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Improved Molecular Detection of *Angiostrongylus cantonensis* in Mollusks and Other Environmental Samples with a Species-Specific Internal Transcribed Spacer 1-Based TaqMan Assay▽

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***Angiostrongylus cantonensis* is the most common cause of human eosinophilic meningitis. Humans become infected by ingesting food items contaminated with third-stage larvae that develop in mollusks. We report the development of a real-time PCR assay for the species-specific identification of *A. cantonensis* in mollusk tissue.**

Angiostrongylus cantonensis is the most common agent associated with eosinophilic meningitis in humans. Young adult worms develop in the brains of rodents and are carried to pulmonary arteries to reach sexual maturity. Eggs are laid in lung tissues, and first-stage (L1) larvae break into air spaces, migrate to the trachea, are swallowed, and are passed with rodent feces. The L1 larvae must infect mollusks to develop into third-stage (L3) larvae; L3 is the infective stage for rodents and other mammals. Humans become infected by ingesting raw produce contaminated with L3 larvae or infected raw or undercooked mollusks or paratenic hosts. The immature worms remain in the human brain, creating tissue damage and inflammation (2, 19, 21).

A. cantonensis is endemic in Southeast Asia, parts of the Caribbean, and the Pacific Islands, including Hawaii (7, 12, 15–17). The worm has been detected in host animals in Louisiana (5, 14) and in one human patient from New Orleans (18), but it is currently unclear to what extent the nematode has spread into other U.S. states (8, 9). Ascertaining the geographic presence of the parasite is important to manage and prevent new cases of eosinophilic meningitis associated with ingestion of infective larvae (12, 18).

Detection of *A. cantonensis* in mollusks can be performed by releasing the larvae from the tissue with pepsin digestion (11). However, that procedure requires access to living mollusks, which complicates analysis of large numbers of samples. After a recent outbreak of angiostrongyliasis in Hawaii (12), we developed a conventional PCR assay and applied it to survey the Hawaiian mollusk population using frozen tissue (20). That PCR assay, as well as morphological identification using pepsin digestion, can only identify the larvae on the superfamily level, so additional molecular work is required for species-specific classification. Here we describe a new real-time PCR assay that allows for a direct detection of *A. cantonensis* at the species level.

The 18S rRNA gene is too conserved among nematode species to allow species-specific detection. The first and second internal transcribed spacers (ITS1 and ITS2) are comparatively more variable than the rRNA coding regions and have thus been used for differentiation of closely related species (1, 4, 6, 10, 22, 23). We PCR amplified and sequenced ITS1 from *A. costaricensis* (two laboratory strains from Costa Rica and Brazil), *A. vasorum* (from naturally infected hosts in United Kingdom), and *A. cantonensis* from three geographical regions (one laboratory strain from Japan plus nine environmental isolates from Hawaii and New Orleans, LA) to assess the variability of this potential PCR target. The oligonucleotide primers used were AngioF1674 (5′-GTCGTAACAAGGTATCTGTAGGTG-3′) and 58SR4 (5′-TAGCTGCGTTTTTCATCGATA-3′). The reaction mixtures contained 0.4 μM each primer and AmpliTaq Gold PCR master mix (Applied Biosystems, Foster City, CA) and were cycled 45 times at 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min. PCR products were cloned into pCR2.1 vectors using the TOPO cloning technique (Invitrogen, Carlsbad, CA) and sequenced on both strands as described elsewhere (20).

