Tropical Biomedicine 31(2): 327–335 (2014)

Identification of *Angiostrongylus cantonensis* and other nematodes using the SSU rDNA in *Achatina fulica* populations of Metro Manila

Constantino-Santos, D.M.A.[1*], Basiao, Z.U.[1], Wade, C.M.[2], Santos, B.S.[1] and Fontanilla, I.K.C.[1]
[1]Institute of Biology, College of Science, University of the Philippines, Diliman, Quezon City, 1101, Philippines
[2]School of Biology, University Park, University of Nottingham, NG7 2RD, United Kingdom
[*]Corresponding author email: daisymay_constantino@yahoo.com
Received 12 September 2013; received in revised form 3 January 2014; accepted 9 January 2014

Abstract. *Angiostrongylus cantonensis* is a parasitic nematode that causes eosinophilic meningitis in humans. Accidental infection occurs by consumption of contaminated intermediates, such as the giant African land snail, *Achatina fulica*. This study surveyed the presence of *A. cantonensis* juveniles in *A. fulica* populations from 12 sites in Metropolitan Manila, Philippines using the SSU rDNA. Fourteen distinct sequences from 226 nematodes were obtained; of these, two matched *A. cantonensis* and *Ancylostoma caninum*, respectively, with 100% identity. Exact identities of the remaining twelve sequences could not be determined due to low percent similarities. Of the sequenced nematodes, *A. cantonensis* occurred with the highest frequency (139 out of 226). Most of these (131 out of 139) were collected in just one area in Quezon City. Nematode infection of *A. fulica* in this area and two others from Makati and another area in Quezon City, respectively, were highest, combining for 95% of the total infection. *Ancylostoma caninum*, on the other hand, was detected in four different sites. *A. caninum* is a canine parasite, and this is the first report of the nematode in *A. fulica*. These results cause public health concerns as both *A. cantonensis* and *A. caninum* are zoonotic to humans.

INTRODUCTION

Angiostrongyliasis is a condition characterized by acute headache, visual disturbances, photophobia, neck stiffness, neck pain, hyperesthesias and paresthesias (Weller & Liu, 1993; Tsai *et al.*, 2003; Tomanakan *et al.*, 2008). This is caused by infection of the rat lungworm, *Angiostrongylus cantonensis* (family Angiostrongylidae) in humans (Cowie & Robinson, 2003; Li *et al.*, 2008). Rats are normally the final hosts of *A. cantonensis (*Anderson, 2000; Graeff-Teixeira *et al.*, 2009), while snails and slugs are used as intermediates (Lee & Yen, 2005). Humans, however, may be accidentally infected by consuming the intermediate host. Such infection is the most common cause of human eosinophilic meningitis. Cases of *A. cantonensis* infection have become prevalent throughout the Pacific, Southeast Asia, Africa, Australia and North, Central and South America (Kliks & Palumbo, 1992).

It has been proposed that *A. cantonensis* was introduced in Asia and the Pacific region during World War II, when its intermediate host, the giant African land snail, *Achatina fulica* (family Achatinidae), was used as an alternative food source by the Japanese (Alicata, 1966; Latonio, 1971). Once introduced, *A. fulica* can easily establish and spread in the area (Raut & Barker, 2002; Cowie & Robinson, 2003; Thiengo *et al.*, 2007). On average, an infected snail carries about 20 parasites. Highly infected ones, however, may harbor 500-2000 juveniles (Caldeira *et al.*, 2007). The ability of *A. fulica*

to carry large numbers of *A. cantonensis* and its dispersal by human means contribute to the spread and zoonosis of *A. cantonensis* not only to its natural hosts but also to accidental hosts, including humans (Cowie & Robinson, 2003; Li *et al.*, 2008). Cases of *A. cantonensis* infection in rodents and mollusks have been documented in the Philippines. Much of these studies detected *A. cantonensis* in several provinces in Luzon (Salazar & Cabrera, 1969; Westerlund & Chamberlain, 1969; Garcia, 1979; Antolin *et al.*, 2006;) and one area in the Visayas (Guerrero & Guerrero, 1972). Less studies, however, has been done to detect *A. cantonensis* in Metro Manila, which is inhabited by more than 11 million people.

