RESEARCH/INVESTIGACIÓN

FIRST COMPREHENSIVE MOLECULAR AND MORPHOLOGICAL IDENTIFICATION OF THE RAT LUNGWORM, *ANGIOSTRONGYLUS CANTONENSIS* CHEN, 1935 (NEMATODA: STRONGYLIDA: METASTRONGYLIDA) IN ASSOCIATION WITH THE GIANT AFRICAN SNAIL, *LISSACHATINA FULICA* (BOWDICH, 1822) (GASTROPODA: PULMONATA: ACHTINIDAE) IN FLORIDA

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ABSTRACT

Smith, T. R., A. C. Howe, K. Dickens, J. D. Stanley, J. A. Brito, and R. N. Inserra. 2015. First comprehensive molecular and morphological identification of the rat lungworm, *Angiostrongylus cantonensis* Chen, 1935 (Nematoda: Strongylida: Metastrongylida) in association with the giant African snail, *Lissachatina fulica* Bowdich (Gastropoda: Pulmonata: Achtinidae) in Florida. Nematropica 45:20-33.

A survey for the rat lungworm, *Angiostrongylus cantonensis*, a nematode parasite of rats and potentially injurious to humans, was conducted in Florida in 2011-2013 in concomitance with an eradication program of the giant African snail, *Lissachatina fulica*, an invasive snail recently found in southern Florida. Twenty-four percent of the giant African snail samples examined were infested by the coiled second- and infective third-stage juveniles of the nematode. These life stages embedded in or dislodged from comminuted snail tissue in water were detected using both molecular quantitative real-time polymerase chain reaction and morphological analyses. Nematode recovery from snail tissues was greater when both methods were concomitantly used on tissue samples from the same giant African snail specimen. More rat lungworms were extracted from the mantel than from the foot tissues of nematode-infested snails. The results of these analyses were validated by the Centers for Disease Control and Prevention in Atlanta, GA. To our knowledge, this is the first identification of the juveniles of *A. cantonensis* infecting *L. fulica* made in Florida using integrated morphological and molecular analyses.

Key words: invasive snail, mechanical maceration, molecular analyses, morphology, morphometrics, nematode parasite of vertebrates, Q-PCR, regulatory programs, sieving method.

RESUMEN

Smith, T. R., A. C. Howe, K. Dickens, J. D. Stanley, J. A. Brito, and R. N. Inserra. 2015. Primera identificación integradora molecular y morfológica del gusano pulmonar de la rata, *Angiostrongylus cantonensis* Chen, 1935 (Nematoda: Strongylida: Metastrongylida) en asociación con el caracol gigante africano, *Lissachatina fulica* Bowdich (Gastropoda: Pulmonata: Achtinidae) en Florida. Nematropica 45:20-33.

Durante los años 2011-2013, se realizó un muestreo en Florida para el gusano pulmonar de la rata, *Angiostrongylus cantonensis*, un nemátodo parásito de las ratas y potencialmente perjudicial para humanos, simultáneamente a un programa de erradicación del caracol gigante africano, *Lissachatina fulica*, un caracol invasivo recientemente encontrado en el sur de Florida. Un veinticuatro por ciento de las muestras de caracol gigante africano examinadas estaban infestadas por el estadios juveniles enroscado segundo e infectivo tercero del nematodo. Estos estadios vitales incluidos o separados del tejido del caracol triturado en agua fueron detectados usando tanto análisis moleculares (reacción en cadena de la polimerasa cuantitativa en tiempo real) como morfológicos. La recuperación de nemátodos a partir de los tejidos del caracol fue mayor cuando ambos métodos fueron usados a la vez sobre muestras de tejido del mismo ejemplar de caracol gigante africano. Se extrajeron más gusanos pulmonares de la rata de los tejidos del manto que de los del pie de caracoles infestados. Los resultados de estos análisis fueron validados por los centros de prevención y control de enfermedades en Atlanta,

GA. Según nuestra información, esta es la primera identificación de juveniles de *A. cantonensis* infestando *L. fulica* en Florida usando análisis morfológicos y moleculares de una forma integrada.

Palabras clave: caracol invasor, maceración mecánica, análisis molecular, morfología, morfometría, nematodo parásito de vertebrados, Q-PCR, programas reguladores, método del tamizado.

