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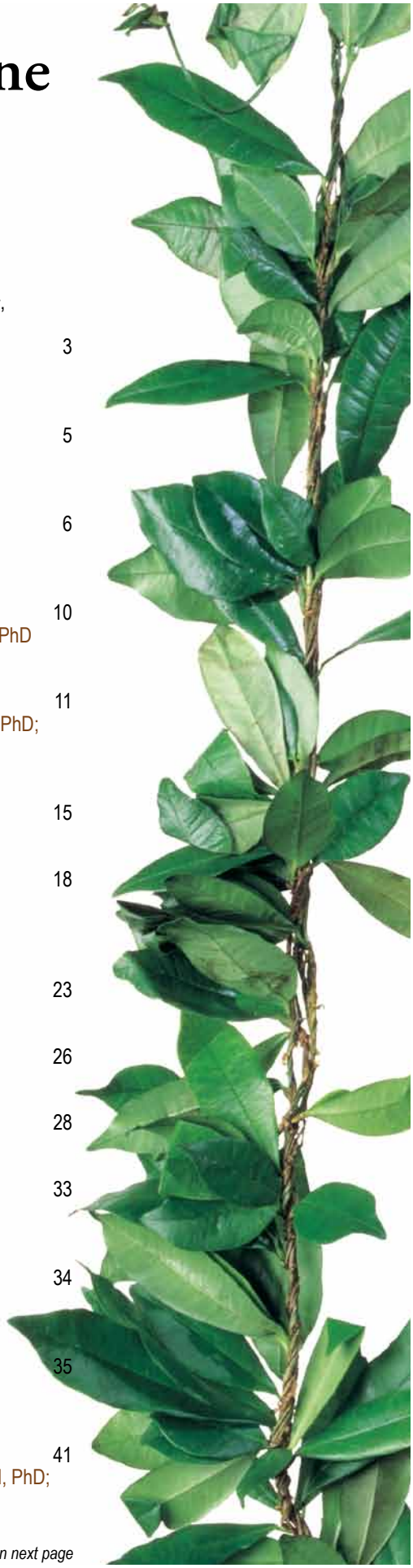

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Guest Editor's Message: Eosinophilic Meningitis Caused by *Angiostrongylus cantonensis*, the Rat Lungworm: Biology, Distribution, Epidemiology, Detection, Diagnosis, Treatment, and Management

Robert H. Cowie PhD

One of the major causes of eosinophilic meningitis is infection by the nematode *Angiostrongylus cantonensis*, the rat lungworm. *Angiostrongylus cantonensis* was first described from southern China, but has now spread to many parts of the world as a result of the human-associated spread of both its definitive and intermediate hosts (rats and snails/slugs, respectively). The first cases in Hawai'i were reported in 1961 but the disease has attracted increasing attention in Hawai'i following outbreaks over the last decade.

The disease is contracted when people ingest the immature worms that are carried by snails and slugs. Ingestion is most often inadvertent - a small baby slug among lettuce leaves for instance. However, in some parts of the world raw snails are a delicacy and their ingestion can lead to disease, and there are cases, including one in Hawai'i, in which people have deliberately eaten uncooked slugs, either on a dare when drunk, or for a bet.

The worms begin their development in rats, which pass them in their feces. The infected feces are eaten by snails and slugs in which the worms develop further. The snails and slugs are then eaten by rats, in which the worms develop further and reproduce, with this, the natural cycle, then repeating.

But if instead of rats, people eat the snails or slugs, the worms develop but die once they reach the central nervous system, particularly the brain. The physical damage to the nervous system and brain caused by the worms' movements, combined with the strong immune reaction caused by the dead worms that results in serious inflammation, can lead to symptoms including headache, stiff neck, numbness, tingling or pain in the skin, fever, nausea and vomiting, blurred vision, weakness, joint pain, and neurological abnormalities. More severe symptoms can include paralysis of the legs, bowel and bladder dysfunction, seizures, coma, and (rarely) death.

