

Autochthonous Transmission of *Angiostrongylus Cantonensis* in a Domestic Rabbit (*Oryctolagus Cuniculus*) in Hawai`i: Detection in Multiple Tissues Using AcanITS1 and AcanR3990 PCR Assays

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Short report

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Abstract

Background: Hawaii is the hotspot for rat lungworm disease (angiostrongyliasis) caused by the nematode *Angiostrongylus cantonensis* in the USA. In humans, PCR of the CSF is typically used for diagnosis, however, collection of CSF requires hospitalization. Here, we evaluate the efficacy of two different PCR tests to detect *A. cantonensis* DNA in multiple tissues including blood from a rabbit presumably infected by eating contaminated lettuce.

Methods: Two different PCR assays (AcanR3990, and AcanITS1) were used comparatively to test DNA extracted from slug and rabbit tissues. Assays were conducted using established protocols and were run in triplicate, with negative (dH₂O) controls included throughout.

Results: A juvenile *Parmarian martensi* (semi-slug) found in local lettuce tested positive for the presence of *Angiostrongylus cantonensis* DNA. A family and their two domestic rabbits (*Oryctolagus cuniculus*) consumed this lettuce twice within the five days preceding testing. One rabbit exhibited symptoms consistent with eosinophilic meningitis 3-6 days after being fed the lettuce. Appropriate veterinary treatment was ineffective and the rabbit was subsequently euthanized. This study comparatively applies two different PCR assays to detect *A. cantonensis* DNA in the peripheral blood, cerebrospinal fluid, brain, heart, and lung tissue of this rabbit, and provides data implicating parasite transmission via contaminated home-grown lettuce.

Six of the nine brain DNA samples, as well as the CSF sample, tested positive in replicate or triplicate for *A. cantonensis* DNA with both PCR assays. The AcanR3990 assay also detected *A. cantonensis* DNA from the lung, heart septum, all nine samples from the brain, and blood products (plasma, EDTA-treated whole blood, and buffy coat/red blood cells) in replicate or triplicate.

Introduction, Methods, And Results

Angiostrongylus cantonensis is the causative agent for neuroangiostrongyliasis (rat lungworm disease). While any slug or snail can serve as an intermediate host, the semi-slug *Parmarion martensi* is a highly efficient intermediate host in Hawai'i [1, 2, 3]. There have been several studies involving experimental infections with *A. cantonensis* in rabbits. These primarily involve the brain or central nervous system (CNS), as rabbits are considered non-permissive hosts and the larvae are not expected to leave the CNS [4]. Wang et al [5] conducted a long-term MRI study of the brains of rabbits infected with 200 *A. cantonensis* third-stage larvae (L3). Changes in the brain were monitored from day 0 to day 207 post-infection (PI). Lesions were first observed on day (D) 22 PI, and severe abnormalities were noted on D35 PI. They reported pathological changes in the brain tissues through D207 PI. Wang et al [6] conducted experimental infections on rabbits to evaluate the effects of albendazole treatment on pathological changes in the brain. Histopathological changes were observed in untreated rabbits infected with 400 *A. cantonensis* larvae between 21-28 days PI; however, data were not collected prior to D21 PI. Alicata [7] evaluated the effects of larval dosage in a study that included four groups of rabbits: groups 1-3 (n=4)

received 100, 200, and 500 larvae respectively, and group 4 (n=2) received 1000 larvae. Animals in group 4 developed paralysis of the hind quarters within 24 hours and died D2 PI, while none of the other groups showed these early, severe neurological symptoms. Jindrak and Magnusson [8] also evaluated the effects of larval dose on symptom onset and severity in rabbits. One rabbit received a dose of 500 larvae and at D3 PI showed mild weakness in its left hind leg. Two other rabbits received a dose of 1000 larvae each and at D4 PI showed a slight heaviness of both hind legs. At a dose of 1500 larvae, another rabbit showed weakness in its hind legs at D3 PI, and by D6 PI showed paralysis in its right forelimb. Two other rabbits received 2000 larvae; one was paralyzed by D3 PI and the other was comatose and paralyzed by D5 PI. These studies suggest a positive correlation between larval dose and neurological symptoms in rabbits.

