Angiostrongylus costaricensis and the intermediate hosts: observations on elimination of L3 in the mucus and inoculation of L1 through the tegument of mollucs

Angiostrongylus costaricensis e hospedeiros intermediários: observação da eliminação de larvas de 3º estágio (L3) no muco e inoculação de L1 na cavidade tegumentar dos moluscos

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Abstract *Human accidental infection with* Angiostrongylus costaricensis *may result in abdominal disease of varied severity. Slugs from the Veronicellidae family are the main intermediate hosts for this parasitic nematode of rodents.* Phyllocaulis variegatus*,* Phyllocaulis soleiformis *and* Phyllocaulis boraceiensis *were experimentally infected to describe the kinetics of L3 elimination in the mucus secretions of those veronicelid species. A maximum of 2 L3/g/day was found in the mucus, while the number of L3 isolated from the fibromuscular tissues varied from 14 to 448. Productive infection was established by inoculations in the hyponotum or in the body cavity, through the tegument. Intra-cavity injection is a less complex procedure and permits a better control of inocula. A preliminary trial to titrate the infective dosis for* P. variegatus *indicated that inocula should range between 1000 and 5000 L1. The data also confirmed the importance of* P. variegatus *as an intermediate host of* A. costaricensis.

Key-words: Angiostrongylus costaricensis. *Veronicellidae. Angiostrongylosis. Host-parasite coevolution.*

Resumo *A infecção acidental humana por* Angiostrongylus costaricensis *pode resultar em doença abdominal de variada gravidade. Veronicelídeos são os principais moluscos hospedeiros intermediários do* Angiostrongylus costaricensis*, nematódeo parasita de roedores. Foi comparada a cinética de eliminação de larvas de terceiro estágio (L3) no muco através da infecção experimental de* Phyllocaulis variegatus*,* P. soleiformis *e* P. boraceiensis*. Um máximo de 2 L3/g/dia foi observado no muco, enquanto o número de larvas isoladas dos tecidos fibromusculares variou 14 e 448. A injeção das larvas no hiponoto ou na cavidade tegumentar estabeleceu infecção produtiva. A via intra-cavitária permite melhor controle de inóculo e envolve procedimento mais simples. Titulação preliminar da dose infectante para* P. variegatus *sugere que os inóculos devem ficar entre 1000 e 5000 L1. Os dados também reforçam a importância de* P. variegatus *como hospedeiro intermediário do* A. costaricensis.

Palavras-chave: Angiostrongylus costaricensis. *Veronicellidae. Angiostrongilose coevolução.*

Angiostrongylus costaricensis is a parasitic intra-arterial nematode that choose veronicelid molluscs as their main intermediate hosts[10]. This metastrongylid worm may produce human abdominal disease with a widespread ocurrance in the Americas, from the southern United States to northern Argentina and southern Brazil[3] [12]. Veronicellid slugs may also represent an agricultural pest as has been documented in Central America[7].

Although the levels of susceptibility and of natural infection have been reported for several species of terrestrial molluscs[5] [8] there are few descriptions of *A. costaricensis*-mollusc's coevolutive status as it is presented in this study. Here we comparatively evaluate the intensity of the *A. costaricensis* infection in three species of the genus *Phyllocaulis* Colosi, 1922. We also show the results of infections by inoculation of first stage larvae (L1) into the molluscan body cavity, via injections through the tegument.

MATERIAL AND METHODS

The strain "Santa Rosa" of *Angiostrongylus costaricensis* has been mantained in the laboratory since 1992, in albino Swiss mice and *Phyllocaulis soleiformis* (D'Orbigny, 1835). L1 were isolated using the Baermann method and then the average number per ml was estimated after counting them in 3 x 0.1ml aliquots of the suspension.

Slices of chayote (*Sechium edule* L., 1758) contaminated with 1.500 L1 were offered individually and *ad libitum* to laboratory reared and starving slugs: five specimens of *Phyllocaulis variegatus* (Semper, 1885) and *P. soleiformis*, and six

specimens of *P. boraceiensis* (Thomé, 1972). 28 days post-infection (PI) three samples of aproximately 1g of mucus were collected daily by scraping the notum or hyponotum with a glass slide. On the 5th day PI, molluscs were killed and their fibro-muscular tissues were digested for larvae quantification[4] [15].