Identification of nematodes in the environment is important to assess their potential risks to humans. Most nematodes present in intermediate hosts, however, are juveniles. In such stages, species are difficult to differentiate based on morphology. As an alternative, a small section of a DNA sequence from a standardized region of the genome can be used to identify species (Dasmahapatra & Mallet, 2006). The 5' end of the small subunit ribosomal RNA gene (SSU rDNA) has been used to differentiate soil nematodes including *Angiostrongylus* species (Floyd *et al.*, 2002; Fontanilla & Wade, 2008). It was proposed that two sequences belong to the same species if they are 99.5%–100% identical for the 450 bp of the 5' end of the SSU rDNA (Floyd *et al.*, 2002). Thus the identity of the species can be determined by matching the sequence to an accessible sequence database such as GenBank. Evaluating the role of *A. fulica* in the spread of *A. cantonensis* and potentially other nematodes is important in the Philippines, especially in the densely populated area of Metro Manila. This would allow proper assessment of the epidemiology of the diseases that they cause. The objectives of this study were to (1) detect the presence of the human-infective third-stage juveniles of *A. cantonensis* in *A. fulica* populations in Metropolitan Manila using the SSU rDNA as a genetic marker; and (2) to determine the prevalence of this nematode in these ubiquitous snails.

MATERIALS AND METHODS

Achatina fulica specimens were collected from 12 sites in Metro Manila (Table 1; Figure 1). These were cut into small pieces and digested overnight in Ash's digestive fluid (Ash, 1970). Nematodes were collected and stored at -20ºC prior to use. The following procedure adapted from Floyd *et al.* (2002) was performed to extract genomic DNA. Nematodes were incubated in 20µl 0.25M NaOH for five hours. After incubation, the tubes were heated for three minutes at 95ºC, cooled at room temperature and centrifuged.

Table 1. Areas sampled in this study

Site	City	District	Area type
CAL	Caloocan City	Camarin	Residential area
LPI	Las Pinas City	Moonwalk	Church grounds
MKT	Makati City	Santa Cruz	Public cemetery
MB1	Malabon City	Potrero	Residential area
MB2	Malabon City	San Agustin	Church grounds
MNL	Manila	Ermita	Hospital grounds
MRK	Marikina City	Tumana	Residential area
PAS	Pasay City	Baclaran	Public cemetery
QC1	Quezon City	Fairview	Residential area
QC2	Quezon City	Novaliches	Park area
QC3	Quezon City	Batasan Hills	Residential area
QC4	Quezon City	Diliman	School grounds

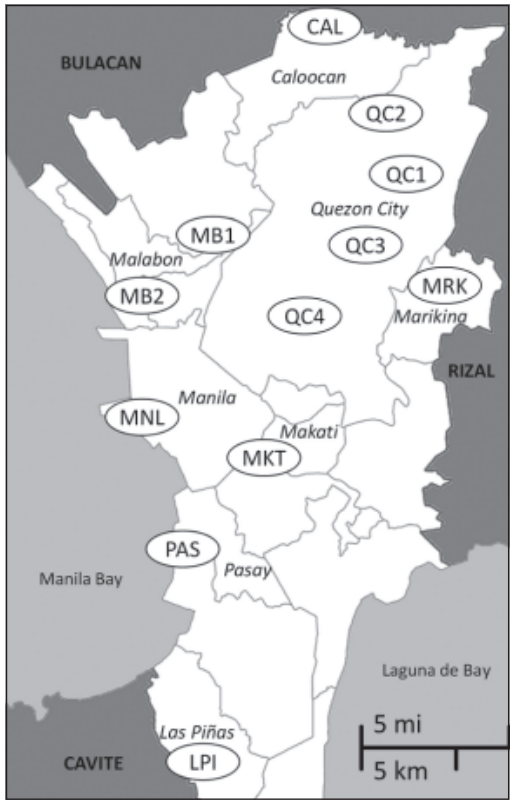

Figure 1. Location of sampling areas. CAL, Barangay Camarin, Caloocan City; LPI, Moonwalk Village, Las Pinas City; MKT, Santa Cruz, Makati City; MB1, Barangay Potrero, Malabon City; MB2, San Agustin, Malabon City; MNL, Ermita, Manila; MRK, Barangay Tumana, Marikina City; PAS, Baclaran, Pasay City; QC1, Barangay Fairview, Quezon City; QC2, Novaliches, Quezon City; QC3, Barangay Batasan Hills, Quezon City; QC4, Diliman, Quezon City