INTRODUCTION

The rat lungworm (RLW), *Angiostrongylus cantonensis* Chen, 1935, is a causal agent of eosinophilic meningitis in humans (Kliks and Palumbo, 1992), a disease that is prevalent in geographical areas where the nematode and its definitive and intermediate hosts are common. The RLW normally parasitizes, completes development, and reproduces in various species of rats (*Rattus* spp.), which are its definitive host. Rats become infested with the nematode by ingesting the nematode's intermediary hosts, which harbor the infective third-stage juveniles (J_3) of the parasite. Third-stage juveniles migrate, after penetration of the intestine, into the circulatory system of the rat and reach the meningeal nerves where they develop into fourth-stage juveniles (J_4) and adults by feeding in the meninges. Females leave the meninges at the time of oviposition and reach the lungs where eggs are deposited. The newly hatched first-stage juveniles (J_1) move from the lungs to the trachea and are swallowed and voided with feces into the environment. First-stage juveniles are not able to find the definitive host by active movement in the soil and need an intermediate gastropod host to initiate their development as second-stage juveniles (J_2) and then as infective J_3 life stages, which are to be vectored into the definitive host in order to complete their life-cycle. In environments populated by nematode-infected rats, the J_1 are ingested with feces by the gastropod host and released in the intestine. They migrate from the digestive system into the tissues of the mantel and foot where they develop into the J_2 and infective and coiled J_3. Rats regularly prey on nematode-infected gastropods acquiring the J_3, which complete their life cycle in the body of these rodents (Ash, 1968, 1976; Mackerras and Sandars, 1955; Maggenti, 1981; Alicata, 1991; Wang *et al.*, 2008; Diaz, 2010). While the initial portion of the nematode's life cycle usually occurs in snails, RLW has been found in many other intermediate hosts such as planarians, amphibians, freshwater shrimp, crayfish, and crabs, which may be used as source of food by rodents and humans (Asato *et al.*, 2004; Lv *et al.*, 2009a; Herwaldt, 2012). The nematode life cycle in humans is similar to that in the rats. Humans can acquire the nematodes by ingesting nematode-infected intermediary hosts, such as raw or undercooked infected snail meat and fluids, or vegetables contaminated with infective J_3 that are present in the slime left by the snails during their feeding and movement (Alicata, 1965; Cross, 1987; Wang *et al.*, 2008).

The geographical distribution of the RLW includes many countries in Africa, the Americas, Australia, South East Asia (China, Malaysia), Japan, and South Pacific Islands. In the United States, RLW has only been reported from Louisiana and Hawaii (Alicata, 1962; Kim *et al.*, 2002; Slom *et al.*, 2002; Hochberg *et al.*, 2007; Diaz, 2008). Gastropods are important intermediate hosts for the RLW. The number of invasive snail species that are becoming established around the world has been steadily increasing over the last 20 yr (Smith and Silagyi, 2009). A well-known intermediate host of RLW is the giant African snail (GAS), *Lissachatina fulica* Bowdich (Aicata, 1966; Wallace and Rosen, 1969). In 1966, GAS was introduced into Miami-Dade County, Florida. Due to the implementation of a 10-yr eradication program at a cost of $7 million (adjusted for inflation), the GAS was successfully extirpated from the state (Sturgeon, 1971; Poucher, 1975). In spite of the occurrence of previous GAS infestations in Florida, there are no records indicating that the RLW (*A. cantonensis*) was present in this exotic snail in the state at that time.

In September of 2011, GAS was found in Miami-Dade County, Florida (Smith *et al.*, 2013). This invasive snail, native to Southeast Africa, has been spread throughout the tropics and subtropics and has been designated as one of the most pestiferous snail species in the world (Raut and Barker, 2002). In addition to being a damaging agricultural pest, GAS represents a threat to human health because of its ability to vector certain parasites involved in brain disorders (eosinophilic meningitis) in humans and other animals (Civeyrel and Simberloff, 1996; Burkett, 2001). The outbreak of GAS infestation in southeastern Florida prompted the enactment of regulatory measures and the implementation of an eradication program by the Florida Department of Agriculture and Consumer Services (FDACS) in conjunction with the United States Department of Agriculture (USDA). This eradication program relied on the manual collection and destruction of GAS specimens in the infested areas and on the application of approved chemical treatments, when possible.

The regulatory measures enacted against GAS included accurate verification of the presence of the RLW in the Florida populations of the newly arrived GAS. Information on the occurrence of the RLW was very important in order to adopt protective measures

for the health of people in the affected areas and the safety of the FDACS and USDA inspectors involved in collecting snails as part of the eradication program. In order to reach this additional objective of the eradication program, a study was conducted to determine: i) the presence of RLW in the GAS populations in Miami areas and ii) the distribution of the nematode in these areas. The RLW detection in the snails in this survey was carried out using comprehensive molecular and morphological analyses, which were conducted in a complementary fashion to confirm nematode identification (Fontanilla, 2010; Nyoike *et al.*, 2012).

MATERIALS AND METHODS

The area surveyed for the presence of RLW included 26 sites indicated as cores (Fig. 1). Each core consisted of an initial detection site surrounded by a 1-mile diameter buffer zone. Numerous snails (146,000) were hand collected from GAS populations in these 26 cores within Miami-Dade County. Snail specimens for this study were obtained from 17 of the 26 cores and processed for presence of RLW juveniles, which were identified using molecular and morphological analyses. In order to determine the effectiveness of these analyses, Hawaiian specimens of the semi slug, *Parmarion martensi* Simroth that were known to be infected with RLW (Hollingsworth *et al.*, 2007), were used as positive controls. These specimens, preserved in 95% ethanol (Omar *et al.*, 2009; Michaud and Foran, 2011), were provided to us by Dr. Robert Hollingsworth (USDA-ARS-PBARC, Hawaii).

All GAS specimens used in this study were collected over a 2-yr period from September 2011 to September 2013. A random number of GAS was collected from as many different cores as possible. Emphasis was placed in preserving the larger and older snails rather than the younger and smaller because they were more likely to be infested with more numerous RLW in their larger body mass than that of the small snails and also because of their more prolonged exposure to and feeding on nematode-infested rat feces (Campbell and Little, 1988).

Based on conventional techniques used to sample for nematodes in other snail species (Lv *et al.*, 2009b; Chen *et al.*, 2011; Jarvi *et al.*, 2012), and on the abundance of RLW juveniles found throughout the foot of the positive control specimens of *P. martensi* from Hawaii during the initial phase of this study, the snail samples analyzed consisted mainly of the muscular foot. The foot was removed at the time of collection in Miami and immediately preserved in 95% ethanol before being shipped to the FDACS- Division of Plant Industry Laboratory in Gainesville, FL. At the end of the first year the sample composition was modified and included both mantle, a thin band of tissue that supports the respiratory and digestive organs where the snail body attaches to the shell, and foot tissues.

