro, RJ, Brasil

[c]Departamento de Imunologia, Microbiologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro – UERJ, Rio de Janeiro, RJ, Brasil

[d]Departamento de Biologia Animal, Instituto de Ciências Biológicas e da Saúde, BR 465, Km 7, CEP 23897-000, Seropédica, RJ, Brasil

[e]Departamento de Parasitologia Animal, Instituto de Veterinária, BR 465, Km 7, CEP 23897-000, Seropédica, RJ, Brasil

[f]Departamento de Ciências Fisiológicas, Instituto de Ciências Biológicas e da Saúde, BR 465, Km 7, CEP 23897-000, Seropédica, RJ, Brasil

*e-mail: jairopinheirodasilva@gmail.com

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(With 4 figures)

Abstract

Snails are essential to complete the life cycle of the metastrongylid nematode *Angiostrongylus cantonensis*, the causative agent of infections in domestic and wild animals, mainly rodents, and also of neural angiostrongyliasis or eosinophilic meningitis in humans. There are many reports of mollusks that can act as intermediate hosts of this parasite, especially freshwater snails and the African giant *Achatina fulica*. The terrestrial gastropod *Bulimulus tenuissimus* is widely distributed in Brazil and other species of the same genus occur in Brazil and other countries, overlapping regions in which there are reports of the occurrence of *A. cantonensis* and angiostrongyliasis. In spite of this, there are no records in the literature of this species performing the role of intermediate host to *A. cantonensis*. The present study analyzed the experimental infection with first-stage larvae of *A. cantonensis*, under laboratory conditions, of *B. tenuissimus*, by using histology and electron microscopy techniques. Three weeks after exposure to L1 larvae, it was possible to recover L3 larvae in small numbers from the infected snails. Developing larvae were observed in the cephalopedal mass (foot), ovotestis, and mantle tissues, being located inside a granulomatous structure composed of hemocyte infiltration, but there was no calcium or collagen deposition in these structures in significant amounts. In the third week post exposure, it was possible observe a sheath around the developing larvae. The infected snails presented reduction in the fibrous muscular tissue in the foot region, loss of the acinar organization in the digestive gland, with increase of amorphous material inside the acini and loss of epithelial pattern of nuclear organization in the acinar cells. However, the ovotestis seemed unaffected by the infection, since there was a large number of developing oocytes and spermatozoa in different stages of formation. The digestion of infected snails allows us the third-stage recovery rate of 17.25%, at 14 days post exposure to the L1. These L3 recovered from *B. tenuissimus* were used to infect rats experimentally, and 43 days post infection first-stage (L1) larvae of *A. cantonensis* were recovered from fresh feces. The results presented constituted the first report of the role of *B. tenuissimus* as an experimental intermediate host to *A. cantonensis* and shed some light on a possible problem, since the overlapping distribution of *B. tenuissimus* and *A. cantonensis* in Brazil and other countries where different species of *Bulimulus* occur enables the establishment and maintenance of the life cycle of this parasite in nature, with wild rodents as reservoirs, acting as a source of infection to humans, causing neural angiostrongyliasis.

Keywords: Bulimulus tenuissimus, Bulimulidae; Metastrogylidae; potential new intermediate host.

Primeiro registro de *Bulimulus tenuissimus* (Mollusca) como hospedeiro intermediário de *Angiostrongylus cantonensis* (Nematoda)

Resumo

Os moluscos são um requisito essencial para a conclusão do ciclo de vida pelo nematoide metastrogilídeo *Angiostrongylus cantonensis*, o agente causador de infecções em animais domésticos e selvagens, principalmente roedores, e também de angiostrongilíase neural ou meningite eosinofílica em humanos. Há muitos relatos de moluscos que podem atuar como

hospedeiro para este parasito, sendo o foco dado aos moluscos de água doce e no gigante africano *Achatina fulica*. O gastrópode terrestre *Bulimulus tenuissimus* é amplamente distribuído no território brasileiro e há outras espécies do mesmo gênero que ocorrem no Brasil e outros países, sobrepondo-se às regiões em que há relatos à ocorrência de *A. cantonensis* e angiostornigilíase. Apesar disso, não há registro na literatura, acerca desta espécie como hospedeiro intermediário para *A. cantonensis*. O presente estudo teve como objetivo verificar a possibilidade de infectar experimentalmente, utilizando larvas L1 de *A. cantonensis*, em condições laboratoriais, o molusco *B. tenuissimus*, utilizando técnicas de histologia e microscopia eletrônica. Três semanas após a exposição às larvas L1, foi possível recuperar larvas L3 dos moluscos infectados, em pequena quantidade. As larvas em desenvolvimento foram observadas na massa cefalopediosa (pé), ovotestis e nos tecidos do manto, sendo localizadas dentro de uma estrutura granulomatosa constituída por infiltração hemocitária, mas não houve deposição de cálcio ou colágeno nessas estruturas em quantidade significativa. Na terceira semana pós exposição, foi possível observar uma bainha ao redor das larvas em desenvolvimento. Os caracóis infectados apresentaram redução no tecido muscular fibroso na região do pé, perda da organização acinar na glândula digestiva, com aumento de material amorfo dentro dos ácinos e perda do padrão epitelial da organização nuclear nas células acinares. No entanto, o ovotestis, pareceu não ser afetado pela infecção, uma vez que houve um grande número de oócitos em desenvolvimento e espermatozóides em diferentes estágios de formação. A digestão dos moluscos infectados nos permitiu a recuperação de larvas de terceiro estágio (17,25%), aos 14 dias após a exposição à L1 de *A. cantonensis*. Estas L3 recuperadas de *B. tenuissimus* foram utilizados para infectar ratos experimentalmente, e 43 dias após a infecção, as larvas do primeiro estágio (L1) foram recuperadas de fezes frescas. Os resultados apresentados representam o primeiro registro do papel de *B. tenuissimus* como hospedeiro intermediário experimental de *A. cantonensis* e trazem alguma luz a um problema, até então silencioso, uma vez que a sobreposição da distribuição de *B. tenuissimus* e *A. cantonensis* no Brasil, e outros países, onde as diferentes espécies de *Bulimulus* ocorrem, torna possível o estabelecimento e manutenção do ciclo de vida deste parasito na natureza, com roedores selvagens como reservatório, agindo como fonte de infecção para humanos e causando a angiostrongilíase neural.