The following reagents were then added: 4µl 1.0M HCl; 10µl 0.5M Tris-HCl; and 5µl 2% Triton X-100. This was followed by another round of centrifugation, heating for three minutes at 95°C, and cooling at room temperature. The DNA extracts were stored at -20°C prior to use.

The first 480-bp from the 5' end of the SSU rDNA was amplified from the extracts (Fontanilla & Wade, 2008). The primers used were as follows: SSU_F_07 (sense) 5'- AAAGATTAAGCCATGCATG-3' and SSU_R_09 (anti-sense) 5'- AGCTGGAATTA CCGCGGCTG-3' (Blaxter *et al.*, 1998). The final volume was 50µl containing 5µL PCR buffer with 1.5mM MgCl2-, 1.0µL 10mM dNTPs, 2.5µL 10µM for each of the primers, 10µL Q buffer (Qiagen™, USA), 0.25µL 1.25 U Taq DNA polymerase (Roche, USA) and 4µl (5-20 ng/µL) DNA sample. The PCR running conditions were as follows: 94°C for 3 min and 43 cycles at 94°C for 30 s, 45°C for 30 s and 65°C for 1 min, and final extension at 72°C for 5 min. PCR products were visualized in 1% agarose gels with ethidium bromide. The PCR products were extracted from the gel using a Qiagen™ Gel Extraction Kit (Qiagen™, USA). The eluted PCR products were sent to First BASE Laboratories (Selangor, Malaysia) for sequencing of the anti-sense strand.

DNA sequences were assembled using the STADEN package version 1.5.3 (Staden *et al.*, 1994) and aligned using the BioEdit Sequence Alignment Editor (BioEdit v. 5.0.5; Hall, 1999) and subjected to Basic Local Alignment Search Tool on Genbank to determine if they matched *A. cantonensis* or any other nematode (Altschul *et al.*, 1990).

The prevalence of nematode infection for each site was computed by taking the percentage of snails infected with nematodes (Roberts & Janovy, 2005). The parasite load (number of nematodes present in infected snails) range of *A. fulica* for each site was also determined. Multiple infections of different parasites were likewise reported.

RESULTS

Nematode infection of *A. fulica* populations

A total of 365 snails were collected from the twelve sites surveyed. Nematode infection of *A. fulica* was observed in all of the sites and details of the infection rate and parasite load of snails per population are summarized in Table 2. Of the 365 snails sampled, 61 snails were infected with nematodes. The *A. fulica* population in QC2 had the highest infection rate with 19 out of 30 snails (63.3%) infected. The remaining populations did not exceed an infection rate of 23.3%. *Achatina fulica* in PAS and LPI had the lowest infection rate (only one snail in 30 or 3.3%) among the 12 populations sampled. A total of 965 nematodes were collected from the 61

Table 2. Percent infection and parasite load per site sampled for *A. fulica*. N, number of *A. fulica* specimens collected per site

Site	N	Infected Snails (% Infection)	Total Nematode Count (Parasite Load Range)
CAL	30	2 (6.7%)	3 (1-2)
LPI	30	1 (3.3%)	1 (1)
MKT	30	7 (23.3%)	202 (1-179)
MB1	30	4 (13.3%)	5 (1-2)
MB2	30	2 (6.7%)	6 (2-4)
MNL	30	3 (10.0%)	5 (1-3)
MRK	30	7 (23.3%)	8 (1-2)
PAS	30	1 (3.3%)	1 (1)
QC1	30	7 (23.3%)	640 (3-340)
QC2	30	19 (63.3%)	70 (1-10)
QC3	50	7 (14.0%)	18 (1-11)
QC4	15	1 (6.7%)	6 (1-5)
Total	365	61 (16.7%)	965 (1-340)