Snail tissues were processed separately for the molecular and morphological analyses. The body of a number of specimens was dissected into pieces, which were processed with both methods, in order to verify their accuracy and efficiency in detecting the RLW in the snail tissues.

Molecular analysis

Several molecular techniques (Carreno and Nadler, 2003; Eamsobhana *et al.*, 2010; Fontanilla and Wade, 2008, 2012) can be used to identify RLW, including quantitative real-time polymerase chain reaction (Q-PCR) (Qvarnstrom *et al.*, 2007, 2010; Jarvi *et al.*, 2012) and loop-mediated isothermal amplification (Chen *et al.*, 2011). In this study, molecular analyses were conducted using the Q-PCR assay, which was repeated for up to five samples from each snail: one sample each from the front, middle, and rear of the foot tissue for all snails collected during the first year, and two additional samples from the anterior and posterior mantle in the second year.

Q-PCR Assay: Tissue sample for this assay was prepared by shaving off the dark epidermal layer with a razor blade to minimize contamination or inhibition of the PCR reaction. Approximately 25 mg of clean tissue was minced on a delicate wiper (Kimwipes) using a scalpel, to enhance cell lysis, and placed in a 1.5-ml sterile centrifuge tube. Tubes were sealed to prevent contamination from other samples. Genomic DNA from the nematode was extracted using the standard protocol recommended in the Qiagen kit (DNeasy Blood & Tissue Handbook, Qiagen, Santa Clarita, CA). The amplification of the 18S rRNA (small subunit (SSU)) gene was achieved using species-specific primers for RLW infective J_3 (Qvarnstrom *et al.*, 2010). Samples were kept in a freezer at – 20°C and analyzed the same day or within 1 wk.

All samples and reagents were vortexed, centrifuged, and put on ice along with Q-PCR tube holders. PCR reaction mix (25 µL) was prepared as follows: 2.0 µL of DNA template (20 ng/µL), 2.4 µL MG water, 12.5 µL Platinum Supermix (Invitrogen, Carlsbad, CA), 2.5 µL each of 2 µM primers AcanITS1F1 (5′-TTCATGGATGGCGAACTGATAG-3′) and AcanITS1R1 (5′-GCGCCCATTGAAACATTATACTT-3′), 0.6 µL of 2 µM TagMan probe AcanITS1P1(5′-6-carboxyfluorescein-TCGCATATCTACTATACGCATGTGACACCTG-BHQ-3′), and 2.5 µL $MgCl_2$ (50 mM) (Invitrogen, Carlsbad, CA) according to Qvarnstrom *et al.* (2010) with modifications. The negative and positive controls consisted of a 2 µL of MG water and 2 µL DNA sample derived from a known RLW-infected semi-slug (Hawaii), respectively. Amplification cycles were performed as described in Qvarnstrom *et al.* (2010) with modifications and included: 50°C for 2 min., initial denaturation at 95°C for 2 min., followed by 40 cycles at 95°C for 15 sec., annealing and extension at 60°C for 1 min. PCR amplifications were carried out in a Cepheid SmartCycler II system (Sunnyvale, CA).