For intra-tegument injections, 50 L1 were suspended in 0.2ml of sterile 0.6% NaCl solution and inoculated in groups of 3 specimens of *P. variegatus*, using a 1ml syringe with 13 x 3.8mm gauge needle. Groups of 3 slugs were injected only with saline. The syringes were laid in a vertical position for at least 5 minutes before injection, to concentrate the L1 into the needle's tip. The puncture into the body's cavitiy or the fibromuscular tissue of the medial notum was perfomed at a 30° angle in an antero-posterior orientation, in the posterior half of the animal. The syringe was immediately rinsed with 0.6% NaCl solution, in order to count the larvae remaining after injection. The actual inoculum was estimated by subtracting the number of larvae left in the syringe from the initial inoculum. Production of L3 was expressed as a ratio of the number of larvae to the weight of individual slugs (L3/g) or by "yield of L3" (total L3/ actual inoculum).

Groups of 3 slugs of *P. variegatus* and *P. soleiform* received intra-cavity injections of 50, 100, 500, 1000, 5000 and 10000 L1 per animal, for a preliminary titration of inocula.

RESULTS

Less than 2 L3/g were esporadically eliminated in the mucus (Table 1). The three

Table 1 - Number of third stage larvae of **Angiostrongylus costaricensis** *eliminated in 1g of mucus of experimentally infected* **Phyllocaulis boraceiensis**, P. soleiformis *e* P. variegatus, *from 28 to 44 days post-infection.*

Species	Days post-infection																
	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44
	-	1	1	-	-	-	-	1	1	1	-	-	-	-	-	-	-
	-	-	-	-	1	-	1	-	-	-	-	-	-	-	-	-	-
P. boraceiensis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
	-	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	1	nd	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	†	†	†	†	†	†	†	†	†	†	†	†
P. soleiformis	-	-	-	-	-	-	-	-	-	1	1	2	-	-	-	-	-
	-	-	-	-	-	†	†	†	†	†	†	†	†	†	†	†	†
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	nd
	-	-	-	-	-	-	-	-	-	-	†	†	†	†	†	†	†
	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-
P. variegatus	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
	-	-	-	2	-	-	-	-	-	-	1	-	-	-	-	-	nd
	-	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-	nd

nd = counting not performed; †: death of the mollusc; one specimen of *P. variegatus* died before the 28th day.

species of veronicelid were infected, showing a large variability in the total number of L3 in the entire body, without a significant average difference between *P. variegatus* and *P. boraceiensis* slugs (Table 2). *P. soleiformis* presented a higher mortality rate (80%) than *P. variegatus* (20%) while

Table 2- Mortality and individual numbers of L3 eliminated in the mucus and isolated from fibromuscular tissues in the infection of 5 Phyllocaulis variegatus, 5 P. soleiformis and 6 P. boraceiensis, exposed to1500 first stage larvae of A. costaricensis (spontaneous ingestion).

	P. variegatus	*P. soleiformis*	*P. boraceiensis*
Maximum output of L3 in the mucus *	1, 1, 2,†,1	†,2,†,0,†	1,1,0,1,1,1
Total L3 in the body	291,95,448,†,28	†,267,†,†,†	14,70,45,78,21,99
	(215.5)**		(54.5)**
L3/gram mollusc	91,25,162,†,22	†,152,†,†,†	4,8,7,15,3,16
	(75)**		(8.8)**
Mortality (%)	20	80	0

* See detailed information in Table 1; † = death of the mollusc; ** (Σ/n-dead animals) not statistically significant (p= 0.09, t Student test).

all *P. boraceiensis* survived (Table 2).

Except for one specimen which was intra-cavity inoculated, the infection could be established in all three methods (Table 3). The individual yield of L3 was quite diverse and it was impossible to calculate in the group submitted to spontaneous

Table 3 - Experimental infection of Phyllocaulis variegatus with 50 L1 of Angiostrongylus costaricensis by means of injection into the body cavity, in the hiponotum and by spontaneous "ingestion".