Table 3. Nematode sequences obtained in this study and their closest match in GenBank. Each Metro Manila Sequence (MMS) refers to a unique sequence

Sequence	Closest match	Identity	Frequency
MMS 1 (GB JX512217)	*Ancylostoma caninum*	100%	11 (4.9%)
MMS 2 (GB JX512218)	*Angiostrongylus cantonensis*	100%	139 (61.5%)
MMS 3 (GB JX512219)	*Oslerus rostratus*	99.5–99.7%	2 (0.9%)
MMS 4 (GB JX512215)	*Rhabditis* sp.	100%	11 (4.9%)
MMS 5 (GB JX512220)	*A. caninum*	99.3%	1 (0.4%)
MMS 6 (GB JX512221)	*Aphelenchoides* sp.	89.1%	1 (0.4%)
MMS 7 (GB JX512216)	*Caenorhabditis briggsae*	98.1%	1 (0.4%)
MMS 8 (GB JX512214)	*Oslerus rostratus*	98.2%	1 (0.4%)
MMS 9 (GB JX512222)	*Oslerus osleri*	99.1%	17 (7.5%)
MMS 10-14 (GB JX512209- JX512213)	*Cephaloboides nidrosiensis*	89.9-96.4%	42 (18.6%)

infected snails. Most of the infected snails carried a small number (1-11) of nematodes. Less than 20 nematodes were collected in each population, with the exception of the *A. fulica* populations in QC2 (70 nematodes), MKT (202), and QC1 (640). Three snails were heavily infected, two of which were from QC1 (212 and 340 nematodes, respectively) and another one from MKT (179 nematodes).

Distinct SSU rDNAsequences identified

Of the nematodes isolated, 226 individuals were subjected to DNA extraction, PCR amplification and sequencing. BLAST results for the SSU rDNA sequences obtained from the nematodes are summarized in Table 3, showing 14 distinct Metro Manila sequences (MMS).

MMS 1 from CAL, QC3, MKT, and MB1 matched the nematode, *Ancylostoma caninum* (GB AJ920347), with 100% percent identity. MMS 2 from QC1, QC4, and MKT matched *A. cantonensis* (GB GQ181114) with 100% identity. MMS3 from MKT matched *Oslerus rostratus* (GB GU946678) with 99.5% and 99.7% identity, respectively. A 100%

match for *Rhabditis* sp. (GB EU196004) was also detected from MMS4 from QC3. For the other sequences that did not have exact match (below 100%), the closest similarity based on BLAST was noted (Table 3). Some of the MMS have slightly different sequences but have the same closest match in GenBank, thus different identity scores were observed (see MMS 10-14; Table 3).

Of the 14 unique MMS detected, MMS 2 (*A. cantonensis*) occurred with the highest frequency. Out of the 226 nematode sequences, 139 (61.5%) matched *A. cantonensis*. The parasite was found in QC1, QC4 and MKT. The most diverse population of nematodes was found in MKT, where six distinct MMS were observed. Three distinct MMS were found in QC3, and only one MMS each in CAL, MB1, QC1 and QC4. MMS 10 – 14 from MRK and QC2 had *Cephaloboides nidrosiensis* (GB EU196020) as the closest match (Table 4).

Occurrence of infection by multiple nematode species

There were five cases observed wherein an individual snail is infected by more than one nematode species. A summary of these cases is shown in Table 5.

Table 4. Nematode infection for each site. Values are numbers of nematodes for each area exhibiting each of the MMS

Area	MMS1	MMS2	MMS3	MMS4	MMS5	MMS6	MMS7	MMS8	MMS9	MMS10 -14	TOTAL
CAL	3	–	–	–	–	–	–	–	–	–	3
LPI	-	–	–	–	–	–	–	–	–	–	0
MKT	1	2	2	–	–	1	–	1	13	–	20
MB1	1	–	–	–	–	–	–	–	–	–	1
MB2	–	–	–	–	–	–	–	–	–	–	0
MNL	–	–	–	–	1	–	–	–	–	–	1
MRK	–	–	–	–	–	–	–	–	–	2	2
PAS	–	–	–	–	–	–	–	–	–	–	0
QC1	–	131	–	–	–	–	–	–	–	–	131
QC2	–	–	–	–	–	–	–	–	4	40	44
QC3	6	–	–	11	–	–	1	–	–	–	18
QC4	–	6	–	–	–	–	–	–	–	–	6