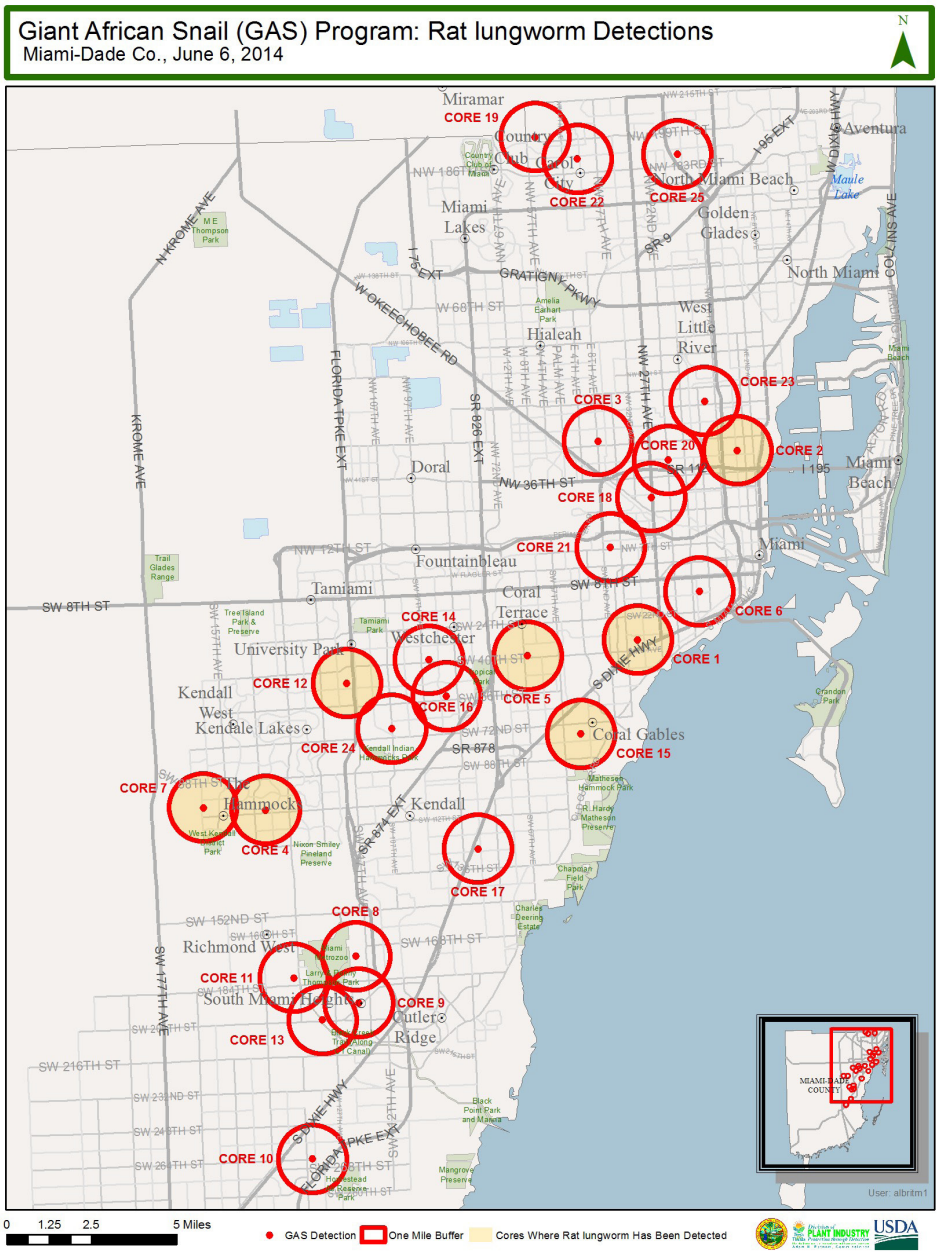

Fig.1. A map of Miami-Dade County, FL showing the distribution of sites or cores (circles) infested with giant African snail. Cores with snails harboring the rat lungworm are shaded in yellow

Morphological analysis

The morphological analysis was conducted on RLW juveniles (J_2-J_3) extracted from GAS tissues using a mechanical maceration method similar to that described by Hooper (1970a) and modified by Hussey and Barker (1973) for the separation of immobile nematodes from plant tissue. This method was first used for the extraction of *A. cantonensis* juveniles from the positive control semi slug, *P. martensi* from Hawaii. The technique involved the maceration of chopped pieces of muscular tissue of the snail in an electric mixer (blender) with revolving knife blades. The maceration time was adjusted according to the type of tissue macerated and for a length of time sufficient to remove the nematode from the tissue without damaging their body. The nematodes were recovered from the macerated tissues by washing through a series of sieves of different apertures (850, 250, and 45 µm). The nematode extraction method that was used for this

study allowed a recovery of approximately 30 RLW juveniles per gram of the positive control semi slug.

The following steps were followed for the extraction of RLW juveniles from the tissues of Florida GAS specimens fixed in 95% ethanol. A representative portion of the foot and (or) mantle was cut into approximately 1 mm^2 pieces. In larger snails (7.5 to 12.5 cm long) about half of the foot was used, and most if not all of the foot was used in smaller snails. Pieces were placed in the cup of a Waring commercial blender. Enough water was added to the blender to allow good contact with the blades. Snail pieces were macerated for 10 sec at the lowest setting followed by 20 sec at high speed. Macerating the material for any longer time caused excessive foam production and damage to the nematode body. The macerated snail suspension was then poured through a series of stacked sieves of decreasing pore diameter as described above.

Almost all of the life stages remained on the 45-µm-pore sieve together with small residues of macerated tissues while the large macerated tissues were retained by the 250-µm-pore sieve. The macerated residues with the RLW juveniles were gently washed into a beaker and in turn poured into a glass Pyrex 50-ml conical glass tube and allowed to settle for approximately 45 min. The final concentrated material was pipetted in small aliquot diluted in water onto a glass slide, covered with a cover slip and observed with a compound microscope at a magnification of 100x for the presence of nematodes. Nematodes observed with the compound microscope were then removed from the water suspension with the aid of a stereomicroscope at a lower magnifications of 50 to 75x and transferred on water agar on a slide for examination and measurements with the aid of a compound microscope at 1000x (Hooper, 1970b; Esser, 1986). Some specimens were also mounted in permanent slides after transferring in glycerin specimens fixed in formalin 4% (Seinhorst, 1962).

Most of the nematodes extracted from the snails and fixed in alcohol were deformed and distorted but still identifiable. Infective J_3 were identified using the morphological characters reported in literature (Mackerras and Sanders, 1955; Alicata, 1963; Ash, 1970; Lv *et al.*, 2009b; Maldonado *et al.*, 2012) and by comparison of their morphology with that of voucher specimens of RLW from Hawaii. Morphological characters of diagnostic value for the J_3 identification included coiled body shape, length and shape of the esophagus, excretory pore-anterior body end distance, tail length, and a pointed tail terminus. Since the morphological characters of juveniles are not sufficient for an accurate identification of the species, after the microscopic examination, the specimens were removed from the water agar blocks and underwent molecular analysis to confirm the morphological identification.

