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Morphometrics of third-stage larvae of the rat lungworm (*Angiostrongylus cantonensis*) in Hawai'i

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Abstract: Rat lungworm, *Angiostrongylus cantonensis*, is a tropical zoonotic, parasitic nematode and the causative agent of the clinical condition known as neuroangiostrongyliasis. Previous morphometric studies on *A. cantonensis* from Hawai'i were conducted about 50 years ago. We revisited the morphometrics of *A. cantonensis* infectious L3 larvae isolated from Hilo, Hawai'i and compared it with previous findings from Hawai'i as well as other locations such as Brazil and Florida, to potentially document any geographically related morphological variations. Nematodes were heat relaxed and body length, maximum width, length of esophagus, tail length, nematode body length/greatest body diameter, body length/esophagus length and body length/tail length were measured. The present morphometric findings are consistent with the previous studies from Hawai'i and Brazil ($P > 0.05$). All morphometric parameters of Hawai'i and Florida isolates, except the nematode body length and body length/esophagus length, were similar ($P > 0.05$).

Key words: angiostrongyliasis, eosinophilic meningitis, morphology, neuroangiostrongyliasis, rat lungworm.

Key Findings

- Morphometrics of *Angiostrongylus cantonensis* from Hawai'i 50 years ago and now are similar
- After the heat relaxation, coiled *A. cantonensis* L3 became semi-coiled and C-shaped
- Nematode morphometrics such as body length, width and ratio variables were documented
- Curved rhabdions and non-overlapped intestines were easily observed nematode parameters
- Conical-shaped and fine pointed tails were additional easily observed parameters

Introduction

The rat lungworm, *Angiostrongylus cantonensis* (Chen, 1935) is a zoonotic parasitic nematode, with mollusks and rats as their intermediate and definitive hosts, respectively (Maldonado et al., 2012; Spratt, 2015). They are primarily found in the tropical and subtropical regions (Smith et al. 2015; Peng et. al., 2017). Humans are thought to become accidental hosts upon consumption of contaminated fresh produce, which can lead to the clinical condition known as neuroangiostrongyliasis, that ultimately results in eosinophilic meningitis (Maldonado et al., 2012). Currently there are multiple reports of human infection from several countries including the United States (Maldonado et al., 2012). The potential of being exposed to *A. cantonensis* from raw produce is an increasing concern in agriculture, food safety and human health (Tsai et al., 2004; Eamsobhana, 2014).

East of Hawai'i Island is considered as the epicenter of neuroangiostrongyliasis in the USA (Johnston et al., 2019). While the parasite has been in the Hawaiian Islands for many decades, a relatively recent increase in the number of cases of neuroangiostrongyliasis has been noted (Wallace and Rosen, 1969; Ash, 1970; Johnston et al., 2019).

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This raises the question of whether the parasite has undergone adaptive changes that could impact its virulence over time especially in Hawai'i. If so, could these changes be reflected on the morphometric parameters?

In 1970, Ash reported a detailed morphometric study on *A. cantonensis* isolated from Hawai'i. After which, there were no other morphometric studies conducted in Hawai'i (Cowie, 2013). However, several morphological studies on *A. cantonensis* were conducted in other geographical locations such as China (Lv et al., 2009), Brazil (Thiengo et al., 2010; Guerino et al., 2017) and Florida, USA (Smith et al., 2015).

The objective of this study was to revisit the morphometric parameters of *A. cantonensis* L3 isolated from wild semi-slugs (*Parmarion martensi*) from the East Hawai'i region and compare the findings with published results.