In humans, early diagnosis of neuroangiostrongyliasis is difficult due to the range of symptoms and variation in incubation rates [9]. In the USA, although symptomatology and exposure history are considered, confirmed diagnosis is primarily via PCR using an ITS1-based assay (AcanITS1) for the detection of *A. cantonensis* DNA in the cerebrospinal fluid (CSF) of humans [10, 11, 12]. Unfortunately for the patient, it takes time for the parasite to reach the CNS and thus become detectable by PCR [13], time during which neurological damage has likely already occurred. Using an AcanITS1 assay developed for qPCR [2], *A. cantonensis* DNA was detected in the peripheral blood of rats at various time points including very early PI [14]. Recent development of a novel qPCR assay (AcanR3990) based on tandem repeats in genomic DNA has been shown to be ~100-1,000 times more sensitive than the AcanITS1 assay [15]. In Sears et al. [15], the AcanR3990 assay detected *A. cantonensis* DNA in CSF, blood, and brain tissues from a horse exhibiting clinical symptoms of infection, whereas the AcanITS1 assay detected *A. cantonensis* DNA only in the brain. Here, we apply both the AcanITS1 and the AcanR3990 assays for the detection of *A. cantonensis* DNA in CSF, various blood products, and multiple organs from a domestic rabbit unintentionally infected through apparent consumption of contaminated local lettuce in a household in Hawai'i.

In the laboratory under sterile conditions, the shell and mantle of the *P. martensi* (semi-slug) were excised and discarded using sterile forceps and scalpel blade. The remainder of the slug (0.2128g) was placed in a 2.0 mL screw top vial with 500 μ L DNA lysis buffer (0.1 M Tris HCl, 0.1 M EDTA, 2% SDS) containing 0.2 g 0.5 zirconia-silica beads (BioSpec Products, Bartlesville, OK, USA) and six 3.0 mm zirconia beads (OPS Diagnostics, Bridgewater, NJ, USA). An Omni Bead Rupter 24 Elite (Omni International, Kennesaw GA, USA) was used for 45 seconds at 8.0m/s to homogenize the tissue. A subsample of 50 μ L of the resulting homogenate was used for DNA extraction using the DNeasy Blood & Tissue Kit (Qiagen) with the Tissue Protocol with one modification: 100 μ L of elution buffer was used instead of the recommended 200 μ L to increase the concentration of eluted DNA. The extraction was quantified by BioSpec-nano (Shimadzu Biotech) at 102.5 μ g/ μ L DNA.

The *P. martensi* DNA sample was subjected to real-time PCR using a Custom TaqMan Gene Expression Assay (Life Technologies, Grand Island, NY) on a StepOne Plus RealTime PCR system (Life Technologies). The cycling conditions, primers (AcanITS1F1 and AcanITS1R1) and probe (AcanITS1P1)

were based on the ITS1 gene region as described [11]. PCR reactions were carried out as described [2] with the modification of 50 cycles instead of 40, in 20 μ L total volume, and included 0.25 μ M probe, 0.9 μ M forward and reverse primers and 1X TaqMan Environmental Master Mix 2.0 (Life Technologies). Another aliquot of the same DNA sample was used as template in quantitative PCR using the AcanR3990 assay as described with minor modification [15]. Briefly, qPCR was performed in 10 μ L reactions containing 1X TaqMan Fast Advanced Master Mix (ThermoFisher Scientific, Waltham MA), and 5 μ L template DNA. Thermocycling was performed on a StepOne system as above with cycling conditions of 95°C for 20 seconds, 40°C for 1 second, and 60°C for 20 seconds for 40 cycles. Negative controls using dH₂O instead of DNA template were run simultaneously for all reactions.