Palavras-chave: *Bulimulus tenuissimus*; Bulimulidae; Metastrongylidae; potencial hospedeiro intermediário.

1. Introduction

Angiostrongyliasis has been recorded in many countries since the main species indicated as causative agents of human disease, *Angiostrongylus cantonensis* (Chen, 1935) and *Angiostrongylus costaricensis* Morera and Céspedes (1971) were described. Nomura and Lin (1945) first recorded a case of human eosinophilic meningitis in Taiwan, in a 15-year-old boy who had 10 *A. cantonensis* worms in his cerebrospinal fluid (CSF) and died because of the infection. Since then, many cases have been recorded every year. The disease caused by this nematode is no longer restricted to Southeast Asia. Reports can be found of the parasite infecting humans and other mammals in 19 countries (Thailand, China (including Taiwan and Hong Kong), Tahiti, French Polynesia, United States, Cuba, New Caledonia, Japan, Australia, Vanuatu, India, Vietnam, Malaysia, Mayotte, Réunion Island-France, Sri Lanka, Cambodia, Samoa, Fiji, Germany, Jamaica, Costa Rica, Indonesia, Belgium, Italy, Côte d'Ivoire, New Zealand, Papua New Guinea, Switzerland, and United Kingdom (Puthiyakunnon and Chen, 2015; Prociv et al., 2000). Flerlage et al. (2017) reported a case of *A. cantonensis*-meningoencephalitis in a 12-month-old boy in Tennessee, USA, who had not traveled outside of southwestern Tennessee or northwestern Mississippi, showing the existence and maintenance of the parasite's life cycle *in loco*. Puthiyakunnon and Chen (2015) reported that until 2012, there were about 3,161 cases of human angiostrongyliasis documented globally, but this number is certainly underestimated, because many cases go unreported due to lack of awareness of this parasite within the medical community. Morassutti et al. (2014) drew attention to neural angiostrongyliasis in Brazil as an emerging disease, which requires more careful investigation by researchers and health professionals.

The giant African snail, *Achatina fulica* (Mollusca, Gastropoda), has been found naturally infected with *A. cantonensis* in the Brazilian states of Pará, Pernambuco, Rio de Janeiro, Espírito Santo, São Paulo and Santa Catarina (Bechara et al., 2018; Oliveira et al., 2015; Moreira et al., 2013; Carvalho et al., 2012; Thiengo et al., 2010; Maldonado Junior et al., 2010; Caldeira et al., 2007). Lv et al. (2009a, 2008) also mentioned this disease as emerging in China as a result of changes in food consumption habits and long-distance transportation of food, stating that the disease occurrence seems to be related to the spread of two invasive snail species (*A. fulica* and *Pomacea canaliculata*). Therefore, the focus now is on *A. fulica* as the main intermediate host of *A. cantonensis*.

Vasquez-Perera (2016) and Puthiyakunnon and Chen (2015) mentioned many species of mollusks as intermediate hosts of *A. cantonensis*, but no species from the *Bulimulus* genus were identified in these reports. Recently and Ramos-de-Souza et al. (2018), in a molecular study, found *B. tenuissimus* naturally infected with *A. cantonensis* in Sergipe state, Brazil. However, these data have not yet been published, and the author just presented the molecular detection of *A. cantonensis* DNA in the snail.

Terrestrial snails of the genus *Bulimulus* (Mollusca, Bulimulidae) are widely distributed throughout the world (South, Central and North America) (Breure, 2016; Parent and Crespi, 2006; Miquel, 1991; Metcalf, 1984). In Brazil, the species *Bulimulus tenuissimus* is widespread in many regions (Carvalho et al., 2012; Simone, 2006). Despite the wide distribution of *B. tenuissimus*, there is only one

report of molecular detection of natural infection of this mollusk naturally infected with *A. cantonensis* (Ramos-de-Souza et al., 2018).

The present study aimed to investigate, under experimental laboratory conditions, the susceptibility of the snail *B. tenuissimus* as a potential intermediate host of *A. cantonensis* by using histological and biological analyses.

2. Material and Methods

2.1. Maintenance of the snails and formation of groups

Bulimulus tenuissimus snails were manually collected in the early morning from vegetable gardens located in the city of Seropédica, RJ, Brazil (Latitude: 22º 44' 38" S; Longitude: 43º 42' 27" W; Altitude: 26m), and taken to the laboratory. The snails were maintained in glass terrariums with the bottom covered by a layer of vegetable soil (2 cm), moistened on alternate days with tap water. The snails were fed with fresh cabbage leaves, cucumber and carrot *ad libitum*, with the food replenished on alternate days.

