Parasitol Res (2003) 89: 89–93
DOI 10.1007/s00436-002-0719-z

ORIGINAL PAPER

**J.M.P. Barçante · T.A. Barçante · S.R.C. Dias
L.Q. Vieira · W.S. Lima · D. Negrão-Corrêa**

A method to obtain axenic *Angiostrongylus vasorum* first-stage larvae from dog feces

Received: 22 April 2002 / Accepted: 10 July 2002 / Published online: 4 September 2002
© Springer-Verlag 2002

Abstract An improved method to obtain a large number of axenic larvae of *Angiostrongylus vasorum* from fecal samples was developed in the present study. The procedure here in reported consisted of obtaining larvae using a modified Baermann technique, followed by an additional filtration step. This isolation technique recovered almost 90% of the living larvae in a clean preparation. Isolated larvae were submitted to decontamination treatments with either sodium hypochlorite or antibiotic cocktail solutions. The axenic status, as confirmed by oral inoculation of decontaminated larvae into germ-free mice, was only achieved using larvae treated with 0.5% sodium hypochlorite solution for 10 min. The isolation and decontamination treatment did not affect larval viability. Treated larvae remained viable and infective to the invertebrate host.

Introduction

Angiostrongylus vasorum is a nematode protostrongylid that parasitizes domestic dogs (*Canis familiaris*) and wild canids such as *Cerdocyon thous*, *Dusicyon vetulus* and *Vulpes vulpes*. The infection is highly prevalent in dogs in the southeast of France, the United Kingdom, Ireland and Uganda (Dodd 1973; Guilhon and Cens 1973; Bwagamoi 1974). Sporadic cases have been reported in the United States, Canada and Brazil (Lima et al. 1985, 1994; Edwards 1995).

Adult worms of *A. vasorum* live in the right ventricle of the heart and the pulmonary artery, where sexual reproduction and oviposition take place. The first-stage larvae (L1) hatch into the alveoli, migrate up the bronchial tree, are swallowed and eliminated to the environment with the host feces. Infection frequently leads to pneumonia, loss of racing performance, coughing and anemia (Jones et al. 1980). Severely infected dogs may develop cardiac insufficiency, pulmonary fibrosis followed by weight loss, hemorrhagic diatheses and death (Dodd 1973; Lombard 1984).

Isolation of nematode larvae from infected sources, such as feces, represents a challenge, especially when the parasitic forms are collected for in vitro cultivation. The positive hydro-/thermotropism observed for live nematode larvae offers an opportunity for the first important separation step using the Baermann method (Morais 1948). However, the resulting preparation contains large amounts of bacteria, fungi and small debris that cannot be present when nematode larvae are to be used for in vitro cultivation, molecular biology studies or antigenic preparation.

Centrifugation on Percoll gradient or antibiotic treatment has been used to obtain clean samples of nematodes from blood (Chandrashekar et al. 1984) or feces (Graeff-Teixeira et al. 1999; Martins et al. 2000). However, these treatments are expensive and may reduce larval viability. In this work, we describe a fast and cheap procedure to obtain axenic live larvae of *A. vasorum* from infected dog feces, using filtration followed by incubation in a sodium hypochlorite solution without any additional antibiotic treatment.

J.M.P. Barçante · T.A. Barçante · S.R.C. Dias
W.S. Lima · D. Negrão-Corrêa (✉)
Departamento de Parasitologia,
Instituto de Ciências Biológicas,
Universidade Federal de Minas Gerais,
Avenida Presidente Antônio Carlos 6627,
Campus Pampulha, CEP 31270-901 Belo Horizonte,
Minas Gerais, Brazil
E-mail: denegrao@icb.ufmg.br
Tel.: +55-31-34992840
Fax: +55-31-34992970

L.Q. Vieira
Departamento de Bioquímica e Imunologia,
Instituto de Ciências Biológicas,
Universidade Federal de Minas Gerais,
Avenida Presidente Antônio Carlos 6627, Campus Pampulha,
CEP 31270-901 Belo Horizonte, Minas Gerais, Brazil

Materials and methods

Parasite

Angiostrongylus vasorum used in the present series of experiments was isolated from a domestic dog (Lima et al. 1985) and

maintained through serial infections of domestic dogs (*Canis familiaris*) and aquatic snails (*Biomphalaria glabrata*) in the Laboratory of Veterinary Helminthology (Department of Parasitology, Universidade Federal de Minas Gerais, Brazil).