Table 5. Individuals infected by more than one nematode species

Site	Species identified	Frequency
MKT (1)	MMS 1 (100% *Ancylostoma caninum*)	1/2 (50.0%)
	MMS 8 (98.2% *Oslerus rostratus*)	1/2 (50.0%)
MKT (2)	MMS 9 (99.1% *Oslerus osleri*)	12/14 (85.7%)
	MMS 4 (100% *Oslerus rostratus*)	2/14 (14.3%)
QC2	MMS 11 (91.1% *Cephaloboides nidrosiensis*)	1/5 (20.0%)
	MMS 9 (99.1% *Oslerus osleri*)	4/5 (80.0%)
QC3 (1)	MMS 1 (100% *Ancylostoma caninum*)	1/3 (33.3%)
	MMS 7 (98.1% *Caenorhabditis briggsae*)	2/3 (66.7%)
QC3 (2)	MMS 1 (100% *Ancylostoma caninum*)	1/2 (50.0%)
	MMS 7 (98.1% *Caenorhabditis briggsae*)	1/2 (50.0%)

DISCUSSION

The SSU rDNA detected *A. cantonensis* and other nematodes in *A. fulica* from Metro Manila

Based on Floyd *et al.*'s threshold of 99.5%-100% identity among sequences belonging to the same species, only *A. cantonensis* and *A. caninum* could be identified with certainty to species level based on GenBank BLAST results. Sequences that matched *Rhabditis* sp. were only assigned to that genus. Although two sequences from MKT matched *Oslerus rostratus* within the desired threshold, they could not be assigned to the said taxon with absolute certainty as less than 450 bases were available due to the poor quality of the sequences. For the remaining sequences, their exact identification could not be determined as of this time. A match below 99.5% is not uncommon because many nematodes are not yet barcoded; the sequences in GenBank are thus far from complete (Floyd *et al.*, 2002).

***Angiostrongylus cantonensis* were found in Quezon City and Makati City populations**

Of the 12 sites sampled, only three areas showed a high infection rate. Approximately 95% of the nematodes collected in this study were found in QC1, QC2, and MKT. These three varied in terms of habitat type (Table 1). The area types of the nine other sites also varied, showing no observable trend in parasite or even host preference.

Although *A. cantonensis* infection was shown to have the highest frequency in this study, this was concentrated in QC1, a residential area in Fairview, Quezon City, where 131 individuals of *A. cantonensis* was identified. Apart from that, only six were identified from QC3, two from MKT, and none from the rest of the areas studied. The abundance of *A. cantonensis* in only one area may be due to the uneven distribution of both the parasite and snail host. For instance, Biseru (1971) observed high variation in infection rates of *A. cantonensis* in *A. fulica* even within a small geographic area in West Malaysia, with even two populations exhibiting no infection. Further, the prevalence of the parasite is subject to the availability of the hosts, both definitive and intermediate, in order for the parasite to complete its life cycle. Thus, the distribution of rodents as definitive hosts may have had an effect on the observed distribution of *A. cantonensis*, but this was not determined in this study. Nonetheless, the high incidence of the nematode observed in certain areas is still a cause for concern.

***Ancylostoma caninum* was detected in four different Metro Manila areas**

This study demonstrates for the first time the presence of the hookworm *Anclylostoma caninum* in *A. fulica*. This nematode utilizes dogs as definitive hosts while its larval stages are free-living in the soil. The larvae infect the definitive host via skin penetration or ingestion (Hotez *et al.*, 1990; Roberts & Janovy, 2005; Franke *et al.*, 2011). These are most likely the same routes by which *A. fulica* is infected. It is alarming that *A. caninum* was detected in four areas of Metro Manila (namely, CAL, MKT, MB1, and QC3), and this finding is significant from an epidemiological perspective because *A. caninum* is also zoonotic to humans via skin penetration or oral ingestion (Hotez *et al.*, 1990). *Ancylostoma caninum* is a facultative skin penetrator, being able to degrade human fibronectin (Hotez *et al.*, 1990), which can cause cutaneous diseases (Croese *et al.*, 1994). Accidental ingestion, on the other hand, may lead to eosinophilic enteritis as manifested by a non-severe form of abdominal pain as *A. caninum* adapts poorly to the human host (Croese *et al.*, 1994).