Angiostrongyliasis is mainly a tropical disease, but with the increasing spread of invasive species, including rats and molluscs, and global warming, which may increase the hosts' potential range, it has become an important emerging infectious disease. Stimulated by the increasing number of cases in Hawai'i during the last decade, an international transdisciplinary scientific workshop on angiostrongyliasis was held August 16-18, 2011, in Honolulu,[1] expanding on a previous workshop in Bangkok in 2010.[2] The workshop convened scientists and clinicians from places as far apart as Brazil, China, Jamaica, Taiwan, Thailand, the mainland United States, and Hawai'i, with expertise in a much broader range of fields than was represented at the Bangkok workshop, spanning ecology, parasitology, epidemiology, detection, diagnosis, treatment, and food safety. The workshop's goal was to develop a rigorous and concerted research agenda to address rat lungworm disease at a global scale through advancing an integrated understanding of all aspects of the disease. A prioritized list of objectives was developed[1] and the top needs in eight areas, as identified in this list, are presented in Table 1.

There is a need to raise awareness and understanding of angiostrongyliasis within the medical community as well as the general public. This special issue of the Hawai'i Journal of Medicine and Public Health is part of this effort. The issue includes 24 articles. Of these, 23 represent or expand on presentations made at the workshop and range from describing the basic life-cycle of the parasite, the diversity of molluscan hosts, and detection of the parasite, to diagnosis and treatment of the disease. An additional article that was not part of the workshop describes research that addresses important food safety aspects of prevention of infection and adds an additional dimension to the diversity of the workshop-derived articles. Most of the articles (18) are full papers, four are extended versions of the original abstracts of the workshop presentations, and two are the original workshop abstracts. All the full papers were reviewed by at least two reviewers; abstracts were reviewed by the guest editor. The articles represent work undertaken in all the major regions of the world in which angiostrongyliasis is emergent.

This is the first time that such a comprehensive diversity of articles on angiostrongyliasis has been brought together in a single publication. As such, it is hoped that it will provide an informative overview of the disease as well as an entry to the rapidly increasing body of literature dealing with all aspects of it.

The workshop website (http://www.hawaii.edu/cowielab/Angio%20website%20home.htm) provides access to all the workshop presentations, although some were modified so that as yet unpublished data are not available. A summary of the workshop has been published in the on-line version of the journal *Emerging Infectious Diseases*.[1]

Conflict of Interest

The author identifies no conflict of interest.

Acknowledgments

Thanks are due to the co-organizers of the workshop: Dr. Alex da Silva (Centers for Disease Control and Prevention, Atlanta, Georgia), Dr. Robert Hollingsworth (US Department of Agriculture, Hilo, Hawai'i), and James Hollyer (College of Tropical Agriculture, University of Hawai'i, Manoa). Thanks are also due to the 28 people who acted as reviewers of the full article manuscripts, some of them reviewing more than one manuscript. The workshop could not have taken place without the administrative and logistical assistance of Vanessa Troegner, Janice Tamanaha, Stacy Yamasaki-Ige, and Dave Au. Funding for the workshop and for this publication was provided by the National Institute of Food and Agriculture, United States Department of Agriculture, through Award No. 2011-65213-29954.

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Table 1. Identified Priorities for Research and Outreach: the Top Five Needs Identified in Each Focal Area (more than five if projects tied for fifth place).