Isolation of *A. vasorum* larvae from dog feces

Feces were collected from dogs infected with *A. vasorum* and the L1 were concentrated using a modified Baermann apparatus. Its modification consisted of a funnel directly connected to a hemolysis tube through a latex hose. Dog fecal samples (50–100 g) were wrapped in surgical gauze and placed on the top of the funnel filled with tap-water at 41 °C. After 12 h, the sediment containing L1 was recovered from the bottom of the hemolysis tube and washed three times with phosphate-buffered saline (PBS; 136 m*M* NaCl, 2 m*M* KCl, 8 m*M* Na_2HPO_4, 10 m*M* KH_2PO_4, pH 7.4) by centrifugation at 250 *g* for 5 min at 20 °C. The active and inactive L1 were counted under a stereomicroscope. *A. vasorum* larvae were resuspended at 1×10^3, 1×10^4, 1×10^5 and 1×10^6 active L1/ml and each larval suspension was placed in a 15-ml conical tube in a final volume of 10 ml. Each tube was covered with one layer of filter paper (S/P brand lens paper, Blaxter, USA), sealed with tape and inverted into a 50-ml conical tube containing PBS at 41 °C, in such a way that the filter paper was kept in contact with the warm PBS. After 6 h, the larvae that migrated through the filter paper were collected from the bottom of the 50-ml tube, washed twice with PBS and re-counted under a stereomicroscope.

In order to estimate the efficiency of this filtration procedure to separate live and dead larvae, tubes containing known proportions of live and dead larvae were covered with filter paper and inverted into warm PBS. The viability of the recovered larvae was estimated as described above.

Microorganism decontamination

Recovered L1 were washed three times with sterile PBS and then exposed in triplicate to four different decontamination procedures. Group 1: 1×10^5 L1 directly recovered from the modified Baermann apparatus, not submitted to filtration or any previous treatment, were incubated for 10 min with sterile PBS. Group 2: 1×10^5 filtered L1 were incubated for 10 min with 10 ml of sterile PBS. Group 3: 1×10^5 filtered L1 were incubated for 10 min with 10 ml of sterile PBS containing a combination of 4 mg gentamicin sulfate/ml (Ariston, São Paulo, SP, Brazil), 1,000 units penicillin/ml (Fort Dodge, Campinas, SP, Brazil) and 500 µg streptomycin/ml (Fort Dodge). Group 4: 1×10^5 filtered L1 were incubated for 10 min with 10 ml of sterile PBS containing 0.5% (v/v) sodium hypochlorite (stock solution containing 10% sodium hypochlorite; Clorosul, Gravataí, RS, Brazil).

After incubation, larvae from each group were washed twice in sterile PBS (250 *g* for 5 min at 20 °C) and tested for viability, infectivity to the intermediate host and microorganism growth.

Viability and infectivity

The viability of larvae from different decontamination treatments was determined by direct observation of L1 movement using a stereomicroscope. Under gentle heating, larvae in movement were classified as active, while immobile larvae in a "C" position were classified as inactive (Richinitti et al. 1999).

To confirm larvae infectivity after decontamination procedure, *A. vasorum* L1 from each treatment group were used to infect snails. Ten *B. glabrata* snails, measuring 10–15 mm in diameter, were individually exposed for 24 h to 2 ml of dechlorinated water containing 1×10^3 L1 of *A. vasorum* from each treatment group. Thirty days after infection, the third-stage larvae were recovered, as described by Ribeiro and Lima (2001), and counted under a stereomicroscope.

Axenic status

Larvae recovered from each decontamination treatment were placed (in triplicate) in thioglycollate medium (Biobrás, Montes Claros, MG, Brazil) and brain–heart infusion broth (BHI; Biobrás) at 37 °C, in RPMI 1640 medium (Gibco BRL Laboratories, Grand Island, N.Y., USA) at 37 °C, in a humidified atmosphere containing 5% CO_2, and in RPMI 1640 medium supplemented with 50 mM Hepes (Sigma Chemical Co., St. Louis, Mo., USA), 100 units penicillin/ml, 100 µg streptomycin/ml at 37 °C, in a humidified atmosphere containing 5% CO_2. Detectable microorganism growth in each culture medium containing larvae was observed for up to 7 days.