Ancylostoma caninum infection in humans has been cited in Australia, United States, South America, Israel and even in the Philippines (Roberts & Janovy, 2005; Croese *et al.*, 1994). Such infections are usually associated with interaction with infected pets or contact with contaminated soil. This study presents another route via handling of contaminated snails.

Other nematodes were also recovered from *Achatina fulica*

All MMS were most similar with species that belong to the order Rhabditida, a

taxonomic group that includes free-living and parasitic species (Smyth, 1994). Of these, only *A. cantonensis* and *O. rostratus* (feline lungworm), both under suborder Strongylida, are known to use *A. fulica* as an intermediate host (Grewal *et al.*, 2003). Two other members of the Strongylida, *A. caninum* and *O. osleri*, are nematode parasites of dogs (Hotez *et al.*, 1990).

Two sequences from this study, MMS 4 and MMS 7, were most similar to two taxa from the family Rhabditidae, *Rhabditis* sp. and *Caenorhabditis briggsae*, respectively, and were collected from QC3. Different species of the genus *Rhabditis* use snails as hosts (Grewal *et al.*, 2003). *Caenorhabditis briggsae*, on the other hand, is a free-living soil nematode. As stated previously, it is unlikely that these nematode sequences are actually what they closely match due to the low percent identities (89.9–96.4%). Some nematodes become available in the soil and thus associate with invertebrate hosts upon entering the dauer stage. The nematode in this stage undergoes changes leading to developmental arrest for survival and dispersal in unfavorable conditions (Inoue *et al.*, 2007; Tissenbaum *et al.*, 2000). This may be true for those that matched *Cephaloboides nidrosiensis*. Members of the family Cephalobidae are usually free-living, and the recovery in *A. fulica* of taxa possibly related to cephalobids may be attributed to the dauer larval stage (Anderson *et al.*, 2009). Furthermore, some parasitic nematodes undergo this stage when the infective larval stages are deposited to the environment. The availability of the host triggers the exit of the larvae in the dauer stage (Tissenbaum *et al.*, 2000). *Ancylostoma caninum* is known to pass through this stage, making itself available in the soil and thus possibly accidentally infecting *A. fulica*.

In conclusion, *A. cantonensis* was detected in *A. fulica* populations in Metro Manila using the SSU rDNA; another nematode species, *A. caninum*, was also identified. Both species are able to infect humans and may pose a threat to public health. This study showed that *A. fulica* populations in Metro Manila are infected with nematodes zoonotic to humans. Their ability to spread and their close association with humans increases the risk of spreading the parasites they harbor and passing them on to humans. The results of this study necessitate an increase in public awareness, particularly in handling these snails. *A. fulica* populations in Quezon City and Makati may be further sampled since it is in these populations where the highest parasite load and highest nematode diversity were observed. Other regions of the Philippines should also be evaluated, particularly Mindanao, where no data is currently available.

Acknowledgements. The authors acknowledge the Office of the Vice Chancellor for Reasearch and Development (OVCRD) for the funding of this project, and logistical support provided by the Institute of Biology, University of the Philippines Diliman. Special thanks to Dr. Lydia R. Leonardo and Dr. Windell L. Rivera for their help in writing this manuscript.

REFERENCES

Alicata, J. (1966). The presence of *Angiostrongylus cantonensis* in the islands of the Indian Ocean and probable role of the giant African snail, *Achatina fulica*, in the dispersal of the parasite to the Pacific islands. *Canadian Journal of Zoology* **44**: 1041-1049.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., & Lipman, D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**: 402-410.

Anderson, R.C. (2000). Nematode Parasites of Vertebrates: Their Development and Transmission. 2nd ed., Wallingford: CABI Pubishing.