The eggs were collected and transferred to new terrariums prepared as described above. The newly hatched snails were maintained until reaching 90 days and at least 5 mm shell length. These snails were grouped in six groups: three control (uninfected) groups (n=90), and three infected groups (n=90), each with 30 specimens. All the groups were formed using triplicates (n_{total}=540).

2.2. Parasites

Third-stage larvae (L3) of *A. cantonensis*, obtained from specimens of *A. fulica* collected in the city of São Gonçalo, RJ, Brazil in 2015, in the area surrounding the home of a patient diagnosed with eosinophilic meningoencephalitis, were inoculated in *R. norvegicus* in the Laboratory of Biology and Parasitology of Wild Mammal Reservoirs (LBPMR) of Oswaldo Cruz Institute (Fiocruz), where the cycle was maintained. The first-stage larvae (L1) utilized in this study were obtained from this experimental cycle maintained under laboratory conditions.

2.3. Infection of the snails

The feces of parasitized *R. norvegicus* were collected and used to obtain the larvae by the technique of Baermann, according to Willcox and Coura (1989). After processing the fecal samples, specimens of *B. tenuissimus* were exposed individually to 1,200 L1 larvae. The L1 larvae of *A. cantonensis* were spread on pieces of fresh cucumber placed in 24-hole plate with moistened filter paper at the bottom. The snails were added on the cucumber pieces. The plates were closed, and the snails were maintained in contact with the larvae. The uninfected snails were treated by the same procedure without L1 larvae. After 24 h, the snails from each group were individually examined under a stereomicroscope to detect larvae (L1 stage) in the plates (Tunholi-Alves et al., 2011). The absence of larvae in the plates ensured the infection and susceptibility of snails under laboratory conditions. Subsequently, the snails were removed from the plates and transferred to terrariums for formation of the experimental groups.

2.4. Recovery of third-stage larvae

Five snails from each group (n_{total}= 45) were maintained until the end of the third week after exposure and dissected. After shell removal, the soft tissues were pooled and cut in small pieces and submitted to artificial digestion, according to Lv et al. (2009b) and Thiengo et al. (2010), to evaluate the presence of L3 larvae. The identification of the development stage was made according Lv et al. (2009b). The recovery percentage was calculated in relation to the number of L1 larvae used to infect the snails (number of L3/number of [1,200] L1).

2.5. Dissection and histological and histochemical analyses

Weekly for three weeks, five snails from each group were randomly chosen, dissected and fixed using Duboscq-Brasil fixative (Fernandes, 1949) for histological and histochemical analyses. The snails were fixed for 24 hours at 4 ºC, after which the shell was removed and the tissues were processed according to routine histological techniques (Humason 1979). Sections measuring 5 µm width were obtained and stained using hematoxylin and eosin (HE), Gomori's trichrome or Von Kossa stain to observe the tissue morphology.

2.6. Electron microscopy of the histological slides

Tissue sections processed as described for the histological analysis were collected on coverslips, deparaffinized, gold-coated (10-15 nm thick layer) and analyzed under an FEI-Quanta 250 scanning electron microscope (SEM) operating in high vacuum mode with an acceleration voltage of 15 kV

2.7. Vertebrate definitive host infection and recovery of first-stage larvae

Four female rats (*Rattus novergicus*), were experimentally infected, by gavage, with 100 L3 larvae of *A. cantonensis* obtained from *B. tenuissimus* experimentally infected as described above. From the second week post infection onward, weekly, fresh feces were examined, by the technique of Baermann, according to Willcox and Coura (1989), to look for L1 larvae. The L1 recovered were examined under light microscope to observe the morphological features and characterize the larval stage according to Lv et al. (2009b) and Hata and Kojima (1990). The Oswaldo Cruz Foundation Ethics Committee on Animal Use (CEUA Number LW-47-14) approved this study.

3. Results

The L3 larvae recovery rate three weeks after exposure was 17.25%, in relation to the initial number of 1,200 L1 larvae used to infect the snails, but the recovery of L3 larvae showed that *A. cantonensis* is able to infect *B. tenuissimus* and complete its life cycle from L1 until L3, the infective stage to the vertebrate definitive host. The L3 larvae were recovered from *B. tenuissimus* experimentally infected from the second week post exposure onward. The morphology of the L3 larvae evidenced the anterior end with oral

aperture, typical expanded knob-like tips and rod-like structure in the anterior end (Figure 1d). Also, it is possible to observe the excretory pore, esophagus bulb, intestine, and anus (Figure 1d-f).

Histological observations revealed developing larvae in different tissues of the snail body. In Figure 2a, it is possible compare the histological structure of the cephalopedal mass of an uninfected *B. tenuissimus*, presenting muscle fibers with many secretory cells in the edge of the region, which secrete mucus to help the snail to move about in the substrate where it lives. Figure 2b presents a section of a similar region of the cephalopedal mass of a one-week old infected mollusk, with disorganization of the fibrous muscular structure of the foot, reduction of the mucous secretory