Larvae recovered from treatments that did not show any detectable contamination in any culture medium were tested for axenic status in germ-free mice. The germ-free mice are better than conventional liquid media for microorganism growth, allowing the growth of very demanding organisms and also being used to detect the very small numbers of microorganisms that might still be present after the larval decontamination procedure. Five 50-day-old female Swiss/NIH mice (Laboratory of Gnotobiology, Department of Biochemistry and Immunology, UFMG, Brazil) were orally inoculated with 1×10^5 decontaminated *A. vasorum* L1 larvae. Experiments with gnotobiotic mice were performed in micro-isolators (UNO Roestvaststaal, Zevenaar, The Netherlands) and handled according to established procedures (Pleasants 1974). Water and autoclavable diet (Nuvital, Curitiba, PA, Brazil) were steam-sterilized and administered *ad libitum*. To confirm the maintenance of the germ-free status of the mice, fecal samples were collected from the inoculated mice 7 days after inoculation and cultured as described above.

Statistical analysis

Data on larval recovery, larval viability and infectivity are reported as means ± standard deviation (SD) and analyzed by the means of one-way analysis of variance (ANOVA) followed by post-hoc analysis through Tukey's multiple comparison test. Differences of $P < 0.05$ were considered statistically significant.

Results

Isolation of *Angiostrongylus vasorum* larvae from dog feces

The isolation of *Angiostrongylus vasorum* larvae using a modified Baermann apparatus followed by a filter paper-filtration procedure allowed the recovery of almost 90% of the active larvae. The filtration step allowed the separation of heavy and light debris, normally observed in fecal suspension, from the living nematode larvae. Different loads of L1 recovered after concentration by the modified Baermann method were used for the filtration procedure. Although no statistical differences were found in the percentage of recovered larvae ($F = 2.918$, $P = 0.1004$), there was a tendency towards a smaller recovery when large number of larvae were used. However, for an initial concentration of 1×10^6 L1, it was possible to recover a high percentage (84%) of living larvae after filtration (Table 1).

Another important improvement that the filtration step added to the isolation of larvae by the modified Baermann method was its capacity to separate dead larvae from the living ones. From an initial larvae

Table 1 Number of active *Angiostrongylus vasorum* first-stage larvae (L1) recovered from different initial larval concentrations (10^3– 10^6 larvae/ml). Experiments were done in triplicate and no statistical difference between the numbers of recovered L1 was observed ($P > 0.05$)

Active L1 load	L1 after filtration (mean ± SD)	Recovery (%)
1×10^3/ml	$(0.94 \pm 0.036)\times10^3$	94
1×10^4/ml	$(0.86 \pm 0.069)\times10^4$	86
1×10^5/ml	$(0.87 \pm 0.031)\times10^5$	87
1×10^6/ml	$(0.84 \pm 0.059)\times10^6$	84

solution containing 17–25% of dead parasite, the filtration procedure resulted in a significant enrichment of the living larvae, with only 3% of dead larvae found in the final filtrate.

Viability and infectivity

The decontamination treatments tested did not influence nematode larval viability. As shown in Table 2, all treatments allowed the recovery of over 90% of active larvae and statistical analysis showed no significant difference ($P > 0.05$) in larvae viability between treatments ($F = 1.023$, $P = 0.4324$).

The infective capability of L1 larvae after the different decontamination treatments, evaluated by the number of L3 larvae recovered from 30 day-infected *Biomphalaria glabrata*, was similar for all treatments. The data show that larvae submitted to each procedure were still viable and capable of infecting the intermediate host (Fig. 1). Statistical analysis (ANOVA followed by Tukey's post-hoc test) confirmed that there was no significant difference ($P > 0.05$) between the number of L3 recovered from *B. glabrata* infected with L1 obtained after the decontamination treatment with sodium hypochlorite, compared with the control groups (group 1 vs group 4, group 2 vs group 4). In contrast, antibiotic decontamination treatment of L1 (group 3) resulted in a significant reduction of L3 recovered from infected snails, compared with the only filtered-treated L1 (group 2 vs group 3; $P < 0.05$), suggesting that antibiotic treatment may interfere with larvae infectivity. However, there was no statistical difference in infectivity

Table 2 Viability of *A. vasorum* L[1] after different decontaminating treatments. *Group 1* L1 without any treatment (control). *Group 2* L1 filtered and washed with PBS. *Group 3* L1 filtered and treated with antibiotic cocktail. *Group 4* L1 filtered and treated with 0.5% sodium hypochlorite. The *Active L1* value is the mean ± SD recovered after decontaminating treatments (done in triplicate). Viable and active larvae showed movement under heat stimulation, while dead ones were immobile and in a "C" position. There was no statistical difference between the groups ($P < 0.05$)