	Injection into the body cavity	Injection into the hiponotum	Spontaneous "ingestion"
Estimated inocula#	33, 19, 45	36, 35, 30	?
Total L3 in the body	6, 1, 0	5, 3, 1	3, 2, 1
	(2.3)*	(3.0)*	(2.0)*
L3 / gram mollusc	2.02, 0.21, 0	1.3, 1.21, 0.31	1.63, 1.78, 0.74
	(0.7)*	(0.9)*	(1.3)*
Yield of L3	0.18, 0.05, 0	0.14, 0.08, 0.03	?
(total L3 / estimated inoculum)	(0.07)*	(0.08)*	

larvae lost in the procedure, subtracted from 50. * Average values, no significant differences (F test, $\alpha = 0.05$).

"ingestion", since the actual inocula were unknown.

Titration of the infecting dosis in *P. variegatus* showed significant increase in the mortality and in the number of L3 when the inocula were higher than 1000 L1 (F Test, $\alpha = 0.05$) (Figure 1a). The yield of L3 was inversely correlated with the inocula (Figure 1b). The mortality, in the experiments with *P. soleiformis*, reached 66.6% with inocula of 50, 100 and 1000 L1 and 33.3% with 500 L1.

The small number of surviving animals limits any further analysis of these results.

The mean percentage of L1 remaining in the syringe during intra-cavity injection was 26.32 with inoculum of 50 L1, 10.8 with 100 L1, 5.6 with 500 L1, 4.6 with 1000 L1, 5.7 with 5000 L1 and 6.4 with 10000 L1.

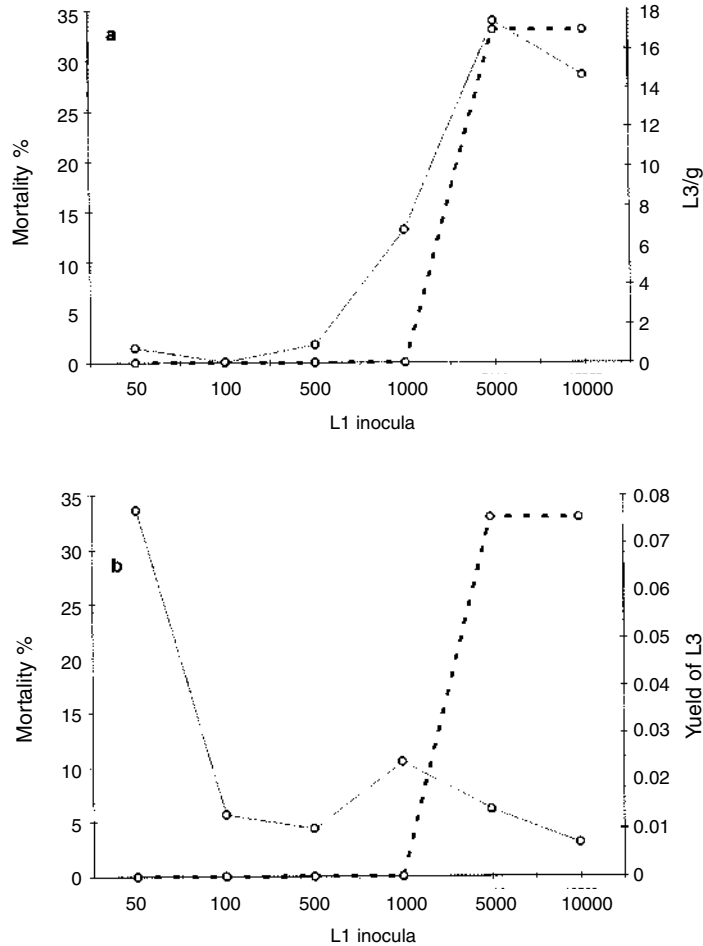No morbidity was observed in the negative

Figure 1 - Results of intra-cavity injection with increasing inocula of L1 in groups of 3 Phyllocaulis variegatus*: a) mean number of L3 (n = 3) adjusted to the weight of molluscs (L3/g) b) resultant yield (L3/actual inoculum) compared to the mortality rate (dotted line).*

DISCUSSION

control groups, injected with 0.6% saline.