One of the two rabbits exposed to the contaminated lettuce, a 4.5 year-old Lionhead neutered male, began exhibiting neurological symptoms (listlessness, progressing to hind limb ataxia and forelimb ataxia) consistent with eosinophilic meningitis three to six days after eating the contaminated lettuce. Exact date of exposure is not known, as the rabbit was fed the lettuce on two different days. Treatment was initiated 5-8 days after presumed exposure with an antibiotic (Baytril Flavor Tabs 22.7 mg/12 hrs), a steroid (PrednisOLONE Suspension 0.4mL/12 hours for 3D, then 0.4mL/24 hours for 3D), and the antiparasitic drug fenbendazole (Panacur 50 mg/ml/24 hours for 5D). The rabbit did not respond to treatment and was euthanized 8-11 days after presumed exposure (3D after initiation of treatment). A necropsy was performed and multiple tissue samples collected. Samples were transported to the lab on ice and stored at -80°C. DNA extraction was performed on subsections of the samples using a DNeasy Blood & Tissue Kit per Tissue Protocol for brain, heart, lung, and pulmonary artery tissue and per Blood Protocol for CSF, EDTA-treated whole blood, plasma, and buffy coat/RBC samples. Amplification of *A. cantonensis* DNA from tissues was carried out as described above using both the AcanITS1 and AcanR3990 assays. Samples were tested in separate PCR runs, using the same conditions in each assay. Negative controls using dH₂O instead of DNA template were run simultaneously for all reactions.

The rabbit in this study developed symptoms between 3-6 days after presumed exposure, and was obtunded and euthanized 5 days after developing symptoms (8-11 days after presumed exposure). The PCR results of the *P. martensi* and *O. cuniculus* tissues tested are provided in Table 1. The negative controls for all reactions remained negative. The *P. martensi* sample was tested with the AcanITS1 PCR assay and found to contain significant amounts of *A. cantonensis* DNA with a relatively low C_T value of 19.36. The tissues collected from the *O. cuniculus* necropsy were tested with both the AcanR3990 and the AcanITS1 PCR assays. Six of the nine brain DNA samples, as well as the CSF sample, tested positive in replicate or triplicate for *A. cantonensis* DNA with both PCR assays. The AcanR3990 assay also detected *A. cantonensis* DNA from the lung, heart septum, all nine samples from the brain, and blood products (plasma, EDTA-treated whole blood, and buffy coat/red blood cells) in replicate or triplicate. Additionally, among the six samples that amplified in replicate or triplicate with both sets of primers, the average C_T value was significantly lower among the samples tested using AcanR3990 (\bar{x} C_T = 23.69) as compared with the C_T values of the same samples tested with AcanITS1 (\bar{x} C_T = 31.69) (P = 0.024). The mean C_T of the 11 samples that amplified with AcanR3990 but not AcanITS1 was relatively high (\bar{x} C_T = 37.06),

suggesting lower *A. cantonensis* DNA concentrations in those samples. The C_T values of the samples that did not replicate were also relatively high (39.05-40.79). PCR results with a minimum of two replicates and a standard deviation (SD) of < 0.5 are graphically depicted in Figure 1.

Table 1

Results of PCR testing of the *Parmarion martensi* and multiple tissues from *Oryctolagus cuniculus* using the AcanR3990 and AcanITS1 PCR assays (0.25 threshold). The number of positive replicates (# Pos Reps), mean CT values and standard deviations (SD) are as indicated. R = right, L = left.

Sample	PCR assay AcanR3990				PCR assay AcanITS1			
	PCR reaction	# Pos Reps	Mean C _T	C _T SD	PCR reaction	# Pos Reps	Mean C _T	C _T SD
P. martensi					279	3	19.36	0.062
O. cuniculus								
R cranial lung	12	2	38.75	5.78*	306	0	-	-
L caudal lung	12	2	39.33	1.205*	306	0	-	-
R cranial lung	12	2	39.64	0.333	306	0	-	-
Pulmonary artery	12	1	39.05*	-	306	0	-	-
R heart	12	1	39.22*	-	306	0	-	-
L heart	12	0		-	306	0	-	-
heart septum	12	2	39.30	0.218	306	0	-	-
Brain 1	12	3	17.55	0.09	306	3	24.99	0.018
Brain 2	12	2	35.04	0.387	306	0	-	-
Brain 3	12	3	26.45	0.113	306	3	34.50	0.139
Brain 4	12	3	19.09	0.117	306	3	26.61	0.023
Brain 5	12	3	32.58	0.336	306	1	39.06*	-
Brain 6	12	2	34.24	0.054	306	0	-	-
Brain 7	12	3	32.61	0.217	306	0	-	-
Brain 8	12	3	27.28	0.335	306	3	36.03	0.458
Brain 9	12	3	22.20	0.073	306	3	29.64	0.112
CSF	12	3	29.59	0.182	307	2	38.40	0.453
plasma	4	2	36.77	0.344	280	1	40.79*	-
EDTA blood	13	2	36.04	0.089	280	0	-	-
Buffy coat and RBCs	13	2	43.35	4.133*	280	0	-	-
*Data with SD > 0.5 or non-replicated results.								

