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Susan I. Jarvi
University of Hawaii at Hilo, jarvi@hawaii.edu

Margaret E.M. Farias
University of Hawaii at Hilo, mefarias@hawaii.edu

Kay Howe
University of Hawaii at Hilo

Steven Jacquier
University of Hawaii at Hilo

Robert Hollingsworth
USDA-ARS-PBARC, robert.hollingsworth@ars.usda.gov

See next page for additional authors

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Susan I. Jarvi[a,*], Margaret E.M. Farias[a], Kay Howe[a], Steven Jacquier[a], Robert Hollingsworth[b], William Pitt[c]

[a] *Department of Pharmaceutical Sciences, College of Pharmacy, University of Hawaii at Hilo, 34 Rainbow Drive, Hilo, HI 96720, USA*
[b] *USDA-ARS-PBARC, 64 Nowelo Street, Hilo, HI 96720, USA*
[c] *Hawaii Field Station, USDA, APHIS, Wildlife Services, NWRC, PO Box 10880, Hilo, HI 96721, USA*

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ABSTRACT

The life cycle of the nematode *Angiostrongylus cantonensis* involves rats as the definitive host and slugs and snails as intermediate hosts. Humans can become infected upon ingestion of intermediate or paratenic (passive carrier) hosts containing stage L3 *A. cantonensis* larvae. Here, we report a quantitative PCR (qPCR) assay that provides a reliable, relative measure of parasite load in intermediate hosts. Quantification of the levels of infection of intermediate hosts is critical for determining *A. cantonensis* intensity on the Island of Hawaii. The identification of high intensity infection 'hotspots' will allow for more effective targeted rat and slug control measures. qPCR appears more efficient and sensitive than microscopy and provides a new tool for quantification of larvae from intermediate hosts, and potentially from other sources as well.

The nematode *Angiostrongylus cantonensis* is a rat lungworm, a zoonotic pathogen which causes a global, emerging infectious disease known as rat lungworm disease (RLWD). This nematode was first discovered in China in 1935 [1], but is now endemic in Asia, Australia, the Caribbean islands and Pacific islands; it has also spread to the American continents with more than 2800 cases of human infection reported in 30 countries [2,3]. Rats are the definitive host, primarily *Rattus rattus* and *R. norvegicus* [4]. When rats eat infected slugs or snails, they ingest third stage (L3) larvae which eventually grow to sexual maturity and reproduce in the heart [5]. Single-celled eggs hatch in the lung, and first stage larvae migrate up the bronchial tree, are swallowed, and 6–8 weeks after infection are excreted with feces [6]. Slugs or snails then eat rat feces and acquire the first stage larvae. Slugs and snails are obligatory intermediate hosts which support parasite development from the first to the third larval stage (L3).

Humans can become infected by ingesting intermediate or paratenic (passive carrier) hosts containing infective L3 larvae. The most important paratenic hosts are crustaceans (such as prawns and land crabs) and predacious land planarians, such as flatworms in the genus *Platydemus* [7]. Once ingested by humans, larvae penetrate the intestinal mucosa and travel through the liver and lungs to the central nervous system (CNS) [2]. RLWD can be a serious threat to human health. Angiostrongyliasis in humans can result in transient meningitis (inflammation of the meninges of the brain and the spinal cord) or a more serious disease involving the brain, spinal cord and nerve roots, with a characteristic eosinophilia of the peripheral blood and cerebrospinal fluid and death in some cases [8]. Humans are a "dead-end" host, meaning the parasites do not reproduce in humans but remain in the CNS or can move to the eye chamber causing ocular angiostrongyliasis, where they remain until parasite death [3].