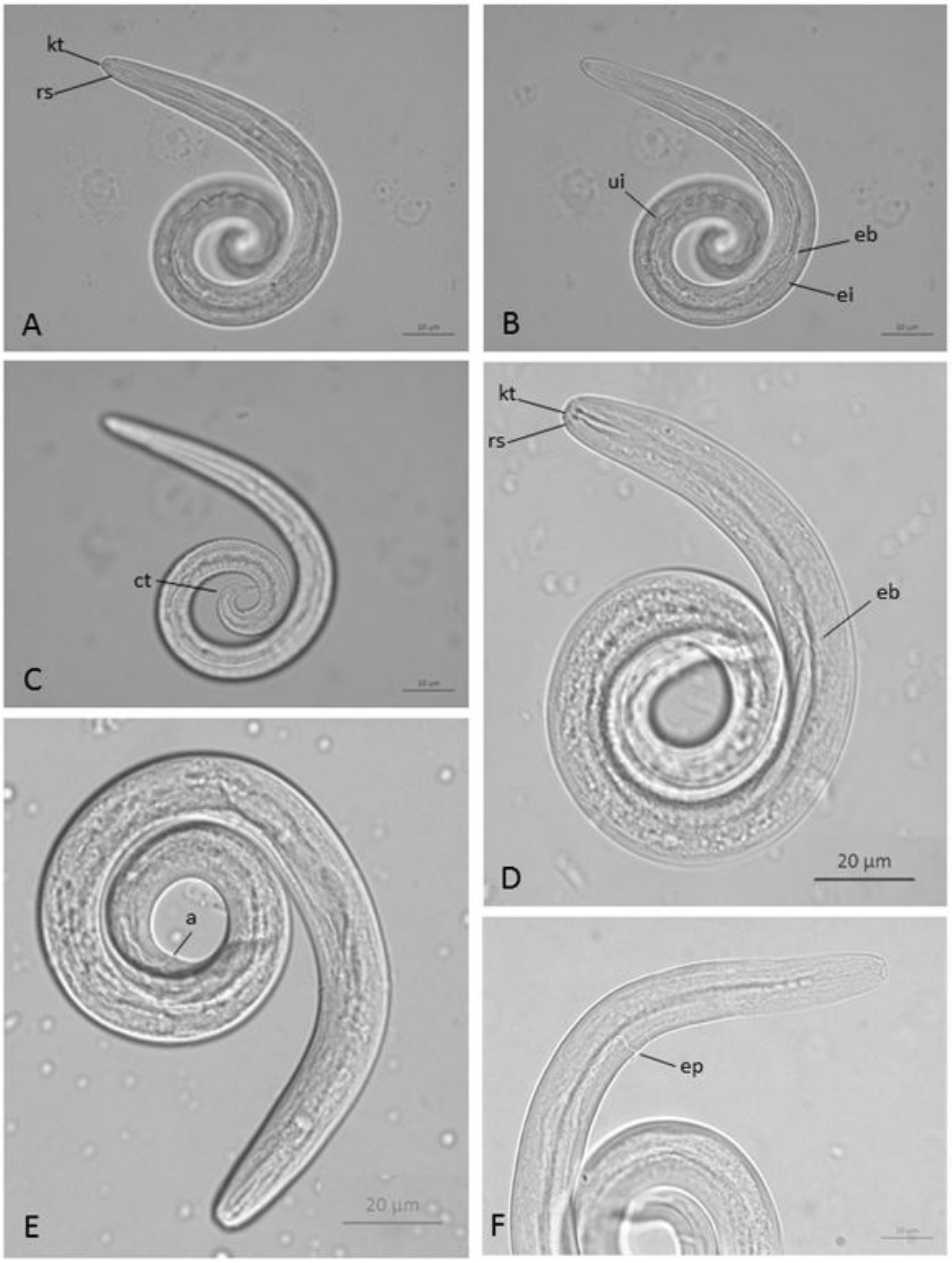

Figure 1. Larval stages of *Angiostrongylus cantonensis* recovered from rats (*Rattus novergicus*) experimentally infected with third-stage larvae (L3) obtained from *Bulimulus tenuissimus* experimentally infected forty-three days post infection. First-stage larvae (L1) in a general view; **A** – L1 larvae, obtained 43 days after infection, presenting the knob-like tips (kt) and rod-like structure (rs) at anterior end of the body; **B** – the esophagus bulb, expanded (ei) and unexpanded intestine (ui) regions. **C** – and the characteristics coiled tail (ct) exhibited during the larval movement. **D** – Third-stage (L3) larvae, obtained 14 days after infection, presenting the anterior end with expanded knob-like tips (kt), rod-like structure (rs) and the esophagus biulb (eb); **E** –the anus (a); and **F** – the excretory pore (ep).

cells, and many granuloma-like structures composed of hemocyte infiltration, but staining with Gomori's trichrome did revealed no collagen deposition or a small quantity of it in these structures around the larvae. Figure 2c, obtained from a snail in the same pre-patent period (one week), shows a section of a region containing part of cephalopedal mass, with a larval profile and similar characteristics observed in Figure 2b and e. The digestive gland is also visible in this figure. Figure 2d shows *B. tenuissimus* in the third week after exposure do *A. cantonensis*, presenting a larva in