Treatments	Active L1	Mean (%)
Group 1	$(0.95 \pm 0.03)\times10^5$	95
Group 2	$(0.96 \pm 0.01)\times10^5$	96
Group 3	$(0.93 \pm 0.02)\times10^5$	93
Group 4	$(0.93 \pm 0.04)\times10^5$	93

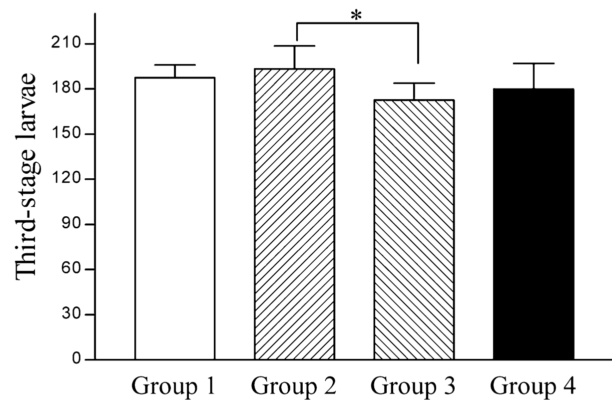

Fig. 1 Mean recovery of third-stage *Angiostrongylus vasorum* larvae from *Biomphalaria glabrata* snails 30 days after infection with 1×10^3 first-stage larvae (L1) of *A. vasorum* obtained from each of the following decontamination treatments. *Group 1* L1 directly recovered by modified Baermann technique and without any treatment (control). *Group 2* L1 filtered and washed with sterile PBS. *Group 3* L1 filtered and treated with an antibiotic cocktail (4 mg gentamicin sulfate/ml, 1,000 units penicillin/ml, 500 µg streptomycin/ml) for 10 min. *Group 4* L1 filtered and treated with 0.5% sodium hypochlorite for 10 min. Each *column* represents the mean ± SD of L3 recovered from ten infected snails (experiments repeated three times with similar results). The *asterisk* indicates a significant statistical difference ($P < 0.05$)

between antibiotic-treated larvae and non-treated larvae (group 3 vs group 1; $P > 0.05$).

Microorganism decontamination

As shown in Table 3, intense microorganism growth was observed as early as 6 h, when larvae recovered directly by the modified Baermann method were washed and added, without decontamination treatment, to different culture media (group 1). Larvae recovered after filtration step (group 2) or larvae filtered and treated with an antibiotic cocktail solution (group 3) did not show microorganism growth in RPMI 1640 medium supplemented with antibiotics. However, microorganism growth was observed when larvae from groups 2 or 3 were cultured in media without addition of antibiotics. The absence of detectable microorganism growth in all culture media was only achieved with *A. vasorum* larvae filtered and incubated with 0.5% sodium hypochlorite solution (group 4 in Table 3).

Oral inoculation of the sodium hypochlorite-treated larvae in germ-free mice confirmed the axenic status of these larvae. Feces collected from germ-free mice 7 days after larval inoculation showed no microorganism growth in any culture media tested for up 7 days (168 h) of incubation (Table 3).

Discussion

The isolation of parasite forms from host tissues or fecal samples in a clean and pure preparation that allows

Table 3 Detectable microorganism growth in culture media inoculated with *A. vasorum* larvae subjected to different decontamination treatments. Decontaminated larvae or feces recovered from germ-free mice inoculated with sodium hypochlorite-treated larvae were cultured for 6, 24 and 168 h in these media: thioglycollate, brain–heart infusion (*BHI*) broth, RPMI 1640 medium and supplemented RPMI 1640 medium. *Feces from germ-free mice* were obtained 7 days after inoculation with L1 treated with 0.5% sodium hypochlorite. + microorganism growth, – absence of microorganism growth

Treatment	Thioglycollate			BHI			RPMI 1640			Supplemented RPMI 1640		
	6 h	12 h	168 h	6 h	12 h	168 h	6 h	12 h	168 h	6 h	12 h	168 h
Group 1 (no treatment)	+	+	+	+	+	+	–	+	+	–	+	+
Group 2 (filtration + PBS)	–	+	+	–	+	+	–	+	+	–	–	–
Group 3 (filtration + antibiotics)	–	+	+	–	+	+	–	+	+	–	–	–
Group 4 (filtration + hypochlorite)	–	–	–	–	–	–	–	–	–	–	–	–
Feces from germ-free mice	–	–	–	–	–	–	–	–	–	–	–	–

excretory/secretory antigen production or nucleic acid extraction is not an easy task (Dalton et al. 1997). The isolation of larvae from infected sources like feces represents a challenge, especially when the parasitic forms are collected for in vitro cultivation.