Focus 1. Detection of *Angiostrongylus cantonensis* in hosts
Genomics / proteomics: sequence genome / develop proteomics for fast detection
Obtain comparative data on sensitivity and specificity of available techniques
Develop methods of parasite detection in fresh human food mainly vegetables and fruits
Sample other potential hosts, notably flatworms and freshwater crustaceans, to assess their potential as hosts and their parasite load
Develop 'low tech' detection methods
Gain a better understanding of the biology of the hosts as it relates to parasite transmission
Focus 2. Control of hosts in the field (rats, slugs/snails, paratenic hosts)
Identify paratenic hosts, their relevance and importance
Gain a better understanding of the basic biology of snails and slugs, including genetics, which could be useful in developing interventions
Undertake surveys of rats in areas where *A. cantonensis* has been reported (eg, south Florida, Rota)
Develop cultural methods of snail/slug control, such as natural barriers (eg, sand)
Gain a better understanding of the environmental variables that affect slug and snail host survival and reproduction, eg, humidity, temperature, etc, and the potential effects of climate change / global warming
Focus 3. Public education to minimize chance of infection
Involve children (ages 7-14) in educational efforts and build education about angiostrongyliasis into science/math (STEM) curriculum (in the United States there may be a National Science Foundation GK-12 grant opportunity)
Require continuing education for health care practitioners
Better define risk factors so that these can be the focus of education
Increase outreach to farmers and farmers' markets. Focus on potential impact on profits
Use social media networks, eg, Facebook, Twitter, etc., and contribute regularly
Define public health messages clearly and consistently
Create an angiostrongyliasis listserve
Focus 4. Control of hosts / larvae on produce (eg, washing / rinsing)
Evaluate different rinse ingredients
In the United States, obtain EPA and/or FDA approval of methods for washing produce, and similarly in Hawai'i get approval from the Departments of Agriculture and Health, as well as other regulatory agencies
Undertake surveys to ascertain the distribution of larvae and hosts, including slugs/snails on different kinds of fresh produce
Develop a hand held LAMP (loop-mediated isothermal amplification) device or other simple methods for detection of *A. cantonensis* in the field
Investigate irradiation of produce as a sanitizing method
Focus 5. Diagnosis
Improve and standardize serology
Develop rapid tests for detection, eg, PCR, antigen detection, chromatography, 'dipsticks'
Standardize clinical criteria for diagnosis
Validate PCR or other molecular methods for detection of *A. cantonensis* in patients
Develop a cooperative network for sharing specimens, antigens and DNA sequences
Focus 6. Treatment
Undertake well thought-out clinical trials
Assess the value of early use of anthelmintics
Standardize the protocol for lumbar puncture (LP), eg, are serial/repeat LPs beneficial; how often should they be done?
Develop guidelines for the use of steroid therapy, eg, when to start, dosage, rate of tapering off
Determine what should be the standard of care?
Focus 7. Pathophysiology
Determine the actual mechanism of neurological injury in humans: (i) increased intra-cranial pressure, (ii) the inflammatory reaction, and if so which cytokines are involved, (iii) mechanical damage from worm migration, or (iv) a combination of these
Develop the best animal model for human disease
Assess the influence of parasite inoculum on incubation period and severity of the illness, in particular how the number of parasites in the inoculum correlates with the number of parasites reaching the brain
Determine how the parasites invade the central nervous system (CNS)
Determine at which larval stage intervention (anthelmintics) will prevent symptoms
Investigate pathophysiology in infected hosts (slugs/snails, rat, paratenic hosts)
Investigate the mechanism by which steroid treatment alleviates symptoms: inflammation reduction or reduction of intra-cranial pressure
Focus 8. Epidemiology
Refine understanding of risk factors
Develop better tools for molecular epidemiology
Standardize the methodology of environmental assessments in terms of location characteristics and the geographical distribution of the parasite and the disease in a region
Develop centralized reporting of epidemiological findings
Determine the relationship between infection and disease: what triggers the disease, how the level of exposure is related to incidence of the disease

The Discovery of Humans in Hawai'i Infected with *Angiostrongylus cantonensis*, and Early Epidemiological Findings

Gordon D. Wallace DVM, MPH

Abstract

An epidemic of eosinophilic meningitis occurred in Hawai'i in 1958, and was presumed to have been caused by a parasitic infection. There were no fatal cases and the source of the infection was not known. In the course of investigating the epidemic, it was learned that two patients who had died at the State Mental Hospital on O'ahu in December 1959 and January 1960 had eosinophilic meningitis. The preserved brain of one patient yielded a number of young adult nematodes identified as Angiostrongylus cantonensis*; the other contained possible nematode remnants.[1] Surveys of rats around the State Mental Hospital showed that 23% were positive for* A. cantonensis*, and, knowing that the life cycle of this parasite involved snails as intermediate hosts, it seemed likely that the two patients had eaten snails.[1] Was this parasite also the cause of the thousands of cases of eosinophilic meningitis then recently reported in French Polynesia? Surveys of rats in the Society Islands showed them to be heavily infected with* A. cantonensis*,[2] and while snails were not eaten raw in the Society Islands, freshwater prawns, which can act as paratenic hosts, were eaten raw and were thought to be the source of infection.[3] Fish were also suggested as possible paratenic hosts that might provide a pathway for human infection.[4] Infected rats were found on many other Pacific islands and cases of eosinophilic meningitis had been reported from some of them. No cases were found on islands where the parasite was absent, consistent with* A. cantonensis *as the etiological agent.[1,3] This early epidemiological research established the association between the presence of rat lungworm (*A. cantonensis*) and cases of human eosinophilic meningitis.*

Keywords

Angiostrongyliasis, Epidemiology, Pacific islands, Tahiti

Conflict of Interest

The author identifies no conflict of interest.