To completely analyze a sample, numerous subsamples of macerated snail material were observed separately in order to make sure that nematodes were not overlooked. Voucher nematode specimens mounted on glass slides were deposited in the nematode collection of Florida Department of Agriculture and Consumer Services, DPI, Gainesville, FL (collection numbers – N12-01104, 1-5). Additional specimens were distributed to the Istituto per la Protezione delle Piante, CNR, Bari, Italy (collection numbers – N12-01104, 6-12), Department of Infectious Disease and Pathology University of Florida, Gainesville, FL (collection numbers – N12-01104, 13), the United States Department of Agriculture Nematode Collection, Beltsville, MD (collection number – N12-01104, 14-15), and University of California Riverside Nematode Collection, Riverside, CA (collection number – N12-01104, 16-17).

Validation of the results of molecular and morphological analyses

PCR products positive and negative for RLW juveniles and J_3 RLW specimens extracted in water with maceration method were submitted to the Centers for Disease Control and Prevention (CDC) in Atlanta, GA, USA to validate the results of the analyses.

RESULTS

A total of 592 snails were analyzed for presence of RLW using molecular and morphological methods.

Molecular analysis

Presence of the RLW RNA caused a positive sample to fluoresce at wavelength 25.60-33.92 nm (Fig. 2); water and tissues negative for the nematode-specific sequence did not fluoresce at that wavelength.

Of 520 GAS analyzed, eight (1.5%) were positive for RLW using the Q-PCR (Table 1). This low RLW detection level was likely due to incorrect tissue sampling procedures from the snail (lack of mantel tissues) used during the first year. In the second year, 88 snails were analyzed using both molecular and morphological methods (Table 3). Twenty-one positive snails (24%) were found in seven different cores (Fig. 1). For these analyses, sampling procedures were improved by including foot and mantel tissues in the analysis. The results of the combination of the molecular and morphological analyses in the second year (Table 3) provide a much more accurate representation of the overall percentage (24%) of RLW occurring in the GAS population in south Florida.

Morphological analysis

A total of 160 snails were processed for morphological analysis. Twenty of these snails were parasitized by the RLW (Table 2). The higher percentage (12.5%) of RLW detection with morphological analysis

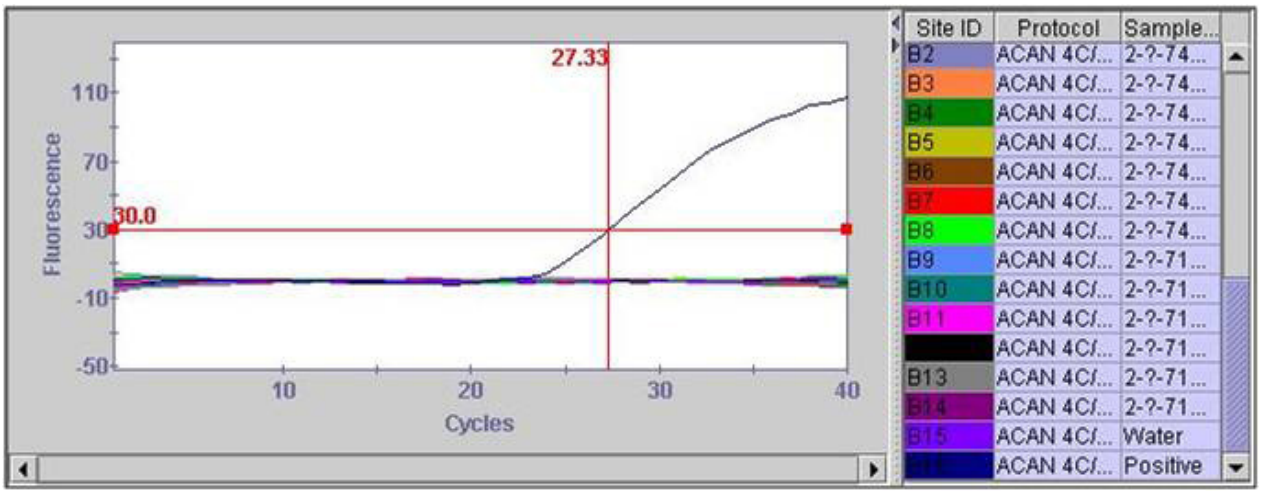

Fig. 2. Q-PCR reference standard obtained from the rat lungworm (*Angiostrongylus cantonensis)* J_3 from semi slug, *Parmarion martensi* used for the detection of rat lungworm J_3 from Florida giant African land snails. The PCR sample is positive for rat lungworm if the curve goes above 30 relative fluorescence units (RFU) at wavelength 25.60-33.92 nm; water and tissues negative for the nematode-specific sequence do not fluoresce at that wavelength.

Table 1. Number, ratio, and percent of *Lissachatina fulica* specimens found infested by *Angiostrongylus cantonensis* in Miami Dade County, FL cores (September 2011 – September 2013) using real-time Q-PCR analysis.

Core	Number of snails tested	Number of snails positive for *A. cantonensis*	Ratio of positive to negative	Infection percentage
1	70	0	0:70	0%
2	83	0	0:83	0%
3	8	0	0:8	0%
4	77	4	4:77	5.2%
5	52	0	0:52	0%
7	5	1	1:5	20%
8	33	0	0:33	0%
9	10	0	0:10	0%
10	54	0	0:54	0%
11	28	0	0:28	0%
12	45	3	3:45	6.7%
13	6	0	0:6	0%
14	5	0	0:5	0%
15	27	0	0:27	0%
17	7	0	0:7	0%
18	9	0	0:9	0%
20	1	0	0:1	0%
Total	520	8	8:520	1.5%

Table 2. Number, ratio, and percent of *Lissachatina fulica* specimens found infested by *Angiostrongylus cantonensis* juveniles (J_2, J_3) in Miami Dade County, FL cores (September 2011 – September 2013) using morphological analysis.