Some nematodes have been successfully decontaminated and cultured in different media, although only a few reports have been published concerning the in vitro cultivation of parasites that belong to the genus *Angiostrongylus* (Hata 1996). In most studies, the decontamination of worms was achieved by incubation with several antibiotics or by gradient centrifugation, that may interfere with larval viability (Hata and Kojima 1991; Hata 1993, 1994, 1996; Graeff-Teixeira et al. 1999; Martins et al. 2000). Furthermore, no confirmation of axenization has been demonstrated in a gnotobiotic model, except by Martins et al. (2000). The authors demonstrated by inoculation of germ-free mice that axenization of *Strongyloides venezuelensis* third-stage larvae is possible by the use of a 0.25% sodium hypochlorite wash, immediately followed by an antibiotic cocktail treatment for at least 30 min.

The bactericidal and disinfectant properties of sodium hypochlorite have previously been demonstrated. In areas with cholera transmission, 1.5% bleach solution was recommended as a disinfectant for vegetable and fruits (Zenebon et al. 1994). Hidalgo et al. (2002), testing a wide range of hypochlorite concentrations (0.00025–0.5%) for antibacterial effect in human fibroblast culture, showed that 0.025–0.25% solutions produced partial antimicrobial activity. Total bactericidal effects of sodium hypochlorite, without fibroblast damage, was observed only for incubation with 0.5% sodium hypochlorite solution.

In this work, we were able to obtain pure, active and viable nematode larvae isolated from dog feces, without any antibiotic treatment. Nematode larval isolation is generally obtained using the Baermann method. However, the resulting preparation contains large numbers of bacteria and debris, which are an important source of contamination of culture media, antigenic preparations, and nucleic acid extracts. In this work, the additional filtration step, followed by larval incubation in a 0.5% sodium hypochlorite solution resulted in an important, improved method for obtaining a large number of pure larvae of *A. vasorum* from fecal samples. Cultivation in BHI, thioglycollate medium and RPMI 1640 did not result in bacterial growth. Moreover, inoculation of germ-free mice with the larvae did not result in detectable growth of microorganisms in the gut. The gut of a germ-free mouse is a richer culture medium than conventional culture media; and these animals are often used as sentinels of a germ-free environment in gnotobiology laboratories (Ducluzeau and Raibaud 1979). It was expected that, if there were low levels of contami nants that survived the passages in poor culture media, these microorganisms would grow in the germ-free gut. Therefore, the larval decontamination obtained after the filtration step followed by sodium hypochlorite treatment resulted in larval axenization.

The isolation and decontamination treatment with sodium hypochlorite did not affect larval viability and infectivity, although the lethal effect of sodium hypochlorite on several parasite forms was reported when a higher concentration was used. Grubb and Oliver-González (1969) demonstrated the inactivation of eggs of *Ascaris* sp. and cysts of *Entamoeba histolytica* with 1.5% sodium hypochlorite. Moreover, Zanini and Graeff-Teixeira (2001) demonstrated the inactivation of infective larvae of *A. costaricensis* with 1 h of incubation in 1.5% bleach solution. As demonstrated in this work, 0.5% sodium hypochlorite is an ideal concentration to achieve, in a short period of incubation, an axenic status of the larvae without affecting viability and infectivity.

The procedure hereby reported is a low-cost and efficient method for the separation and axenization of larvae from dog fecal debris. Moreover, it is an improvement over the modified Baermann method for the isolation and purification of different nematode larvae for molecular and immunological purposes.

Acknowledgements The work was partially supported by CAPES and CNPq, Brazil: J.M.P.B. and T.A.B. are recipients of CAPES fellowships, S.R.C.D. is the recipient of a CNPq fellowship and L.Q.V., W.S.L. and D.N.-C. are recipients of CNPq PQ fellowships. The authors wish to thank Dr. Paulo Marcos Zech Coelho and the technical staff of the Schistosomiasis Laboratory (GIDE-UFMG) for supplying the *Biomphalaria* snails used in the experiments. The authors are grateful to Dr. Jacques Nicoli and

Dr. Roger Orcutt for useful discussions. The authors also thank Edna Maia and Andrea Maçal for useful suggestions and technical advice.