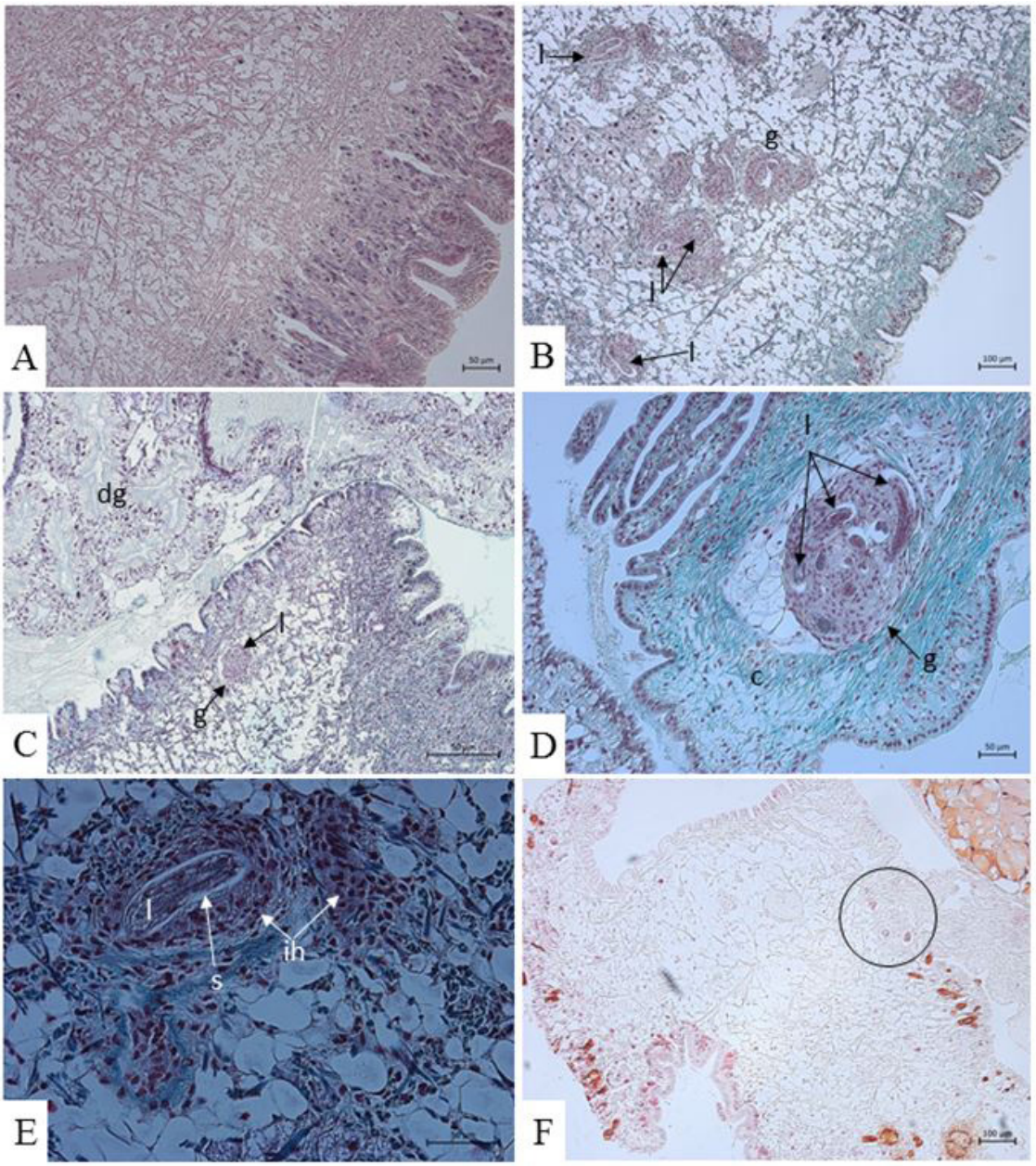

Figure 2. Histological section of the cephalopedal mass (foot) region of the *Bulimulus tenuissimus* uninfected and experimentally infected with *Angiostrongylus cantonensis*. **A** – Uninfected snail with normal morphology; **B - F** – infected snails. **B** and **C** – one-week post-exposure snails, presenting many granulomatous structures (g) containing developing larvae (l), note the increase of interstitial spaces among the muscle fibers in the tissue, and part of digestive gland (dg); **D** and **E** – three-week post-exposure snails, presenting a granuloma (g) with many larval profiles (l) and a greater amount of collagenous fibers in the tissue near the granuloma, and another larva in a longitudinal cross section showing part of the sheath (s) around the larval body and intense hemocyte infiltration (ih); **F** – A general view of the cephalopedal mass region, with granulomatous structures with larval profiles in them (circle) without calcium presence. **A –** hematoxylin-eosin; **B - E** – Gomori's trichrome; **F –** Von Kossa's technique.

longitudinal section where it is possible observe a sheath, which separates the larva from the capsule formed by the hemocyte infiltration. It is also possible to see a higher amount of collagenous material deposited around the granuloma region. The calcium histochemistry, by the von Kossa technique, did not reveal deposition of these ions around or in the granulomatous structure formed around the developing larvae (Figure 2f).

The MEV analysis of the cross sections allowed observing a developing larva in a longitudinal section immersed in muscular tissue of the cephalopedal mass (Figure 3a), with the presence of a capsule, probably originated from