Core	Number of snails tested	Number of snails positive for *A. cantonensis*	Ratio of positive to negative	Infection percentage
1	23	3	3:23	13.0%
2	21	2	2:21	9.5%
3	3	0	0:3	0%
4	46	10	10:46	21.7%
5	11	1	1:11	9.0%
7	1	1	1:1	100%
8	4	0	0:4	0%
9	5	0	0:5	0%
10	9	0	0:9	0%
11	3	0	0:3	0%
12	28	1	1:28	3.5%
15	4	2	2:4	50%
17	1	0	0:1	0%
21	1	0	0:1	0%
Total	160	20	20:160	12.5%

Table 3. Prevalence of *Angiostrongylus cantonensis* juveniles (J_2, J_3) in both foot and mantel tissues of *Lissachatina fulica* snails, within the cores of Miami, using the combined real-time Q-PCR and morphological analyses (October 2012 - June 2013).

Core	Number of *L. fulica* sampled	Number positive for *A. cantonensis*	Percent positive for *A. cantonensis*
1	10	3	30%
2	9	2	22%
3	2	0	0%
4	23	11	48%
5	5	1	20%
7	1	1	100%
9	2	0	0%
10	3	0	0%
12	28	1	4%
15	3	2	67%
17	1	0	0%
21	1	0	0%
Total	88	21	24%

Table 4. Number of *Angiostrongylus cantonensis* juveniles (J_2, J_3) detected in *Lissachatina fulica* foot and mantel tissues using morphological analysis (October 2012-June 2013).

Snail number	Number of *A. cantonensis* juveniles in foot	Number of *A. cantonensis* juveniles in mantle	Recovery from mantle as % of total
1	9	664	99%
2	0	2	100%
3	2	3	60%
4	0	20	100%
5	0	12	100%
6	0	1	100%
7	0	2	100%
8	0	1	100%
9	0	16	100%
10	0	2	100%
11	1	0	0%
12	0	1	100%
13	0	5	100%
Total	12	729	98%

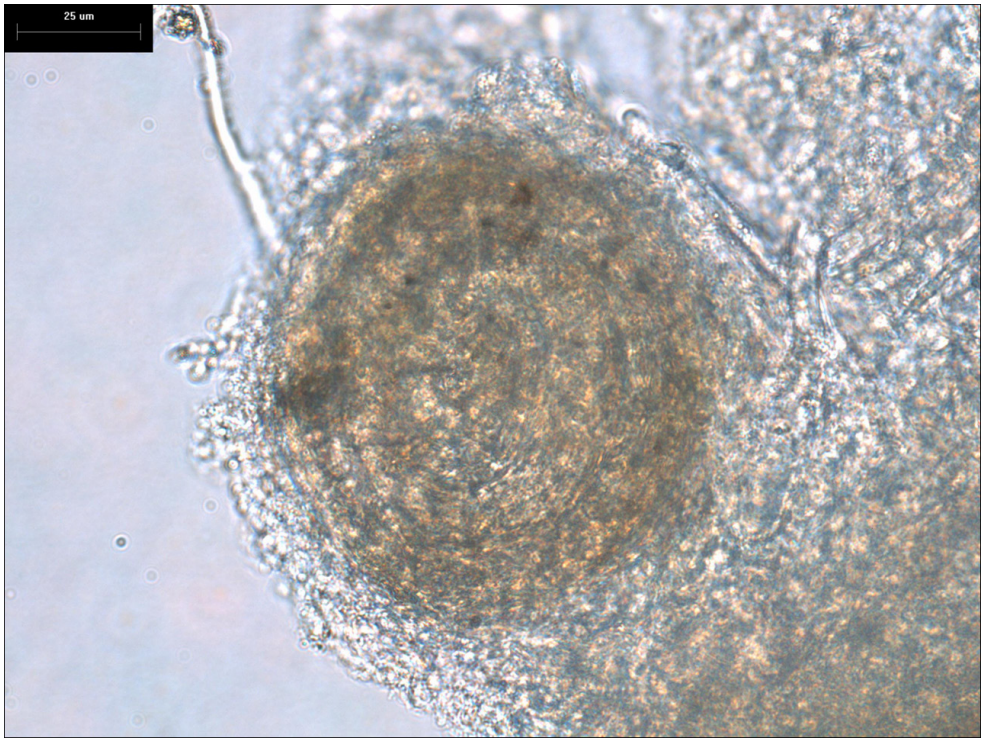

Fig. 3. A coiled and quiescent third-stage juvenile of the rat lungworm (*Angiostrongylus cantonensis*) embedded in the foot tissues of a giant African snail (*Lissachatina fulica*) collected in Miami-Dade County, FL.