Discussion

Angiostrongylus cantonensis DNA was detected with replication in nearly every tissue tested using the AcanR3990 qPCR assay including heart septum, lung, all nine samples from brain, CSF, and blood tissues, while the AcanITS1 assay [2, 11] detected DNA in five of nine brain samples and CSF. Both PCR assays have been shown to amplify DNA from *A. cantonensis* as well as from a closely related species *A. mackarrasae* [15]. While there currently are no PCR-based tests available that distinguish between these two species, detection in this study is likely restricted to *A. cantonensis*, as *A. mackarrasae* has yet to be documented in Hawai`i.

It should be noted that the salad in which the infected slug was found was reported to have been prepared according to recommendations by the Hawai`i State Department of Health, including the use of running municipal water to thoroughly wash the lettuce and the inspection of each leaf independently for signs of slugs or feeding damage. In addition, the lettuce was soaked in water after inspection and was subsequently dried in a salad spinner. This procedure failed to remove the infected slug. A recent study evaluating multiple produce-washing treatments for their ability to kill *A. cantonensis* found low larvicidal efficacy among consumer-grade fruit and vegetable washes, botanical extracts, and acidic solutions as compared with alkaline solutions and oxidizers [16]. Studies of other treatments involving UV light and/or ozone to reduce or kill *A. cantonensis* larvae are currently underway.

Multiple previous studies involve experimentally-infected rabbits which, like humans, are non-permissive hosts [4, 5, 6, 7, 8]. These studies have been crucial in documenting the long-term harmful effects of *A. cantonensis* exposure on the brain (up to 207 days PI); these effects may be implicated in the long-term sequelae reported in some human infections. The previous studies are also important in their documentation of positive correlation between larval exposure (dose) and severity of symptoms, which may also have relevance in human infections. The current study does not involve experimental infection. The semi-slug found in the locally-grown lettuce carried a high parasite burden as evidenced by the C_T value of the sample processed. While both rabbits seemingly ate this lettuce, only one became symptomatic shortly thereafter (though another undocumented, previous exposure cannot be entirely discounted); thus, this appears to be an autochthonous *A. cantonensis* infection of a domestic rabbit in Hawai`i.

The AcanR3990 assay appears more sensitive than the AcanITS1 assay in detecting *A. cantonensis* DNA from lung, heart septum, brain, and peripheral blood products collected from this rabbit. *Angiostrongylus cantonensis* DNA in peripheral blood has previously been detected in rats using the AcanITS1 assay [14] and in a horse using the AcanR3990 assay [15]. The AcanR3990 assay has been shown to be up to 1000 times more sensitive than the AcanITS1 assay [15]. Typically, lower C_T values correlate with higher concentrations of DNA extracted from biological tissue samples. Among samples that were detected by both assays in this study, lower C_T values were consistently observed using the AcanR3990 assay, reflective of the improved detection level of this assay. An early-detection blood-based diagnostic tool, based on the AcanR3990 (or another similarly sensitive) assay is, no doubt, forthcoming.

Abbreviations

ITS1: Internal transcribed spacer 1; CNS: Central Nervous System; CSF: Cerebral spinal fluid; L3: third stage larvae; PI: post-infection; D: day; EDTA: Ethylenediaminetetraacetic acid; PCR: polymerase chain reaction; C_T : cycle threshold.