A. cantonensis has been documented as a parasitic disease of humans in Hawaii and other Pacific islands since the early 1960s [9]. The flatworm *Platydemus manokwari* and the semi-slug *Parmarion martensi* (hereafter referred to as semi-slug) had recently immigrated to Japan and were thought to be the probable cause of an outbreak of angiostrongyliasis there in the year 2000 [10]. The semi-slug is also a recent immigrant to the Hawaiian Islands [11] and is thought to be responsible for a recent outbreak of angiostrongyliasis cases on the Island of Hawaii [12]. In the district where the disease outbreak occurred (Puna district of the Island of Hawaii), >75% of *P. martensi* were found to be infected with *A. cantonensis*. In certain areas, semi-slugs were very numerous and some were heavily infected with L3 *A. cantonensis* larvae [12]. A recently published species-specific real-time PCR allows detection, but not quantification, of *A. cantonensis* in environmental samples [13]. The main goal of this study is to establish a quantitative PCR (qPCR)

* Corresponding author. Tel.: +1 808 933 2954; fax: +1 808 933 3889.
E-mail address: jarvi@hawaii.edu (S.I. Jarvi).

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assay that will provide a reliable, relative measure of parasite load in intermediate hosts (e.g. semi-slugs).

A. cantonensis larvae were isolated from naturally infected semi-slugs collected from the Puna district of the Island of Hawaii. Larvae were harvested from slugs via HCl (0.7%)–pepsin (0.5%) digestion (~2 h, 37 °C), filtered using a modified Baermann technique [14], and counted by direct examination microscopy. The total number of larvae was estimated by counting a minimum of three 10 mm^2 fields and using the average to calculate the total number of larvae based on the area of the petri dish. After counting, samples were pooled and centrifuged (750 rpm, 10 min). Supernatant was discarded, and tissue and larvae were resuspended in DNA lysis buffer (0.1 M Tris–HCl, 0.1 M EDTA, 2% SDS). Genomic DNA was extracted from an estimated 4935 larvae using the DNeasy Animal and Tissue Kit (Qiagen, Valencia, CA) with multiple elutions followed by ethanol precipitation and resuspension in a total of 700 μl buffer AE (Qiagen) yielding a final gDNA concentration equivalent to 7.1 larvae/μl. DNA was quantified using a Bio-Spec Nano (Shimadzu). A standard curve was generated by using multiple volumes of undiluted DNA as well as 1:10, 1:20 and 1:100 dilutions of gDNA to create a range of starting template equivalent to 0.071–63.9 larvae per reaction. Samples were then subjected to real-time PCR using a Custom TaqMan Gene Expression Assay (Life Technologies, Grand Island, NY, assay ID A139RIC) on a StepOne Plus RealTime PCR system (Life Technologies). The cycling conditions, primers (AcanITS1F1 and AcanITS1R1) and probe (AcanITS1P1) were those described by Qvarnstrom et al. [13]. PCR reactions were carried out in 20 μl total volume and included 0.25 μM probe, 0.9 μM forward and reverse primers and 1X TaqMan Environmental Master Mix 2.0 (Life Technologies). C_T values from these reactions were then plotted against known larvae numbers (based on microscopy) to create the standard curve shown in Fig. 1 (red/dark squares). A negative control (containing no DNA) was included in all reactions and remained negative, indicating no amplification for at least 40 cycles. All PCR reactions were replicated for verification. The C_T (cycle threshold) values for this concentration range (0.071–63.9 larvae/sample) were between 16 and 27 cycles, suggesting that a much larger dynamic range is possible.

In order to validate the accuracy of the standard curve and robustness of our assay, samples containing a wide range of nematode concentrations were prepared by using multiple volumes or dilutions of a stock solution estimated to contain a total of 4250 nematodes resuspended in 960 μl buffer AE. These dilutions of extracted DNA were then subjected to blind qPCR analysis in the same reaction as the standard samples above. Samples were run in replicate and all reactions included negative controls which remained negative. The C_T values for these samples were then plotted on the standard curve in Fig. 1 (validation samples or green/light squares) and used to estimate larvae numbers. Comparisons were then made of the number of larvae estimated by microscopy with larval estimates based on qPCR (Table 1). The qPCR larval estimates closely paralleled those by microscopy; this high degree of correlation validates the estimates derived from the standard curve.