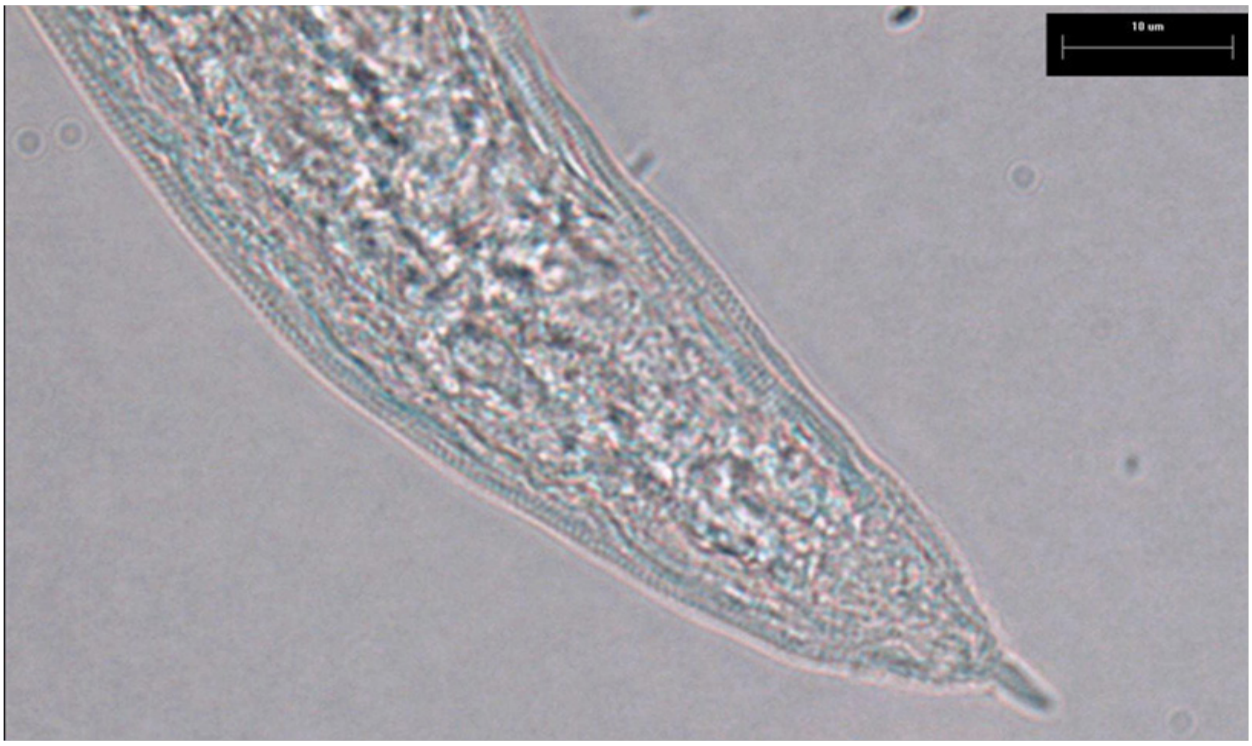

Fig. 4. Posterior body portion of a third-stage juvenile of the rat lungworm (*Angiostrongilus cantonensis*) showing a digitate tail with round terminus.

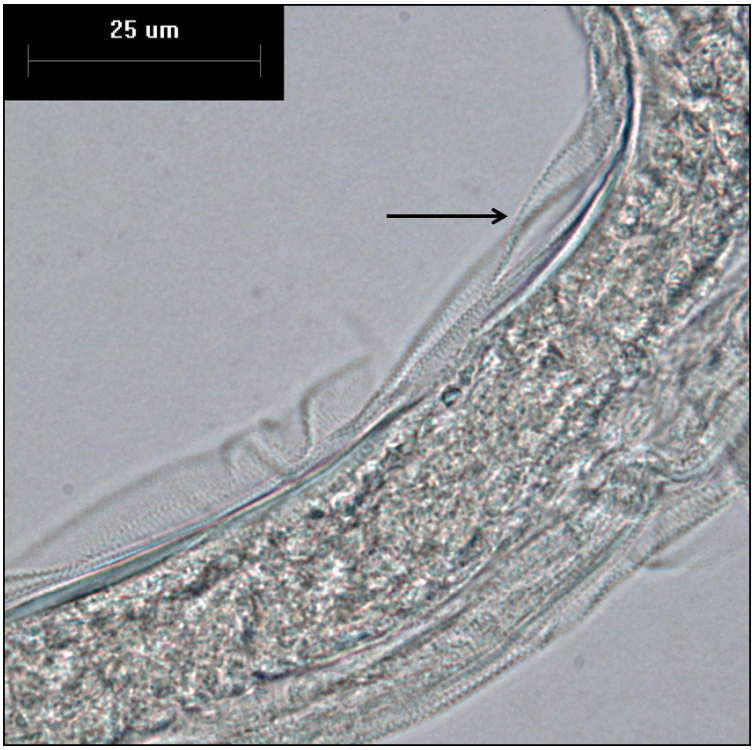

Fig. 5. A portion of the middle body of a third-stage juvenile of the rat lungworm (*Angiostrongylus cantonensis*) encased in the cuticles (arrow) of the first- and second-stage juveniles.