Methodology

Angiostrongylus cantonensis L3 were isolated from wild-caught semi-slugs, which are known to be heavily infected with the nematode in the eastern region of the Hawai'i Island (Hollingsworth et al., 2007). As described in Howe et al. (2019), semi-slugs were immersed in 50 mL tap water in Falcon tubes and incubated for 72 hours at room temperature, which allowed the L3 to leave the drowned host and accumulated at the bottom of the tube. An aliquot of 15 mL from the bottom of the tube was then transferred to a glass culture plate (100 mm × 15 mm). The presence of L3 was observed under a dissecting microscope (Wild Heerbrugg, Switzerland) at 10–40× magnification (Jarvi et al., 2019; Howe et al., 2019). The L3 of *A. cantonensis* were identified based on their diagnostic 'Q' and 'S'-like motions (Lv et al., 2009), and were transferred to another glass culture plate (100 mm × 15 mm) in clean water using a 20-μL pipette. Twenty nematodes were then individually transferred to single cavity microscope slides (Karter Scientific Labware Manufacturing, Lake Charles, LA, USA), heat relaxed, and observed under a Leica™ Inverted Microscope (Leica Microsystems Co., Wetzlar, Germany). De Man Formulae (the De Man Indices): nematode body length, maximum width, length of esophagus, anus to tail length, and excretory pore from anterior length (De Man, 1876; De Man, 1881) were recorded. Rhabdion length and width were also measured. Moreover, ratios of nematode body length/greatest body diameter (a), body length/esophagus length (b) and body length/tail length (c) were calculated according to De Man (1876, 1881). All measurements were taken using a calibrated ocular micrometer. The mean ± standard error of the means (SEM) of each parameter was calculated. These parameters were similar to those used by Ash (1970). Photographs of the nematodes were taken at 400× magnification. Sketches of *A. cantonensis* were developed through a free online software (Snapstouch) and were compared with the previously published sketches in Ash (1970).

Statistical analysis

Nematode morphometrics from the current research were compared with similar results obtained from four other geographic *A. cantonensis* isolates (Ash, 1970; Thiengo et al., 2010; Smith et al., 2015; Guerino et al., 2017). The lowest and highest values of each parameter were considered as two separate observations. Data of each parameter from all isolates were checked for normality using Proc Univariate in SAS version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Wherever necessary, data were normalized using log10 (x + 1) for length parameters or *arsin* (sqrt(x/100)) for ratio variables. Morphometric parameters were subjected to one-way analysis of variance (ANOVA) using Proc GLM in SAS. Untransformed arithmetic means of data were separated by Waller-Duncan K-ratio (K=100) t-test. Data range with the lowest and highest data and their mean separation values were presented.

Results

Morphometric measurements

Nematode body length (L), maximum width, length of esophagus, anus to tail length, excretory pore from anterior, rhabdion dimensions and values of the ratio variables: L/greatest body diameter (a), L/esophagus length (b) and L/tail length (c) are given in Table 1. Nematode body length of Hawaiian isolates is similar to that of Brazilian isolates ($P > 0.05$) and is greater than that of Florida isolates ($P \leq 0.05$). The nematode body length/esophagus length of Florida isolates is significantly less than the other isolates ($P < 0.05$). However, all the other morphometric parameters were similar to Hawaiian and Brazilian isolates ($P > 0.05$) (Table 1).

Table 1. Comparison of morphometric measurements ± standard errors, and the lowest and highest data values (in bold) of third-stage larvae of the rat lungworm (*Angiostrongylus cantonensis*) from Hilo Hawai'i (current research) followed by published values of *A. cantonensis* from Ash, 1970 (Hawai'i), Thiengo *et al.*, 2010 and Guerino *et al.*, 2017 (Brazil), and Smith *et al.*, 2015 (Florida) isolates.