The gDNA reference standards were then amplified in the same PCR reaction with gDNA extracted from tissue samples collected from 10 naturally infected semi-slugs. Tissue samples were collected from different parts of the slugs (e.g. tail, back, midsection or visceral mass, head and slime) to evaluate parasite distribution within semi-slugs. gDNA extractions and qPCR were carried out as described above starting with 17.7–28.3 mg tissue samples from Koa'e, 11.6–100.9 mg tissue samples from Kapoho and 5.8–22.5 mg tissue samples from Hawaiian Paradise Park (HPP). Side-by-side sampling of tissues from each slug allowed a comparison of larval estimates by qPCR with visual counts made by microscopy. Tissues for microscopy from semi-slugs collected from Koa'e were processed and larvae counted as above, with digestion in 5 ml

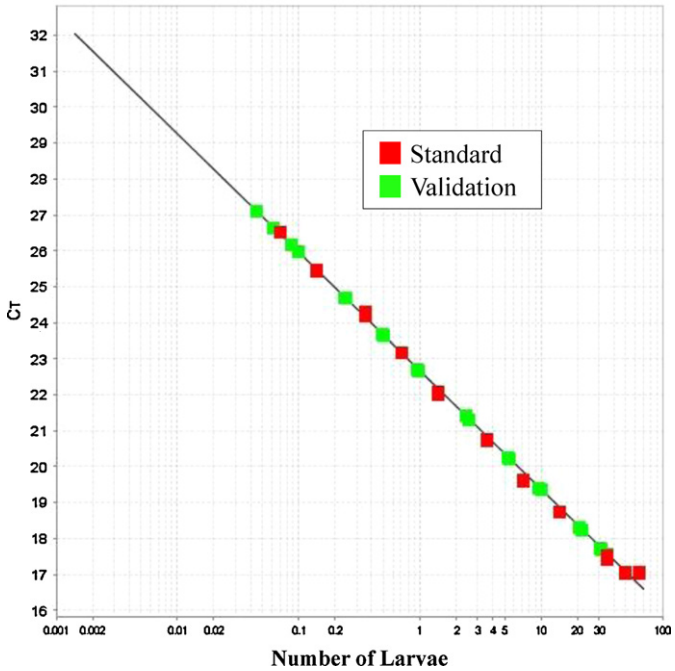

Fig. 1. Standard curve with the cycle threshold (C_T) values on the *Y*-axis and number of larvae on the *X*-axis. The samples used to generate the standard curve were multiple volumes and dilutions of gDNA extracted from a stock solution containing an estimated 4935 larvae. These samples are depicted as red/dark squares and ranged from 0.071 to 63.9 larvae/sample. C_T values of PCRs using multiple volumes and dilutions of a separate DNA extraction based on 4250 larvae are plotted on the standard curve and shown as green/light squares ("validation" samples). These C_T values (*Y*-axis) were then used to estimate larvae numbers (*X*-axis) for these samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

HCl–pepsin buffer (ranging from 40 to 415 mg tissue samples). Tissues from semi-slugs from Kapoho (samples ranging from 13.4 to 30.8 mg) and HPP (ranging from 10.5 to 29.8 mg) were digested in 500 μl to 1 ml HCl–pepsin buffer, and larvae were counted directly without filtering. qPCR reactions were replicated using two different volumes of starting template (2 μl and 5 μl) and the average larval concentration (normalized for volume) is shown in Table 2. We detected larvae in all tissues sampled from infected slugs with the estimated number of larvae based on both qPCR and microscopy highest in the midsection and the tail, and lowest in the slime the slug exuded in the collection vial. Linear regression (Minitab 16.2.1) of qPCR estimates vs microscopy estimates suggests that samples taken from either the midsection or the tail should be reliable as indicators of infection (R^2 of all samples = 0.62%, R^2 of tail

Table 1
Comparison of larval counts by microscopy and by qPCR amplification of different concentrations of gDNA "validation" samples completed in replicate (qPCR1 and qPCR2).

