ORIGINAL ARTICLE

EVALUATION OF TECHNIQUES FOR RECOVERY OF *Angiostrongylus vasorum* FROM *Achatina fulica*, A POTENTIAL INTERMEDIATE HOST

Aytube Lucas Coaglio, Lanuze Rose Mozzer, Deborah Negrão Corrêa, Cíntia Aparecida de Jesus Pereira and Walter dos Santos Lima

ABSTRACT

Mollusks are intermediate hosts of *Angiostrongylus vasorum*, which is a parasite with zoonotic potential for which canids are the definitive host. In this study, 180 specimens of *Achatina fulica* that had been experimentally infected with 1,000 L_1 of *A. vasorum* were finely chopped to evaluate four tissue digestion techniques: 1% potassium hydroxide (group I); 3% hydrochloric acid (group II); 1% hydrochloric acid and pepsin (group III) with procedures at 37 ºC in a double boiler; and Baermann at 42 °C (group IV). On the first, eighth and thirtieth days after infection, L_1, L_2 and L_3 were obtained, and 240 L_3 recovered from group III were offered to a dog. The results were the following: group I: 59% L_1, 7% L_2, 13% L_3; group II: 31% L_1, 13% L_2, 27% L_3; group III: 23% L_1, 22% L_2, 30% L_3; and group IV: 0.3% L_1, 0.02% L_2, 5% L_3. Live larvae were observed in group III: 15.5% L_1, 8.22% L_3; group II: 9.8% L_3; and group IV: 100% L_1, L_2 and L_3. Larvae were detected 56 days after the dog became infected. All larvae stages of *A. vasorum* were recovered and identified through the techniques evaluated, thus confirming the potential of *A. fulica* as an intermediate host of the nematode.

KEY WORDS: *Achatina fulica*; *Angiostrongylus vasorum*; *Canis familiaris*; parasitology; snails.

RESUMO

Avaliação de técnicas para recuperação de *Angiostrongylus vasorum* em *Achatina fulica*, um potencial hospedeiro intermediário

Moluscos são hospedeiros intermediários de *Angiostrongylus vasorum*, parasito com potencial zoonótico que tem os canídeos como hospedeiro definitivo. Neste trabalho, foram seccionados os tecidos de 180 espécimes de *Achatina fulica* infectados com 1.000 L_1 de *A. vasorum*, os quais foram submetidos às seguintes técnicas: hidróxido de potássio 1% (grupo I), ácido clorídrico 3% (grupo II), ácido clorídrico e pepsina 1% (grupo III) em banho-maria a 37 °C e Baermann a 42 °C (grupo IV). No primeiro, oitavo e trigésimo dias após infecção (dpi), foram obtidos L_1, L_2 e L_3, e 240 L_3 recuperadas do grupo III foram fornecidas a um cão. Os resultados mostraram

Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Brazil

Corresponding author: Walter dos Santos Lima, Av. Presidente Antônio Carlos 6627, Zip Code 31270-901, Belo Horizonte, MG, Brazil. E-mail: wlima@icb.ufmg.br

no grupo I: 59% L_1, 7% L_2 e 13% L_3; no grupo II: 31% L_1, 13% L_2 e 27% L_3, no grupo III: 23% L_1, 22% L_2 e 30% L_3 e no grupo IV: 0,3% L_1, 0,02% L_2 e 5% L_3. Observaram-se larvas vivas no grupo III: 15,5% L_1 e 8,22% L_3; no grupo II: 9,8% L_3 e no grupo IV: 100% L_1, L_2 e L_3. No 56° dpi, encontrou-se L_1 nas fezes do cão. Pelas técnicas avaliadas, foram recuperados e identificados todos os estádios de *A. vasorum* intramolusco, confirmando o potencial de *A. fulica* como hospedeiro intermediário do nematoide.

DESCRITORES: *Achatina fulica*; *Angiostrongylus vasorum*; *Canis familiaris*; parasitologia; caramujos.

INTRODUCTION

About 18 species of *Angiostrongylus* Kamensky, 1905, have been described parasitizing domestic and sylvatic animals (Maldonado et al., 2012). *Angiostrongylus cantonensis* Chen, 1935, and *Angiostrongylus costaricensis* Morera & Céspedes, 1971, cause zoonoses such as eosinophilic meningitis and abdominal angiostrongyliasis, respectively. *Angiostrongylus vasorum* (Baillet, 1866) Kamensky, 1905, is a pathogenic parasite of domestic and sylvatic canids with worldwide distribution, including in Brazil (Caldeira et al., 2007; Guilhon & Cens, 1973; Lima et al., 1985; Morgan & Shaw, 2010).