Anderson, R.C., Chabaud, A.G. & Willmott, S. (2009). Keys to the Nematode Parasites of Vertebrates. Wallingford: CABI Publishing.

Antolin, M.M., Joshi, R.C., Sebastian, L.S., Marquez, L.V. & Duque, U.G. (2006). Endo- and ecto parasites of the Philippine rice field rat, *Rattus tanezumi* Temminck, on PhilRice farms. *International Rice Research Notes* **31**: 26-27.

Ash, L.R. (1970). Diagnostic morphology of the third-stage larvae of *Angiostrongylus cantonensis*, *Angiostrongylus vasorum*, *Aelurostrongylus abstrutus*, and *Anafilaroides rostatus* (Nematoda: Metastrongyloidea). *Journal of Parasitology* **56**: 249-253.

Bisseru, B. (1971). The prevalence of *Angiostrongylus cantonensis* larvae collected from the giant African snail, *Achatina fulica*, in West Malaysia and Singapore. *Southeast Asian Journal for Tropical Medicine and Public Health* **2**: 523-526.

Blaxter, M.L., De Ley, P., Garey, J.R., Liu, L.X., Scheldeman, P., Vierstraete, A., Vanfleteren, J.R., Mackey, L.Y., Dorris, M., Frisse, L.M., Vida, J.T. & Thomas, W.K. (1998). A molecular evolutionary framework for the phylum Nematoda. *Nature* **392**: 71-75.

Caldeira, R.L., Mendonca, C., Goveia, C.O., Lenzi, H.L., Graeff-Teixeira, C., Lima, W.S., Mota, E.M., Pecora, I.L., De Medeiros, A.L.Z. & Carvalho, O.D. (2007). First record of molluscs naturally infected with *Angiostrongylus cantonensis* (Chen 1935) (Nematoda: Metastrongylidae) in Brazil. *Memorias Instituto Oswaldo Cruz* **102**: 887-889.

Cowie, R.H. & Robinson, D.G. (2003). Pathways of introduction of non-indigenous land and freshwater snails and slugs. *In*: G. Ruiz & J.T. Carlton, J.T. Invasive Species: Vectors and Management Strategies. Washington DC: Island Press; 93-122.

Croese, J., Loukas, A., Opdebeeck, J. & Fairley, S. (1994). Human Enteric Infection with Canine Hookworms. *Annals of Internal Medicine* **120**: 369-374.

Dasmahapatra, K.K. & Mallet, J. (2006). DNA barcodes: recent successes and future prospects. *Heredity* **97**: 254-255.

Floyd, R., Abebe, E., Papert, A. & Blaxter, M. (2002). Molecular barcodes for soil nematode identification. *Molecular Ecology* **11**: 839-850.

Fontanilla, I.C. & Wade, C.M. (2008). The small subunit (SSU) ribosomal (r) RNA gene as a genetic marker for identifying infective 3rd juvenile stage *Angiostrongylus cantonensis*. *Acta Tropica* **105**: 181-186.

Franke, D., Strube, C., Epe, C., Welz, C. & Schneider, T. (2011). Larval migration in PERL chambers as an *in vitro* model for percutaneous infection stimulates feeding in the canine hookworm *Ancylostoma caninum*. *Parasites and Vectors* **4**: 7.

Garcia, E.G. (1979). *Angiostrongylus cantonensis* in the Philippines: a review. *In*: J.H. Cross. Angiostrongyliasis in Eastern Asia and Australia. Taipei: NAMRU-2-SP-44; 53-56.

Graeff-Teixeira, C., Da Silva, A.C.A. & Yoshimura, K. (2009). Update on Eosinophilic meningoencephalitis and its clinical relevance. *Clinical Microbiology Reviews* **22**: 322-348.

Grewal, P., Grewal, S., Tan, L. & Adams, B.J. (2003). Parasitism of molluscs by nematodes: types of associations and evolutionary trends. *Journal of Nematology* **35**: 146-156.

Guerrero, L.A. & Guerrero, R.I. (1972). *Angiostrongylus cantonensis* in commercial rats in Dumaguete City, Negros Oriental. *Acta Medica Philippina* **8**: 33-35.

Hall, T. (1999). Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98.