Host-parasite coevolutionary status may be described based on morbidity or mortality. Adaptation may evolve over time with the attenuation of the parasite's pathogenicity and host reactivity, but this conception may not apply to a number of situations. The kinetics of the number of parasitic forms offered for transmission would be a more adequate indicator of the coevolutionary status, as reviewed by Garnik[2].

Most studies on intermediate hosts of *A. costaricensis* deal with the identification of susceptible species[5 6 7 8 9]. However, it is important to evaluate the degree of adaptation of a given host species and its consequent contribution to the mantainance of the parasite's species and its eventual transmission to man.

Application of the titer-time curve model[2] in molluscs experimentally infected with *A. costaricensis*' larvae was not possible due to the low number of L3 eliminated in the mucus (Table 1). Similar results were obtained in a previous evaluation (Zanini e Graeff-Teixeira, unpublished data), in the titration experiments using higher inocula (Figures 1a and 1b) and in animals with

high parasitic burden (Table 2). The use of laboratory reared molluscs reduces the possibility that this low yield would result from unspecifically activated resistence mechanisms due to coinfections when molluscs are collected from nature[1].

The procedures for the mantainance of the parasite in the laboratory include exposure of molluscs to L1 placed over small fragments of lettuce (*Lactuca sativa* L., 1758) or chayote (*Sechium edule*). This is based on the original description of the cycle by Morera[10] who has emphasized the spontaneous ingestion as the only way molluscs get the infection. The yield of L3 obtained was always quite variable and the number of inoculated larvae could not be controlled. Based on Thiengo[13], injection of L1 through the tegument was carried out, as an alternative mode of experimental infection, allowing the control of inocula.

Both intra-cavity and intra-notum injections resulted in positive infections, but the former proved to be less difficult and more easily controlled procedure for experimental infections, since injection into the notum results in a high loss of L1 extravasation, due to the intense contraction of the animal as the needle is introduced into the fibromuscular tissue. Intra-cavity injection diminishes the extravasation and consequently the loss of larvae.

Titration of the infecting larvae in *P. variegatus* (Figures 1a and 1b) using intra-cavity injection clearly showed that yield of L3 was not directly related to the inocula, being more or less constant in the inocula range from 100 to 1000 L1, showing only a small peak with 1000 L1. This fact suggests that the mollusc has a limited ability to harbor and provide a developmental environment to the larvae. Alternatively, intra-cavity penetration may not be the usual mode of entry for L1 into the mollusc. Inocula should be at the range of 1000-5000 L1, considering the high mortality rate and the stabilization of the curve showing inocula/L3 production (Figure 1a).

The pattern of L3 elimination in mucus in experimental conditions may not represent what happens in nature. Observations on the elimination of L3 in mucus from naturally infected slugs would be very important. Rambo e Graeff-Teixeira[11] have demonstrated that the parasitic burden in naturally infected molluscs is low, with 50% of the animals harboring less than 10 L3 in the entire body. It is reasonable to expect that the number of L3 eliminated in mucus is also low.

The mortality data in the comparative study of the three species (Table 2) and in the titration experiment, indicates that *P. variegatus* is the best adapted host, followed by *P. boraceiensis* and *P. soleiformis*. This conclusion is reinforced by the analysis of L3/g/day produced in *P. variegatus* (75.7) and *P. boraceiensis* (9.4) (Table 2).

P. boraceiensis was never found naturally infected and its susceptibility is documented here for the first time. It occurs on the coast of southern Brazil, from Minas Gerais to Santa Catarina[14]. Human disease has been regularly documented in those States, but usually restricted to western continental areas.

Besides the L3 titer-time curve, quantification of the total parasitic (larval) burden may be of value as an expression of intermediate host-parasite coevolutionary status.

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