Angiostrongylus vasorum is a heteroxenous nematode that parasitizes the right ventricle and the pulmonary arteries and their branches, where oviposition takes place. Its first-stage larvae (L_1) hatch in the alveoli, migrate up the bronchial tree and are swallowed and then excreted into the environment along with the host's feces. L_1 actively penetrate or are ingested by their intermediate hosts (which are gastropods), where they reach the third stage (L_3). This is the phase in which they infect canids (Guilhon & Afghahi, 1969; Willesen et al., 2008), and infection frequently leads to pneumonia, coughing, anemia and loss of capacity to run performance (Jones et al., 1980; Spratt, 2015).

Achatina fulica Bowdich, 1822, is a gastropod that is geographically widely distributed around the world (Fontanilla et al., 2014), including in Brazil (Albuquerque et al., 2008; Thiengo et al., 2007). It has been described as an intermediate host of nematodes of the families Metastrongylidae and Angiostrongylidae (Ohlweiler et al., 2010; Sauerländer & Eckert, 1974; Thiengo et al., 2010), and its habitats include localities where *A. vasorum* is known to occur (Duarte et al., 2007; Lima et al., 1985).

The most common technique for detecting infected mollusks in the environment is to use pure hydrochloric acid with pepsin (Graeff-Teixeira & Morera, 1995; Neuhauss et al., 2007; Wallace & Rosen, 1969). However, there is disagreement regarding the methodologies for diagnosing parasites in nematode-infected mollusks (Graeff-Teixeira & Morera, 1995; Laitano et al., 2001; Wallace & Rosen, 1969;). Over recent years, molecular techniques such as the polymerase chain reaction (PCR) (Qvarnstrom et al., 2007) and its

derivative, isothermal amplification of nucleic acids (LAMP) (Liu et al., 2011), have been used to identify larvae of *Angiostrongylus* spp. in mollusks.

In the present study, we evaluated the effectiveness of techniques for recovering the larval stages of *A. vasorum* from experimentally infected *A. fulica*, using hydrochloric acid with pepsin, hydrochloric acid alone, potassium hydroxide and the Baermann technique, with the aim of enabling morphological identification of the stages of the parasite. The feasibility of using L_3 from *A. fulica* to infect canids was also tested.

METHODS

Parasite

A. vasorum L_1 specimens were obtained from the cycle that is maintained in the Veterinary Helminthology Laboratory of the Federal University of Minas Gerais, with consecutive passages through snails (*Omalonyx matheroni* Potiez and Michaud, 1835) and dogs. This strain was first isolated from a dog in Caratinga, Minas Gerais, by Lima et al. (1985). The feces of infected dogs were collected and L_1 were recovered by means of the Baermann technique, as modified by Barçante et al. (2003a).

Infection of mollusks

A total of 180 specimens of *A. fulica*, with a mean shell length of 30 mm, were used in three independent experiments. All of the mollusks used in the experiments hatched in our laboratory. In each experiment, 60 specimens of *A. fulica* were used, divided into four groups of 15 mollusks each. These were individually infected with 1,000 L_1 of *A. vasorum* that had been obtained from dog feces by means of the Baermann technique. The L_1 were washed, quantified, resuspended in 1 mL of tap water and placed in a polystyrene container (4 cm x 5 cm). The exposed parts of the mollusks were then individually immersed in the suspension for 24 h. The container was sealed with gauze and adhesive tape. Afterwards, they were transferred to maintenance wells (9 cm x 26 cm x 17 cm) containing lettuce, water and autoclaved soil. The containers used in the infection process were analyzed in order to count the remaining L_1 and thus to ascertain the real infection rate among the mollusks (Pereira et al., 2006).

Techniques used for larval recovery of A. vasorum from A. fulica

Recovery of the *A. vasorum* larvae at the different stages, i.e. first-stage larvae (L_1), second-stage larvae (L_2) and third-stage larvae (L_3), was done on the 1st, 8th and 30th days after infection, respectively.

Four solutions were used to recover larvae. For group I, potassium hydroxide (KOH) was used at a concentration of 1% (1 mM), modified from Cheever (1968). For group II, hydrochloric acid (HCl) was used at a concentration of 3% (300 mM), as described by Lima (1998). For group III, digestion with pepsin was used at a concentration of 1% (VETEC, 100 units/mL), in a solution of HCl at a concentration of 1% (100 mM), modified from Wallace & Rosen (1969). For group IV, finely chopped tissues were subjected to the Baermann technique for 12 h, as described by Mozzer et al. (2011).