The sequence analysis revealed high interspecific and low intraspecific variability. A TaqMan assay targeting ITS1 was then designed using Primer Express version 2.3 (Applied Biosystems, Foster City, CA). The real-time PCR assay was performed in a 20-μl total volume containing Platinum qPCR Supermix (Invitrogen, Carlsbad, CA), 0.2 μM (each) primers AcanITS1F1 (5′-TTCATGGATGGCGAACTGATAG-3′) and AcanITS1R1 (5′-GCGCCCATTGAAACATTATACTT-3′), and 0.05 μM the TaqMan probe AcanITS1P1 (5′-6-carboxyfluorescein-ATCGCATATCTACTATACGCATGTGACACCTG-BHQ-3′). The standard cycling conditions for

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TABLE 1. Comparison of conventional and real-time PCR for detection of *Angiostrongylus cantonensis* in mollusks and nematode samples

Biological origin of DNA sample	Geographic origin	No. of samples tested	No. of samples positive by:	
			18S rRNA-based conventional PCR	ITS1-based TaqMan PCR
Parmarion martensi	Hawaii	112	75	83
Veronicella cubensis	Hawaii	50	23[a]	22
Laevicaulis alte	Hawaii	5	3	4
Achatina fulica	Hawaii	6	4	5
Other/unidentified mollusks	Hawaii	16	4	5
Flatworms	Hawaii	2	2	2
Slime from infected slugs	Hawaii	13	1	1
Pomacea insularum	Louisiana	31	5	5
A. costaricensis	Brazil, Costa Rica	2	2[b]	0
A. vasorum	United Kingdom	2	2[b]	0
Other nematodes[c]	CDC collection	14	0	0
Total		253	121	127

[a] This number includes three samples positive by PCR but later identified as non-*Angiostrongylus* nematodes by DNA sequencing analysis of the amplicons (20). These three samples were negative in the real-time PCR assay.

[b] The conventional PCR detects other *Angiostrongylus* species besides *A. cantonensis*.

[c] Two stool samples containing *Strongyloides* worms, eight environmental samples containing unclassified free-living nematodes and one of each of the following parasitic nematodes: *Dipetalonema* sp., *Toxocara cati*, *Dracunculus medinensis*, and *Ascaris lumbricoides*.

TaqMan assays were used (i.e., 40 cycles of 95°C for 15 s and 60°C for 1 min).

We evaluated the real-time PCR assay with a set of 26 *Parmarion martensi* slugs from Hawaii. Seventeen slugs were positive for L3 larvae as determined by pepsin digestion, and nine slugs were negative. DNA was extracted from approximately 25 mg of tissue of each slug using the DNeasy tissue and blood DNA extraction kit (Qiagen, Inc., Valencia, CA). The real-time PCR performed on this set of samples returned an identical result to the morphological analysis. The real-time PCR amplified only DNA from *A. cantonensis* and did not react with DNA from other nematode species (Table 1). The detection limit of the assay was determined by serially diluting a recombinant plasmid containing the ITS1 sequence to less than 1 copy per μl of sample. The real-time PCR reliably detected down to 10 plasmid copies in the reaction.

The real-time PCR assay was then used to analyze a larger set of naturally infected host animals from Hawaii, partly described elsewhere (13, 20), and Island Apple snails (*Pomacea insularum*) from New Orleans, LA. All samples had previously been characterized by the conventional PCR followed by DNA sequencing analysis (20).

Table 1 summarizes the PCR findings and highlights the enhanced performance of the real-time PCR in comparison to the conventional PCR. In addition, the real-time PCR assay was more practical to use since it did not require DNA sequence confirmation to rule out false positives.

The findings from Island Apple snails from New Orleans infected with *A. cantonensis* concur with previous reports about the potential for angiostrongyliasis transmission in this area (5, 14). Another interesting finding was the positive PCR results in two samples of flatworms from Hawaii. Predatory flatworms that ingest infected mollusks are known to be paratenic hosts of *A. cantonensis* and have been suspected to be an important source of infection for humans in Japan because they hide in leafy vegetables (3).

In conclusion, this real-time PCR assay can be a useful tool for environmental surveys of local wildlife to determine the geographic distribution of this reemerging human parasite.

Nucleotide sequence accession numbers. A representative set of ITS1 sequences has been deposited in GenBank under accession no. GU587745 to GU587762 and GU733321 to GU733325.

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