Larvae/reaction			
Microscopic count	Template amount (μl)	qPCR1	qPCR2
39.60	9.00	34.66	35.06
30.80	7.00	30.91	31.53
22.00	5.00	20.79	21.68
8.80	2.00	9.57	10.05
4.40	1.00	5.38	5.48
2.20	5 (1:10)	2.52	2.37
0.88	2 (1:10)	0.98	0.95
0.44	1 (1:10)	0.50	0.49
0.22	1 (1:20)	0.24	0.24
0.09	2 (1:100)	0.10	0.09
0.04	1 (1:100)	0.06	0.04

Table 2
Semi-slug (*P. martensi*) identification, site of origin on the Island of Hawaii (HPP, Hawaiian Paradise Park), origin of the tissue samples, and estimated number of larvae/mg tissue based on qPCR and microscopic counting. GPS coordinates for The Koa'e site are N19.523019 W-154.85884, the Kapoho Site (Kua O Ka La) are N19.467262 W-154.833597, and the HPP site are N19.584092 W-155.006782; ND, no data.

Semi-slug	Site of origin	25 mg tissue	qPCR larvae/mg	Microscopic counting larvae/mg
1	Koa'e	Midsection	49.26	15.03
1	Koa'e	Tail	41.34	17.04
1	Koa'e	Back	13.69	4.19
1	Koa'e	Head	10.39	4.32
1	Koa'e	Slime	0.83	0.21
2	Koa'e	Midsection	3.46	0.45
2	Koa'e	Tail	6.42	2.90
2	Koa'e	Back	1.80	0.12
2	Koa'e	Head	1.80	0.32
2	Koa'e	Slime	0.11	0.00
	Kapoho	Midsection	6.57	0.884
3	Kapoho	Tail	2.85	1.940
3	Kapoho	Head	2.11	0.584
	Kapoho	Slime	0.10	ND
4	HPP	Tail	56.02	44.76
4	HPP	Midsection	60.00	13.39
5	HPP	Tail	3.12	3.03
5	HPP	Midsection	15.25	2.34
6	HPP	Tail	3.83	0.12
6	HPP	Midsection	0.45	0.00
7	HPP	Tail	25.02	2.24
7	HPP	Midsection	38.34	11.75
8	HPP	Tail	31.55	22.05
8	HPP	Midsection	56.80	13.01
9	HPP	Tail	47.20	27.24
9	HPP	Midsection	23.09	1.46
10	HPP	Tail	29.61	15.32
10	HPP	Midsection	28.50	2.18

only = 0.82%, R^2 of midsection only = 0.85%). For sample collection, taking a small portion of the tip of the tail allows more consistency between samples and in no cases did we detect larvae in the midsection and not the tail. Given that R^2 values do not differ substantially between midsection and tail suggests that qPCR of tissue samples from the tail should be a good indicator of infection.

We suspected that qPCR estimates would be higher than estimates made by microscopy because qPCR can detect gDNA from both live and dead larvae. This is what we observed in all comparisons. Another possible reason for higher qPCR estimates may relate to the efficiency of extraction of live larvae from semi-slug tissues. The Koa'e samples were filtered for microscopic counting which requires that larvae be alive in order to be collected and presumably not all larvae are successful in escaping the partially digested tissue and migrating down through the thin paper filter to the bottom of the funnel. Factors other than filtering may also play a role as the Kapoho and HPP samples were not filtered, but we observed large disparities between the two methods as well. Other factors might include incomplete or over-complete digestion, or human error in counting (due to difficulty in seeing nematodes within the particulate matrix of partially digested tissue). qPCR offers several advantages over microscopy especially in laboratories where microscopy is not routinely employed. qPCR is more efficient because samples can be collected and stored frozen until analyzed, and is less labor intensive. Quantification with known reference template standards provides a relative measure of parasite load that facilitates comparative analyses by minimizing variability between tests. It also provides a tool for checking the efficiency of microscopic methods. Third-stage larvae, which can be shed in mucus [15] or can exit the bodies of dead or dying hosts [7], are a likely source of *A. cantonensis* found in fresh produce (such as lettuce). This qPCR method can be used experimentally with various produce washes to determine the degree of contamination associated with different sources and effectiveness of produce washes that may provide justification for carrying out definitive infection studies in feeding trials in a rat model. Finally, quantification of the levels of infection of intermediate hosts will provide the data needed to develop a detailed geographic map of RLW intensity on the Island of Hawaii. These data can then be used as a basis for implementation of targeted rat and slug control measures, as well as providing a baseline against which the efficacy of targeted rat and slug control measures may be evaluated.

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