First, the mollusk shell was broken and the snail was removed individually and finely chopped into small pieces, with the aid of surgical scissors. These tissues were transferred individually to 50 mL tubes containing 25 mL of the solution to be tested, in a double boiler at 37 ºC. Every 15 minutes, the solution was manually homogenized until the tissues had become partially dissociated. While observing the partial dissociation of mollusk tissues, small fragments were mechanically macerated using a plastic pestle and a steel sieve of mesh size 1 mm, until the remainder of the tissue had been broken down. All of the content in the 50 mL tube and the remaining tissues that had been macerated using the sieve were centrifuged at 117g/5 min and after it, they were washed with tap water, this was repeated eight times. Thereafter, larvae of *A. vasorum* that had been obtained by means of the different techniques were identified in the sediment and were quantified using a stereoscopic microscope (25X), in accordance with the descriptions of Bessa et al. (2000) and Barçante et al. (2003b).

Infection of Canis familiaris

The viability of the L_3 of *A. vasorum* that were obtained from the experimentally infected *A. fulica* specimens was analyzed using larvae obtained from digestion with 1% HCL-pepsin (group III). A two-year-old male mongrel dog weighing four kilograms, which was free from helminth infections, was used. This dog became infected through oral ingestion of 240 L_3. Starting on the 30th day after infection, feces were collected daily and the modified Baermann technique was used until L_1 was detected. After determining the prepatent period, feces were collected every seven days. The experiment was approved by the Ethics Committee for Animal Research of the Federal University of Minas Gerais (CETEA/UFMG), under protocol number 147/2011.

Statistical analysis

Data on the recovery of larval stages were analyzed by means of the ANOVA variance test with one criterion, followed by the Tukey post-test. Differences of $p < 0.05$ were considered to be statistically significant.

RESULTS

In the containers used for infecting the mollusks, the mean proportion of L_1 that remained was 25%. Thus, the mean number of L_1 that penetrated the mollusks was 750 L_1.

The larval recovery of stages of *A. vasorum* in *A. fulica* in each group is shown in Figure 1. The time taken to achieve larval recovery varied according to the process used. For group I, one hour was needed for larval recovery, from which the mean numbers obtained were 446 ± 79.43 (59%) L_1, 54 ± 13 (7%) L_2 and 101 ± 31 (13%) L_3. For group II, the larvae were recovered in one hour and thirty minutes and the mean numbers obtained were 234 ± 65 (31%) L_1, 98 ± 14 (13%) L_2 and 203 ± 33 (27%) L_3. For group III, the recovery time was thirty minutes and the mean numbers recovered were 175.4 ± 26.42 (23%) L_1, 170.4 ± 43.26 (22%) L_2 and 231.4 ± 62.97 (30%) L_3. For group IV, the larvae were recovered in twelve hours and the mean numbers recovered were 2.4 ± 2.9 (0.3%) L1, 0.2 ± 0.3 (0.02%) L_2 and 34 ± 39 (5%) L_3. There were statistical differences ($p < 0.05$) between the recovery techniques.
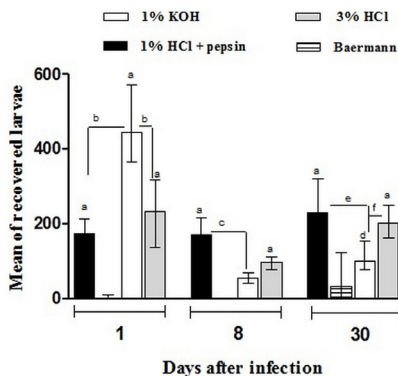

Figure 1. Larval recovery of *Angiostrongylus vasorum* from *Achatina fulica*. At each point, the letter (a) ($p< 0.001$) shows the difference between digestions: 1% HCL + pepsin, 1% KOH and 3% HCl, compared with Baermann; first day after infection (b) ($p< 0.001$), difference between 1% KOH and 1% HCl + pepsin, and 3% HCl; eighth day after infection (c) ($p< 0.001$), difference between 1% HCl + pepsin and 1% KOH; thirtieth day after infection (d) ($p< 0.05$), difference between 1% KOH and Baermann; (e) ($p< 0.001$), difference between 1% HCl + pepsin and 1% KOH; and (f) ($p< 0.01$), difference between 3% HCl and 1% KOH.

Through the techniques used, living larvae were recovered from group II, comprising 9.8% of L_3, and in group III, comprising 15.5% of L_1 and 8.22% of L_3 out of the total number of larvae recovered. For group IV, the technique does not use chemical products and, thus, all the larval stages were alive (Table).