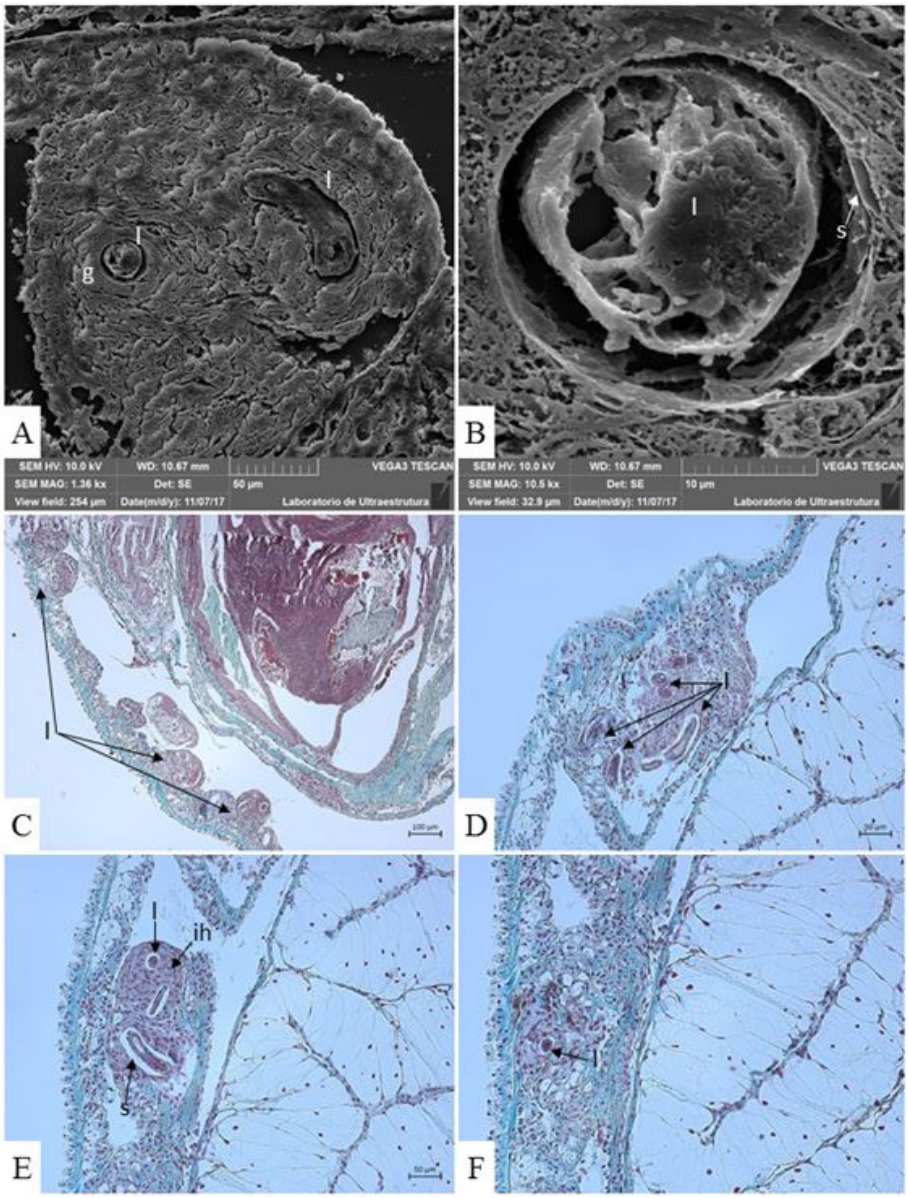

Figure 3. Cross section of *Bulimulus tenuissimus* experimentally infected with first-stage larvae of *Angiostrongylus cantonensis*. **A** and **B** – Cephalopedal mass tissue observed by scanning electron microscopy (SEM) showing the muscular fibrous tissue with the granulomatous (g) formation presenting larval profiles (l) in transversal and longitudinal sections, and a detailed view of the larval body (l) inside the granuloma, showing part of the sheath (s), which separates the larva from the snail tissue; **C** and **D** – mollusks in the first week after exposure, presenting a large amount of granulomatous structures in the mantle tissues with many larval (l) profiles in different planes of tissue sections; **E** and **F** – Snails in the third week after exposure, still evidencing a large number of granulomas with numerous larval (l) profiles in the mantle tissues, intense hemocyte infiltration (hi) and the presence of part of the sheath (s) around one developing larva. A-B: scanning electron microscopy; C-F: Gomori's trichrome.

host cells, surrounding the larval body, within which the larva is maintained (Figure 3b).

The mantle region (Figure 3c-f) presented a large number of developing larvae starting in the first week after exposure (Figure 3c). Also, in the granulomatous structure formed it is possible observe different larval profiles, indicating the formation of more than one developing larva, but the structures in the mantle do not have collagenous material deposited in or around them in a significant amount. The Figure 3e presents a sheath enveloping the developing larva inside the granuloma structure.

In Figure 4a, the digestive gland section of an uninfected snail reveals normal acinar organization of the epithelium. One week after exposure to *A. cantonensis* L1 larvae (Figure 2c, 4b and c), the acinar organization is lost, with a large amount of amorphous material in the acini, vacuolization of acinar cells, nuclei with irregular shape and location (not visible in some cells), and increase