Hotez, P., Haggerty, J., Hawdon, J., Milstone, L., Gamble, H.R., Schad, G. & Richards, F. (1990). Metalloproteases of infective *Ancylostoma* hookworm larvae and their possible functions in tissue invasion and ecdysis. *Infection and Immunity* **58**: 3883-3892.

Inoue, T., Ailion, M., Poon, S., Kim, H.K., Thomas, J.H. & Sternberg, P.W. (2007). Genetic analysis of dauer formation in *Caenorhabditis briggsae*. *Genetics* **177**: 809-818.

Kliks, M.M. & Palumbo, N.E. (1992). Eosinophilic meningitis beyond the Pacific Basin: the global dispersal of a peridomestic zoonosis caused by *Angiostrongylus cantonensis*, the nematode lungworm of rats. *Social Science & Medicine* **34**: 199-212.

Latonio, A.A. (1971). The giant African snail, *Achatina fulica*: a new threat to public health. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **65**: 22.

Lee, J. & Yen, C. (2005). Protease secreted by the infective larvae of *Angiostrongylus cantonensis* and its role in the penetration of mouse intestine. *American Journal of Tropical Medicine and Hygiene* **72**: 831-836.

Li, H., Xu, F., Gu, J. & Chen, X. (2008). Case Report: A severe eosinophilic meningoencephalitis caused by infection of *Angiostrongylus cantonensis*. *American Journal of Tropical Medicine and Hygiene* **79**: 568-570.

Raut, S.Y. & Barker, G.M. (2002). *Achatina fulica* Bowdich and other Achatinidae as pests in tropical agriculture. *In*: G.M. Barker. Molluscs as Crop Pests. Hamilton: CABI Publishing; 55-114.

Roberts, L. & Janovy, J.J. (2005). Gerald D. Schmidt and Larry S. Roberts' Foundations of Parasitology. 7th ed., USA: McGraw–Hill Co.; 728.

Salazar, N.P. & Cabrera, B.D. (1969). *Angiostrongylus cantonensis* in rodent and molluscan hosts in Manila and suburbs. *Acta Medica Philippina* **6**: 20-25.

Smyth, J.D. (1994). Introduction to Animal Parasitology. 3rd ed., Australia: Cambridge University Press; 572.

Staden, R., Beal, K.F. & Bonfield, J.K. (1994). The Staden package. *Methods in Molecular Biology* **132**: 115-130.

Thiengo, S.C., Faraco, F.A., Salgado, N.C., Cowie, R.H. & Fernandez, M.A. (2007). Rapid spread of an invasive snail in South America: the giant African snail, *Achatina fulica*, in Brasil. *Biological Invasions* **9**: 693-702.

Tissenbaum, H.A., Hawdon, J., Perregaux, M., Hotez, P., Guarente, L. & Ruvkun, G. (2000). A common muscarinic pathway for diapause recovery in the distantly related nematode species *Caenorhabditis elegans* and *Ancylostoma caninum*. *Proceedings of the National Academy of Sciences* **97**: 460-465.

Tomanakan, K., Srisurach, N., Sae-Tung, S. & Pengpinich, C. (2008). Detection of circulating antibody of *Parastrongylus cantonensis* in sera with Eosinophilic meningitis by Dot-Blot ELISA. *Journal of the Medical Association of Thailand* **91**: 1082-1086.

Tsai, H.C., Liu, Y.C., Kunin, C.M., Lai, P.H., Lee, S.S., Chen, Y.S., Wann, S.R., Lin, W.R., Huang, C.K., Ger, L.P., Lin, H.H. & Yen, M.Y. (2003). Eosinophilic meningitis caused by *Angiostrongylus cantonensis* associated with eating raw snails: correlation of brain Magnetic Resonance Imaging scans with clinical findings. *American Journal of Tropical Medicine Hygiene* **68**: 281-285.

Weller, P.F. & Liu, L.X. (1993). Eosinophilic meningitis. *Seminars in neurology* **13**: 161-168.

Westerlund, N.C. & Chamberlain, M. (1969). Further observations on *Angiostrongylus cantonensis* in the Philippines. *Acta Medica Philippina* **6**: 3-11.