	Values for *A. cantonensis*[1]				
	Hawai'i isolates		Brazil isolates		Florida isolates
Morphometric parameters	Current research (n=20)	Ash, 1970 (n=35)	Thiengo *et al.*, 2010 (n=29-34)	Guerino *et al.*, 2017 (n=60)	Smith *et al.*, 2015 (n=7)
Body length (µm)	492.6± 7 **(420-545) AB**	474 **(425-524) AB**	460.4±31.8 **(460.3-544.6) A**	450.8±3.1 **(410.5-493.6) B**	- **(310-410) C**
Maximum width (µm)	24.5± 0.4 **(21.3-30) A**	26 **(23-34) A**	24.8±3.8 **(18.5-31.9) A**	21.1±0.7 **(13.1-38.5) A**	- **(20-22) A**
Esophagus length (µm)	180.1±1.9 **(157.5-190) A**	181 **(167-194) A**	168.4±11.2 **(129.6-189.0) A**	168.7±1.1 **(149.3 - 185.4) A**	- **(155-165.9) A**
Anus to tail length (µm)	39.9±0.9 **(27.5-43.8) A**	39 **(34-44) A**	41.2±10.4 **(27.7-89.3) A**	35.3±0.5 **(28.8 - 44.6) A**	- **(33-39.6) A**
Excretory pore from anterior end (µm)	97± 1.4 **(77.5-102.5) A**	93 **(78-105) A**	157.9±28.8 **(79.6-200) A**	86.0 ± 0.6 **(77.9 - 93.2) A**	- **(60-82) A**
Body length/greatest body diameter (a)	20.3± 0.5 **(16.6-23.1) A**	18.2 **(15.4-18.5)**^{z} **A**	18.5 **(17.1-24.9)**^{y} **A**	21.3 **(12.8-31.3)**^{x} **A**	- **(15.5-18.6)**^{w} **A**
Body length/esophagus length (b)	2.8±0.0 **(2.6-3.1) AB**	2.6 **(2.5-2.7)**^{z} **BC**	2.7 **(2.9-3.6)**^{y} **A**	2.7 **(2.7-2.7)**^{x} **B**	- **(2.0-2.5)**^{w} **C**
Body length/tail length (c)	12.4±0.3 **(10.9-16.4) A**	12.2 **(11.9-12.5)**^{z} **A**	11.2 **(6.1-16.6)**^{y} **A**	12.8 **(11.1-14.3)**^{x} **A**	- **(9.4-10.4)**^{w} **A**
Rhabdion length (µm)	19.7 ± 0.6 **(12.5-22.5)**	-	-	-	-
Rhabdion width (µm)	3.3 ± 0.2 **(2.5-5.00)**	-	-	-	-

[1] Data range with the lowest and highest data values in the same row followed by same letter(s) do not differ according to Waller-Duncan *K*-Ratio (*K*= 100) *t*-test.

$^{z, y, x}$ and ^{w} = Calculated based on provided data (Ash, 1970, Thiengo *et al.*, 2010, Guerino *et al.*, 2017 and Smith *et al.*, 2015, respectively).

- = Data not provided (Ash, 1970, Thiengo *et al.*, 2010, Guerino *et al.*, 2017 and Smith *et al.*, 2015).

The least and the most variable parameters in this study, as indicated by coefficient of variation (%), were the body length (L)/esophagus length ratio (4.70%), and the rhabdion width (21.91%). The coefficient of variation of all the parameters except rhabdion width was ≤ 11.97%.

Nematod shape

Before heat relaxation, the nematodes were coiled (Fig. 1A). Heat relaxation changed the shape of the nematode to semi-coiled (Fig. 1B; Fig. 5B). Heat relaxed nematodes were further turned to C-shaped (Fig. 2; Fig. 5C). The nematode tail was conical-shaped and terminally pointed (Fig. 3A). The maximum body width was at the juncture of the esophagus and intestine. The intestine and esophagus were not overlapped (Fig. 4B).

Figure 1. *A. cantonensis* L3 larva before (A) and immediately after (B) heat relaxation. Photographs are taken with the aid of an inverted microscope and an attached camera at 400x magnification.

Rhabdions, other easily visible body parts and nematode configuration

The width-to-length ratio of the rod-like structure at the mouth (rhabdion) was 1:6.3 (Fig. 3B and 3C). Additionally, nematode rhabdions were slightly curved (Fig. 4A). The anus (Fig. 3A) and excretory pore (Fig. 3C; Fig. 4A) of heat-relaxed nematodes, and the diagnostic "Q"-like motion of live nematodes (Fig. 4A-C; Fig. 5A) were easily visible.

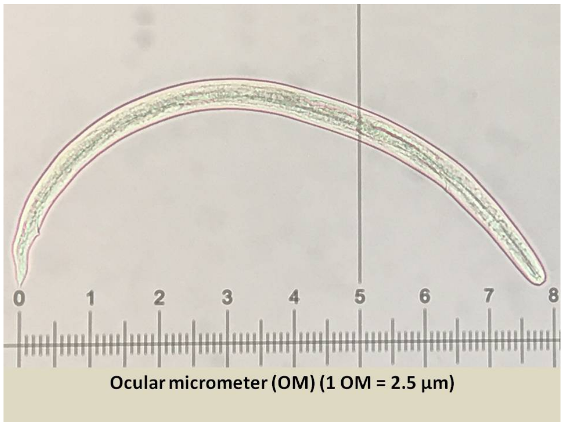

Figure 2. *A. cantonensis* L3 larva, post-heat relaxed. Photograph was taken using microscope camera at 400x magnification (1 smallest ocular micrometer division = 2.5 µm).