Table. Mean number of live larval stages of *Angiostrongylus vasorum* recovered in *Achatina fulica* using different techniques

Group	Technique	Mean number of living larval stages		
		1st dpi (L1)	8th dpi (L1/L2)	30th dpi (L3)
I	1 %KOH	0	0/0	0
II	3 % HCl	0	0/0	20
III	1% HCl/pepsin	27	2/0	29
IV	Baermann	2	0.5/0.2	34

Living larvae in stage L_1 were recovered through the 1% HCl/pepsin and Baermann techniques, stage L_2 only through Baermann and stage L_3 through 1% HCl/pepsin, 3% HCl and Baermann (Figure 2).
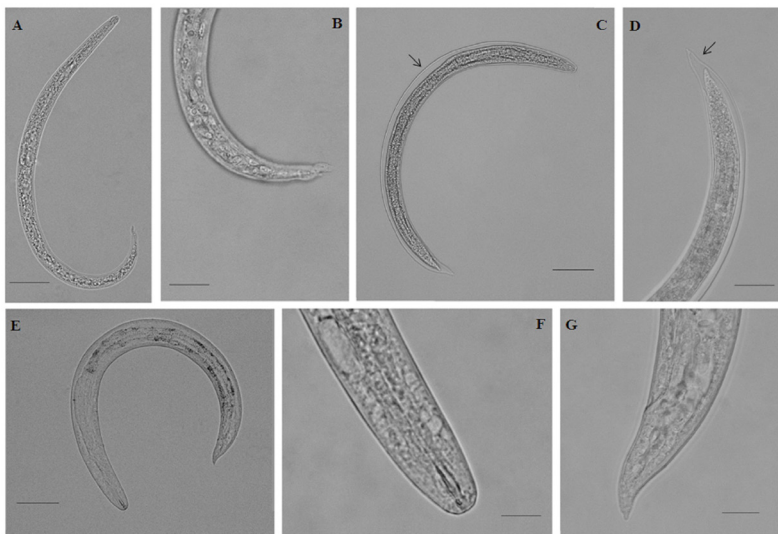

Figure 2. Live larvae of *Angiostrongylus vasorum* recovered by means of the techniques 1% HCl/pepsin, Baermann and 3% HCl. A. L_1 live recovery. B. The posterior end of the larva is sharply pointed and has a distinct notch on the dorsal surface. C. L_2 lies inside the cuticle of L_1 and intestines look dark brown because they are composed of large cells loaded with granules. D. The posterior end of the L_2 demonstrates the second cuticle. E. L_3 live recovery. F. L_3 with two rod-shaped structures in the anterior part. G. The tail tip of L_3 is pointed and has a distinct fingerlike structure. The arrow indicates the second cuticle. Bar: 50μm

Dog infection

   First-stage larvae were detected in the dog feces on the 56th day after infection. The peak elimination occurred on the 84th day, with a total of 131 L_1 per gram of feces.

   The quantity of L_1 found in feces varied among the collections, with peaks and troughs over the course of the 133 days of the experiment. However, throughout this period, there was no absence of larvae in the feces (Figure 3).
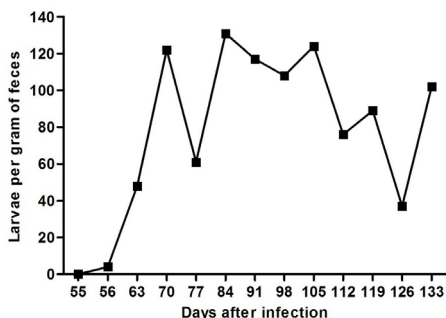

Figure 3. Number of larvae per gram of feces recovered from an experimentally infected dog infection with 240 L_3.

DISCUSSION

   Use of chemical digestion for nematode-larval recovery from naturally or experimentally infected mollusks has frequently been described (Wallace & Rosen, 1969; Graeff-Teixeira & Morera, 1995; Neuhauss et al., 2007; Thiengo et al., 2008; Moreira et al., 2013). When we used 1% HCl/pepsin, three larval stages of *A. vasorum* were recovered in half an hour, which was a shorter time than had previously been reported by other authors who used this technique at lower concentrations (Wallace & Rosen, 1969; Sauerländer & Eckert, 1974; Graeff-Teixeira & Morera, 1995; Neuhauss et al., 2007). In the present study, the larval recovery of *A. vasorum* was higher than that of Sauerländer & Eckert (1974), who infected *A. fulica* with *A. vasorum* and found an L_3 recovery rate of 4%. Neuhauss et al. (2007) used 0.7% HCl + 0.03% pepsin on *A. fulica* infected with *A. cantonensis* and obtained L_3 recovery of 0.5%. Therefore, the concentration used in the present study was shown to be a possible methodology for recovering nematodes from mollusks. Giannelli et al. (2014) recovered larval stages after 35 minutes, and the undigested tissue debris of *Helix aspersa* was removed from the study material. In contrast, in the present study, use of 1% HCL with 1% pepsin allowed recovery of the larval stages, with very little tissue debris, such that when the material was macerated through a sieve, it was easily diluted. This enabled recovery of larvae that may have been present in the tissue.