compared to that (1.5%) of the molecular method was due to a better tissue sampling procedure than that used for the molecular method during the first year. However, nematode detection increased (24%) when the two methods were used in combination for the same snail samples (Table 3). The majority of the RLW juveniles recovered from the macerated snail tissues on the 45-µm-pore sieve were coiled (Fig. 3) and consisted of J_3 encased in the cuticles of J_2 and sometime in those of J_1 and J_2 (Fig. 4). No J_1 were detected in this study. The morphometric values of these J_3 (n = 7) encased in the cuticles of J_2 were smaller than those reported for *A. cantonensis* J_3. The length and width of their body was 310 to 410 and 20 to 22 µm vs. 425 to 524 and 25 to 34 µm of *A. cantonensis*. Their esophagus length, excretory pore anterior body distance, and tail length ranged 155 to165.9, 60 to 82 and 33 to 39.6 µm, respectively, vs. 167 to 194, 78 to 105, and 34 to 44 µm of *A. cantonensis* (Ash, 1970). The genital primordium in the extracted J_3 in ethanol was not observable. These smaller morphometric values observed in our populations were due to the adverse effect of ethanol on the nematode body tissue that caused shrinkage and distortion of body organs. The J_3 tails were digitate (*sensu* Frederick and Tarjan (1989)) with round terminus (Fig. 5) as reported for *A. cantonensis* J_3 (Alicata, 1963). In spite of the shrinkage and distortion of the J_3 specimens recovered in ethanol, their morphological characters were congruent with those of *A. cantonensis* reported in literature. Identification of *A. cantonensis* based only on the morphological characters of the J_3 is not reliable and requires validation using molecular analysis. Our morphological identifications were all confirmed by molecular analyses after the examination of the specimens.

The results of molecular and morphological analyses of RLW indicated that the preferred snail tissues for the colonization by the nematode were those of the mantel rather than those of the foot of the snail. The percent of RLW juveniles detected in the mantel with both methods was 98% greater than that in the foot (Table 4). These findings support the observations made by Knapp (1966), who suggested that certain regions of the snail body were much richer in RLW than the foot and are in agreement with the findings of Brockelman *et al.* (1976), who stated that 85% of infective RLW juveniles found in GAS are located in the mantle tissue.

While the Q-PCR test procedure may be considered a more rapid method to detect and identify RLW present within a single sample of the fully minced mantle tissue, we could better verify the nematode presence in multiple snail samples through the combination of the PCR analysis with the morphological method.

Validation of the results of molecular and morphological analyses

Submitted positives PCR products (C4-10280; C4-14266-L1; C4-14266-L1) to CDC were confirmed as *A. cantonensis*. Absence of *A. cantonensis* was reported for the negative PCR products (C4-14266-R1). The submitted J_3 specimens extracted by the maceration method were not analyzed. The results of our analyses and their validation by the CDC provide definitive evidence of the presence the RLW, *A. cantonensis*, in GAS, in Florida. This is the first comprehensive identification of this pathogenic nematode infecting GAS in the state.

DISCUSSION

Our findings were used for a Press Release by FDACS on October 12, 2012, to inform the public and the operators of the GAS eradication program about the detection of the RLW in Florida GAS populations. In the interest of public health in the quarantine area of Miami, information on preliminary and unverified molecular detections of RLW in four specimens of GAS from 12 of the 19 cores in the Miami area was divulged early in the survey by the first author in a cooperative publication with Teem *et al.* (2013). This early unverified information is now validated by our data.

The presence of the rat lungworm in Florida was suspected even before the most-recent introduction of GAS. This assumption was based in part, on the death of a white-handed gibbon (*Hylobates lar*) at the Miami Zoo in 2003 (Duffy *et al.*, 2004) that died of complications caused by the ingestion of RLW. It is possible that this animal ate either contaminated produce or an infected snail from in or around its outdoor cage; however, follow-up surveys of gastropods in the immediate environs of the Miami Zoo yielded no positive snails (Teem *et al.*, 2013). Nonetheless, these authors speculated without providing data and citing a personal communication in 2010 with Dr. Christine Miller (Miami, Metro Zoo) that black rats presumably infected by RLW were found around the cage where the gibbon died in 2004.

Based on this report, the earlier findings, and as a public safety precaution, it was assumed from the beginning of the GAS eradication program that RLW was present in the GAS population. Therefore, a field handling and collection protocol, that included wearing gloves, frequent hand washing, and avoiding contact between hands and mouths, noses, or eyes after handling snails (Herwaldt, 2012; Capinera and Walden, 2013) was established to keep FDACS inspectors and researchers, as well as the general public from coming into contact with RLW. In addition, the general public was cautioned to carefully wash or cook all produce that may have been in contact with snails before eating (Zanini and Graeff-Teixeira, 2001). To date, no human illness due to rat lungworm has been reported in Miami-Dade County, but our results provide further proof of the importance of the precautionary measures in handling GAS in south Florida.

The higher number of positive PCR products from

mantle samples compared with those from the foot samples has led to a modified, and more precise, snail sampling procedure for the future in Florida. Snail sampling should include mainly tissues from portions of the mantle, rather than foot tissues in order to increase the probability of detecting RLW. Our morphological examination was likely more practically useful than the molecular detection method in this survey because it allowed for the quantification of the life-stages and total number of RLW in a sample. The use of ethanol for preserving RLW-infested snail tissues was likely not the best method for morphological identification of the nematode. The fixative formalin would probably have provided better RLW specimens for nematode identification. However, although recovery of life stages and overall quantification of nematodes may have been more practical with the morphological examinations, mechanical maceration of snail tissue in water combined with microscopic examination could fail to detect fragmented RLW bodies, which can be easily overlooked in the aliquot of water suspension being examined with the microscope. Therefore, the PCR method is more sensitive in detecting the presence of RLW in survey samples. Consequently, the combination of the two methods provides more reliable results than either technique alone.

As a final comment, we would like to point out that, based on our study, the distribution of the RLW appears to be limited to the areas in Miami where the GAS has been confined by the implementation of the eradication program. However, as implied by Teem *et al.* (2013), we cannot exclude the possibility that the RLW has other intermediary hosts among the numerous species of snails and other mollusks present in southern Florida.

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