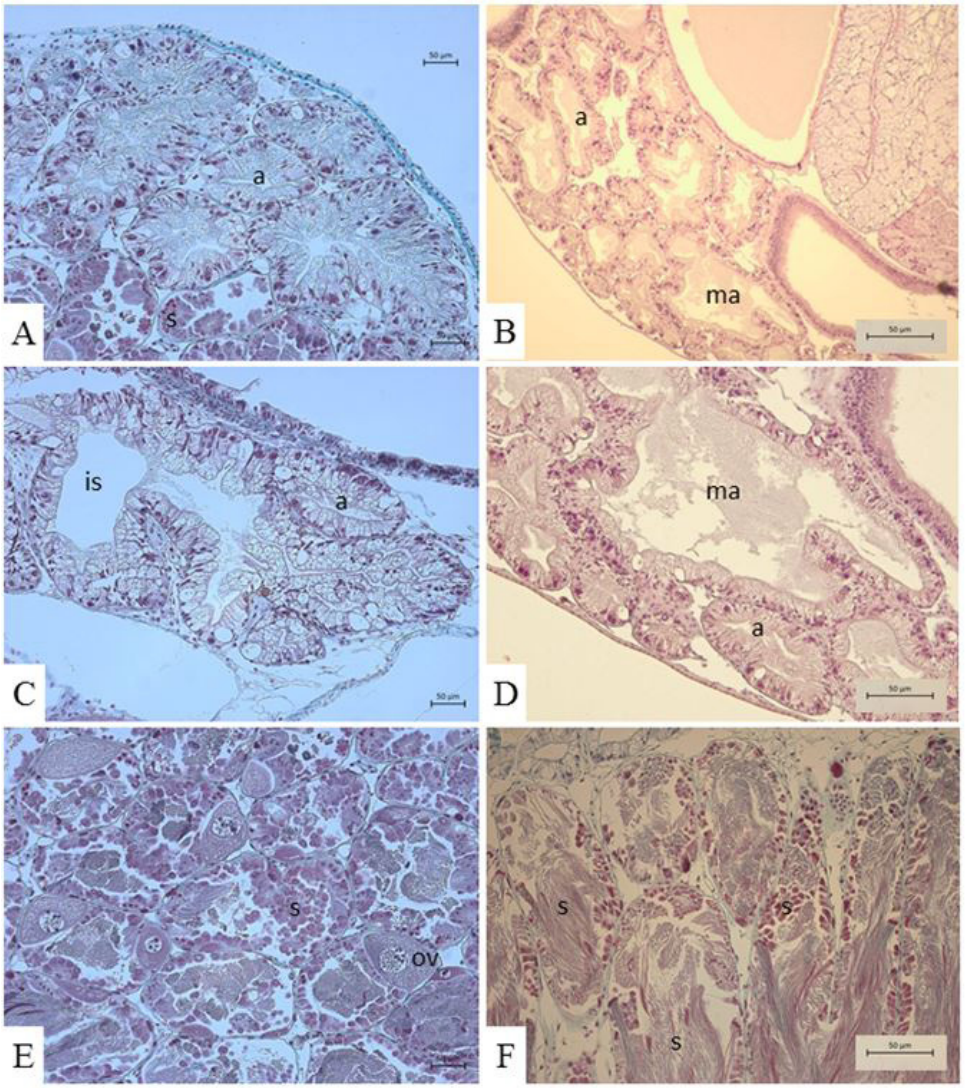

Figure 4. Histological section of the digestive gland and ovotestis tissues of *Bulimulus tenuissimus* uninfected and experimentally infected with *Angiostrongylus cantonensis*. **A** – Uninfected snail with normal morphology, with typical acinar organization and part of ovotestis with spermatozoa (s); **B - F** – infected snails. **B** and **C** – one-week post-exposure snails showing the loss of acinar (a) organization, with a large amount of amorphous material (ma) in the acini, vacuolization of acinar cells, the irregular nucleus (not visible in some cells), and increase of the inter-acinar space; **D –** Snails in the third week after exposure, in which histopathological changes observed in the early of prepatent period are still present; **E** – Gonadal tissues of an uninfected snail with normal organization and gametogenesis activity, indicated by the large number of spermatozoa and oocytes (ov) in different developmental stages; **F** – Ovotestis of infected snail (third week after exposure), presenting normal tissue organization, with many developing spermatozoa (s). **A –** Gomori's trichrome; **B - F –** hematoxylin-eosin.

of the inter-acinar space. In the third week post exposure (Figure 4d), the inter-acinar space seems is larger than in the first period analyzed, and the changes observed first remain visible in the acini along with the cellular organization.

The gonad of uninfected animals shows intense gametogenesis, with a significant number of oocytes and spermatozoa in different development stages (Figure 4a and e). During the larval development period analyzed (first to third weeks after exposure), the gamete production seems not to be affected, presenting male and female cells in different development stages (Figure 4f).

The feces examination showed the presence of L1 larvae forty three days after infection with L3 larvae (Figure 1a-c). The larvae harvested from fresh feces presented the same knob-like tips and rod-like structure in the anterior end previously observed in third-stage larvae (Figure 1d), the unexpanded and expanded gut regions (Figure 1b) and the coiled tail (Figure 1c). These morphological characteristics allow us identifying them as early-stage L1.

4. Discussion

The results obtained in the present study represent the first report of *B. tenuissimus* as an experimental intermediate host of *A. cantonensis*. The infection of the snail with L1 larvae of this nematode, with subsequent L3 recovery, undoubtedly demonstrates the role of this mollusk species as intermediate host of *A. cantonensis*. The infection rate noted here is lower than observed by Lima et al. (2016), who reported larval (L3) recoveries of 32% and 20% for *Biomphalaria straminea* and *Biomphalaria tenagophila*, respectively, experimentally infected with *A. cantonensis*. Also, our results shows the recovery of L3 viable and infectious from the second week post exposure onward, a prepatent period shorter than those recorded by Lima et al. (2016) and Kim et al. (2002), who reported the duration of 21 days to larval intramolluscan development of *A. cantonensis* in different species of snails host.

Richards and Merritt (1967) observed that *Biomphalaria glabrata* was experimentally infected by ingestion of infected rat feces, and first-stage larvae were observed penetrating the wall of the intestine, indicating a passive infection. The host snail presented a cellular reaction to developing larvae in tissue nodules.