Declarations

Ethics approval and consent to participate

The owners of this animal provided informed consent to conduct this research.

Data availability statement

All data generated or analyzed during this study are included in this published article.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

SIJ and AM conceived of this project, KAS, and AJ, conducted the bench experiments, WJS provided an alternative PCR test and AM, ML, and SO conducted the necropsy and tissue collection. All authors contributed to the writing of this manuscript.

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Figures

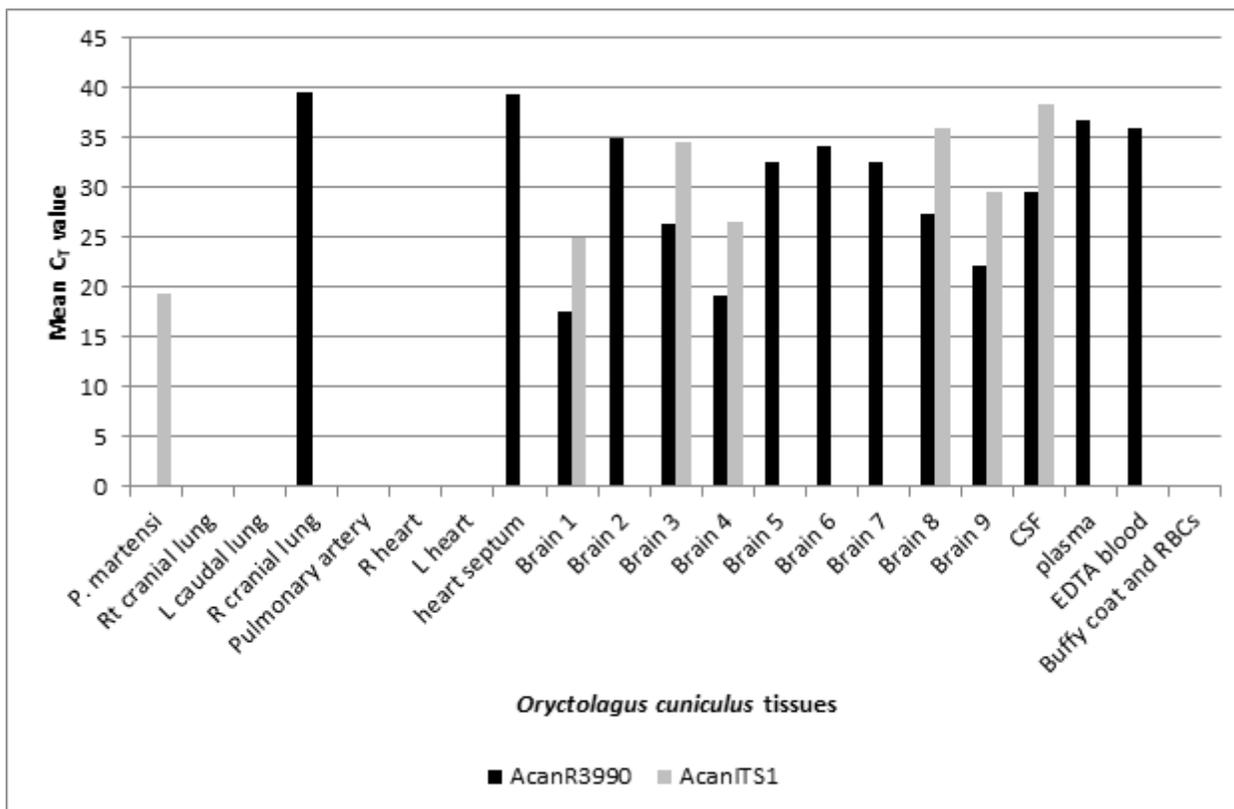


Figure 1

Mean C_T values of *Parmarion martensi* and *Oryctolagus cuniculus* tissues using the AcanR3990 and the Acan ITS1 PCR assays. Included are results with a minimum of at least two replicates and SD of < 0.5. R = right, L = left