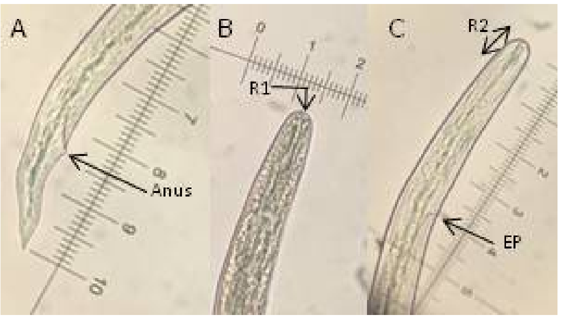

Figure 3. Anus (A), rhabdion width (B) and rhabdion length (C) of heat relaxed *A. cantonensis* L3 larva (R1= rhabdion width, R2 = rhabdion length and EP = excretory pore). Photographs were taken using microscope camera at 400x magnification (1 smallest ocular micrometer division = 2.5 µm).

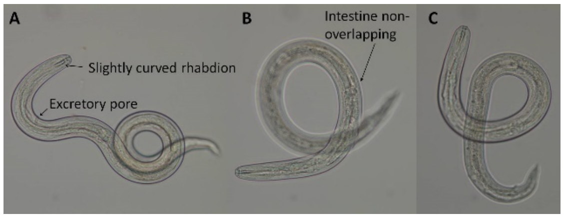

Figure 4. The "Q"- like motion *A. cantonensis* L3 larva (A, B and C) with a slightly curved rhabdion (A) and non-overlapping intestine at the oesophagus-intestine juncture (B). Photographs are taken using microscope camera at 400x magnification.

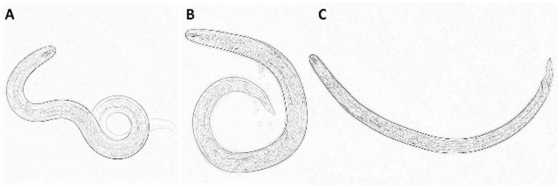

Figure 5. "Q"-shaped (A), semi-coiled (B) and C-shaped (C) sketches of *A. cantonensis* L3 larva. Sketches are developed through a software (Snapstouch, 2015). Nematode photographs were taken with the aid of an inverted microscope and an attached camera.

Discussion

All the morphometric measurements of *A. cantonensis* L3 from this study are statistically similar to those reported in previous studies (Ash, 1970; Thiengo et al., 2010; Guerino et al., 2017). The maximum nematode body width, nematode tail shape and their positions on the nematode body were consistent with the observations by Ash (1970). In addition, the length of L3 of *A. cantonensis* (L) is also consistent with Alicata (1963) who documented it as 460 to 510 μm. Nematode body length and body length/esophagus length of this study is significantly greater than those reported from Florida by Smith et al. (2015). However, Smith et al. (2015) hypothesized that the ethanol used in the nematode isolation process could have reduced those morphometric values by shrinking the nematode. Thus, this difference does not necessarily indicate variation between Hawai'i and Florida isolates.

Lv et al. (2009) described the presence of two well developed, rod-like structures with expanded knob-like tips at their anterior end under the buccal cavity and other specific body parts such as anus and excretory pore of *A. cantonensis*. This study also documents similar structures with additional investigation of body length, width and slightly curved shape of those rod-like structures (rhabdions). Though Lv et al. (2009) did not report morphometric values, the "Q"-like motion that Lv reported was consistent with our findings.

Our results are consistent with the findings of Ash (1970) from Hawai'i isolates of *A. cantonensis*. In addition, our findings are also consistent with the other studies from Brazil (Thiengo et al., 2010; Guerino et al., 2017) and Florida (Smith et al., 2015).

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