   From the technique using 3% HCl, 30% of the larval stages of *A.*

vasorum were recovered over a digestion period of one hour and 30 minutes. This was shorter than the time taken by Moreira et al. (2013) and Graeff-Teixeira & Morera (1995), who used 0.7% HCl and recovered larvae after 6 h and 24 h of digestion, respectively.

KOH has been used to recover *Schistosoma mansoni* eggs from rodent tissues (Cheever, 1968). In the present study, it was tested for the first time on mollusks, and it was found to be possible to recover and identify larval stages of *A. vasorum* in tissues from *A. fulica* using this technique. Although the larvae were not recovered alive, this technique may nonetheless be used to recover nematode larvae from mollusks.

The larval stages recovered by means of these techniques presented characteristics similar to those observed in other studies (Bessa et al., 2000; Barçante et al., 2003b; Mozzer et al., 2011).

Through the Baermann technique, the larval stages were recovered in smaller quantities than those obtained by Mozzer et al. (2011), who were able to recover 80% of the L_3 of *A. vasorum* from *O. matheroni*. One of the factors that may explain this high rate of larval recovery is the greater flaccidity of the tissue of this mollusk. This makes it more easily macerated than *A. fulica*. Therefore, studies on the immune response of infected mollusks need to be conducted, since other factors may interfere with the susceptibility to infection.

Other more sophisticated methods, such PCR assay and the LAMP method for specific detection, have been used to diagnose the parasite species found in mollusks (Qvarnstrom et al., 2007; Chen et al., 2011; Liu et al., 2011). These molecular techniques have high sensitivity and specificity, but have the disadvantage of requiring specific equipment. In comparing the results obtained from these molecular techniques with those from digestion methods, it can be seen that there are advantages in chemical digestion, given that this allows morphological identification, since it is easier to perform in study areas and in locations that have limitations on financial resources.

For the molecular techniques, it was necessary to find a minimum molecular amount, while for the proposed digestion techniques, it was possible to recover only one larva from the mollusk (data not shown). The chemical digestion showed the larval stages of the parasite, which would enable morphological studies.

In the present study, the samples were serially washed before being analyzed, which favored identification of the larval stages, whereas in Wallace & Rosen (1969), there was no washing and thus there was difficulty in viewing the larvae. The technique using 3% HCl produced larval recovery that was as good as that using 1% HCl/pepsin. Although the digestion time was three times longer, the use of only one reagent makes this technique an alternative. The Baermann technique has the advantage of enabling a high rate of recovery of live larvae.

Larvae were observed in the feces of the experimentally infected dog 56 days after infection. These results corroborate those of Bessa et al. (2000),

Barçante et al. (2003b), Mozzer et al. (2011) and Paula-Andrade (2012), who observed a prepatent period ranging from 28 to 108 days. Studies on infection of dogs with L_3 of *A. vasorum* coming from different mollusks (Bessa et al., 2000; Barçante et al., 2003; Pereira et al., 2006; Mozzer et al., 2011; Mozzer et al., 2014) have corroborated the idea that some nematodes show nonspecificity with regard to their intermediate host. Since the mollusk *A. fulica* is present in all Brazilian regions, the possibility that the biological cycle of these nematodes may have become established in this mollusk cannot be ruled out.

The mollusk *A. fulica* has been shown to have great capacity for maintaining the dispersion of outbreaks of *A. cantonensis* infection in Asia, while natural infection has been reported in Brazil (Moreira et al., 2013; Caldeira et al., 2007; Shan et al., 2009; Vitta et al., 2011). Thus, such events are a danger to public health in Brazil, given that a large diversity of nematodes of importance in human and veterinary medicines are present in this country, such as the parasites of the genus *Angiostrongylus*.

The present study corroborates the findings reported in the literature. It demonstrates the nonspecificity of *A. vasorum* towards intermediate hosts, given that the parasite developed in *A. fulica* and that stage L_3 recovered from these mollusks was able to induce canine angiostrongyliasis. Furthermore, through the techniques tested, it was possible to recover and identify the stages L_1, L_2 and L_3 of *A. vasorum*, thus suggesting that studies on nematodes in mollusks can be conducted using an accessible method.

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