References

Bwagamoi O (1974) Renal lymphoid and pulmonary lesions in naturally acquired canine angiostrongylosis in Uganda. Bull Epizoot Dis Afr 22:55–68

Chandrashekar H, Rao UR, Rajsekariah GR, Subrahmanyam D (1984) Separation of viable microfilariae free of blood cells on Percoll gradients. J Helminthol 58:69–70

Dalton JP, Day SR, Drew AC, Brindley PJ (1997) A method for the isolation of shistosome eggs and miracidia free of contaminant in host tissues. Parasitology 115:29–32

Dodd K (1973) *Angiostrongylus vasorum* (Baillet, 1866) infestation in a greyhound kennels. Vet Rec 92:195–197

Ducluzeau R, Raibaud P (1979) Ecologie microbienne du tube digestif. Masson, Paris

Edwards NJ (1995) ECG manual for the veterinary technician. Sauders, Philadelphia

Graeff-Teixeira C, Geiger S, Walderich B, Hoffmann W, Abrahams E, Schulz-Key H (1999) Isolation of *Angiostrongylus costaricensis* larvae from rodent feces on a Percoll gradient. J Parasitol 85:1170–1171

Grubb TC, Oliver-González J (1969) The effect of sodium hypochlorite on *Ascaris* eggs and cysts of *Entamoeba histolytica*. Rev Inst Med Trop Sao Paulo 32:359–363

Guilhon J, Cens B (1973) *Angiostrongylus vasorum* (Baillet, 1866): Étude biologique et morphologique. Ann Parasitol Hum Comp 48:567–569

Hata H (1993) In vitro cultivation of the third-stage larvae of *Angiostrongylus cantonensis*. J Vet Med Sci 55:345–348

Hata H (1994) Essential amino acids and other essential components for the development of *Angiostrongylus costaricensis* from third-stage to young adults. J Parasitol 80:518–520

Hata H (1996) In vitro cultivation of *Angiostrongylus costaricensis* eggs to first-stage larvae in chemically defined medium. Int J Parasitol 26:281–286

Hata H, Kojima S (1991) *Angiostrongylus costaricensis*: culture of third-stage larvae to young adults in a defined medium. Exp Parasitol 73:354–361

Hidalgo E, Bartolome R, Dominguez C (2002) Cytotoxicity mechanisms of sodium hypochlorite in cultured human dermal fibroblasts and its bactericidal effectiveness. Chem Biol Interact 139:265–282

Jones GW, Neal C, Turner GRJ (1980) *Angiostrongylus vasorum* infection in dogs in Cornwall. Vet Rec 26:83

Lima WS, Costa HMA, Guimarães MP, Leite ACR (1985) *Angiostrongylus vasorum* (Baillet, 1866) Nematoda: Prothostrongylidae em cães de Minas Gerais, Brasil. Mem Inst Oswaldo Cruz 80:233–235

Lima WS, Guimarães MP, Lemos IS (1994) Occurrence of *Angiostrongylus vasorum* in the lungs of Brazilian fox *Dusicyon vetulus*. J Helminthol 68:87

Lombard CW (1984) Normal values of canine M-mode echocardiogram. Am J Vet Res 45:2015–2018

Martins WA, Melo AL, Nicoli JR, Cara DC, Carvalho MAR, Lana MA, Vieira EC, Farias LM (2000) A method of decontaminating *Strongyloides venezuelensis* larvae for the study of strongyloidiasis in germ-free and conventional mice. J Med Microbiol 49:387–390

Morais RG (1948) Contribuição para o estudo do *Strongyloides stercoralis* e da angiostrongiloidíase no Brasil. Rev Saude Publica 1:507–524

Pleasants JR (1974) Gnotobiotics. In: Melby EC, Altman NH (eds) Handbook of laboratory animal science. CRC Press, Cleveland, pp 119–174

Ribeiro VM, Lima WS (2001) Larval production of cats infected and re-infected with *Aelurostrongylus abstrusus* (Nematoda: Protostrongylidae). Revue Med Vet 152:815–820

Richinitti LMZ, Fonseca NA, Graeff-Teixeira C (1999) The effect of temperature on mobility of *Angiostrongylus costaricensis* third-stage larvae. Inst Med Trop Sao Paulo 41:225–228

Zanini GM, Graeff-Teixeira C (2001) Inactivation of infective larvae of *Angiostrongylus costaricensis* with short time incubations in 1.5% bleach solution, vinegar or saturated cooking salt solution. Acta Trop 78:17–21

Zenebon O, Duarte-de-Maio F, Sakuna AM, Rosa E (1994) Determinação de mercúrio em águas sanitárias comercializadas em região do sudeste do Brasil. Rev Saude Publica 28: 127–130