Rachford (1976) observed that *Lymnaea palustris* infected with *A. cantonensis* larvae presented histopathological changes, characterized by mechanical damage to cells and nonspecific cellular responses to the larvae, with amebocytes (hemocytes), fibroblasts, and pigment cells accumulated around larvae, encapsulating them in nodules. The authors also observed that the intensity of the response was variable.

Harris and Cheng (1975) stated that the most common route of infection of *B. glabrata* experimentally exposed to first-stage larvae of *A. cantonensis* was via penetration of the gastric and prointestinal walls after ingestion, with migration through the kidney and rectal ridge to the mantle collar and head-foot of the host, where they become lodged. They also observed that the parasites were encapsulated in *B. glabrata* by 24-48 h post-infection. Encapsulation is a two-phase process involving: I - initial infiltration and aggregation of hemolymph cells around the parasite; and, II - posterior transformation of cellular aggregates into more fibrous-appearing nodules. The small number of parasites, which were found in tissues of the rectal ridge, kidney or vascular connective tissue near the gonad, were similarly encapsulated.

The results of these authors bear some similarities with our findings. Although some authors stated that the infection of snails by first-stage larvae of *A. costaricensis* can occur through an active pathway, by penetrating the snail tegument (Thiengo et al., 2005; Banevicius et al., 2006), the larval distribution in the infected snail suggested passive infection, where the L1 larvae, once ingested, penetrate through the intestinal wall of the mollusk. However, the first research group studied the relationship of *A. cantonensis* with *B. glabrata* or *L. palustris*, freshwater snails, and in the present study the snail host was a terrestrial gastropod.

Recognitionally, the vertebrate host of *A. cantonensis* is a terrestrial rodent, which leads us to infer that the life cycle of this parasite would be more likely to be completed if the invertebrate intermediate host was a terrestrial mollusk than if it were an aquatic mollusk. Despite the habits of the definitive vertebrate host, rodents, the greatest contribution in the literature about the relationship between snail host and larval *A. cantonensis* was made in studies using a freshwater snail species, mainly *B. glabrata* a freshwater snail species that was never found naturally infected with *A. cantonensis* (Tunholi-Alves et al., 2014a, 2014b). Puthiyakunnon and Chen (2015) cited 15 terrestrial species of mollusks, in a total number of 29 species, reported in the literature as intermediate hosts of *A. cantonensis*.

Why does this matter? Other researchers have cited fibroblast aggregation and transformation of cellular aggregates into a more fibrous structure around the encapsulating larvae, unlike observed in the present study. This may be explained by the fact that the tissues in freshwater snails are softer and contain more water than those terrestrial mollusks. For this reason, we found no intense transformation of cellular aggregates into fibrous material, as reported by Harris and Cheng (1975).

In addition, there were larvae in the tissues of the muscular cephalopedal mass, mantle and gonad (ovotestis), resulting in a wider distribution of the developing parasites in the snail than mentioned in previous studies with freshwater snails. Similar results were observed by Tunholi-Alves et al. (2011), who stated that the infection of *B. glabrata* with *A. cantonensis* larvae did not significantly change the gonadal tissues of the snails when compared to those from uninfected snails. In both, the structure of the ovotestis seemed to be preserved, where the process of gamete formation was evident, showing a functional structure of this organ.

Lv et al. (2009b), in a histological study of the development of *A. cantonensis* in the snail *Pomacea canaliculata*, failed to observe the presence of two sheaths for L3, only

observing one folded sheath. In our images, it was possible to observe only one sheath in the developing larvae in the snail tissues. Even in the electron microscopic images, it was not possible observe more than one sheath. As stated by Lv et al. (2009b) regarding the *A. cantonensis*/*P. canaliculata* interface, in the system analyzed in the present study, there was no other sheath adhered to the wall of the cavity where the larvae develop.

The L1 recovery from fresh feces of experimentally infected rats in the present study proved, in this way, that the larvae L3 of *A. cantonensis* obtained from larval development in *B. tenuissimus* are infectious and viable, being able to infect the definitive vertebrate host and continue its development until completing the entire evolutionary cycle, reaching the adult stage and sexual maturity. The duration of the prepatent period is in accordance to the literature; once that Mackerras and Sandars (1955) stated that prepatent period in the rat usually lies between 42 and 45 days.

In the present study, it was possible to observe the knob-like tips and rod-like structure in the anterior end. Lv et al. (2009b) did not cite before these structures in the description of the morphology of first-stage larvae obtained from *Pomacea canaliculata*. The observation of an intestine divided in an expanded and unexpanded region allow to identify these larvae as an early L1, according the same authors (Lv et al., 2009b).

The results of the master's dissertation of Ramos-de-Souza et al. (2018) have not been published yet and the content of the dissertation is not available for consultation. Only the abstract can be found. For this reason, the present study brings a large amount of new information about the experimental infection of *B. tenuissimus* with *A. cantonensis*, giving support to answer the initial question, indicating that this snail is a potential new intermediate host, at least under experimental conditions, for larval development of *A. cantonensis*.

By the first time, the role of *B. tenuissimus* as a possible experimental intermediate host is proved. Based on our results, and by comparing them to other reports in literature, we conclude that the mollusk *B. tenuissimus* can be used as intermediate host for larval development of *A. cantonensis* under experimental conditions The wide distribution of this snail along with the overlapping areas of occurrence of the snail and *A. cantonensis*, both in humans and mollusks, is a warning of the risk of maintenance of the life cycle of the parasite in a previously unknown intermediate host snail. This provides strong support of the infection, since it presents slight histological changes throughout the intramolluscan larval development period.

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