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Biology, Systematics, Life Cycle, and Distribution of *Angiostrongylus cantonensis*, the Cause of Rat Lungworm Disease

Robert H. Cowie PhD

Abstract

Angiostrongylus cantonensis *is a metastrongyloid nematode in the family Angiostrongylidae. It is the cause of angiostrongyliasis (rat lungworm disease), which manifests as eosinophilic meningitis. First described in 1935 from rats in China,* A. cantonensis *was placed in the genus* Parastrongylus *in 1986, but most workers have not adopted this treatment. The taxonomy of* A. cantonensis *and related worms is largely based on adult morphology, notably of the male bursa. However, identification of infective third stage larvae is more difficult. The natural life cycle involves rats as the definitive host and snails or slugs as the intermediate host. Human infection, as accidental hosts, results in worms maturing in the brain, but dying there instead of moving back into the bloodstream, as in rats, thereby leading to eosinophilic meningitis. The disease is an emerging infectious disease;* Angiostrongylus cantonensis *continues to be reported in new regions beyond its native range.*

Keywords

Angiostrongyliasis, Angiostrongylus cantonensis*, Emerging infectious disease, Eosinophilic meningitis, Parasitology, Rat lungworm disease, Slugs, Snails*

Introduction

The parasitic nematode (roundworm) *Angiostrongylus cantonensis* (Chen, 1935) is responsible for the human disease known as angiostrongyliasis or rat lungworm disease, a major cause of eosinophilic meningitis (or meningoencephalitis),[1-3] and with symptoms ranging from mild headache, through a range of neurologically induced debilitation, to coma and occasionally death,[4,5] the severity probably being related to the number of worms present, their exact location, and the intensity of the host's inflammatory reaction. It is an emerging infectious disease[6,7] and it is important that understanding of all aspects of its biology, epidemiology, diagnosis, and treatment is increased and made more widely known. This short paper reviews key aspects of the basic biology, parasitology, and geographic spread of the causative agent, *Angiostrongylus cantonensis*, providing an avenue into the more detailed literature.

Systematics

Classification and Diversity

Angiostrongylus cantonensis is a nematode (phylum Nematoda) in the superfamily Metastrongyloidea and family Angiostrongylidae.[8] The systematics of the Angiostrongylidae is not well understood, with many species inadequately described and probably others not yet recognized, a situation that has advanced but little since Anderson[8] expressed the hope over 30 years ago that this might improve. There are around 20 species in the genus *Angiostrongylus* globally[9,10] Two of these cause disease in humans: *Angiostrongylus costaricensis* Morera & Céspedes, 1971, which causes abdominal angiostrongyliasis, and which is a problem especially in South and Central America,[10,11] and *Angiostrongylus cantonensis* (Chen, 1935), which causes eosinophilic meningitis, is spreading rapidly to many parts of the world, and is the subject of this paper.

Angiostrongylus cantonensis was first described from rats in China by Chen in1935[12] and placed in the genus *Pulmonema*, as *Pulmonema cantonensis*. The same species was also described a short time later in 1937 as *Haemostrongylus ratti* by Yokogawa[13], who did not realize that it was the same species that had been described already by Chen. The genus *Pulmonema* was subsequently synonymized with *Angiostrongylus* and the species name *ratti* was synonymized with *cantonensis*.[14] These nomenclatural changes were widely accepted so that the most common and widely used name for the species became *Angiostrongylus cantonensis*.

However, in 1986, Ubelaker[15] split the genus *Angiostrongylus* into five genera, based largely on their anatomy, assigning species to each of the genera, based on morphology but also on the definitive host, as follows: *Angiostrongylus* (found in carnivores, eg, dogs, foxes, cats), *Parastrongylus* (murids, eg, mice and rats), *Angiocaulus* (mustelids, eg, martens), *Gallegostrongylus* (gerbils and one murid), *Stefanskostrongylus* (insectivores, eg, shrews, tenrecs, etc.). *Angiostrongylus cantonensis*, the definitive host of which is rats, was thus transferred to the genus *Parastrongylus*, becoming *Parastrongylus cantonensis*. However, although occasionally used,[16,17] this classification has not been widely adopted and the species continues to be referred to most widely as *Angiostrongylus cantonensis*.

Morphology and Identification

Angiostrongylids are roundworms (nematodes) with thin cylindrical bodies. Research has focused primarily on *Angiostrongylus cantonensis* and species closely related to it.[2,10,15,18-22] *Angiostrongylus cantonensis* and *A. mackerrasae* (which was misidentified[20] as *A. cantonensis* by Mackerras and Sandars[18] in their detailed study) are extremely similar in size and anatomy,[20] and the following data of Mackerras and Sandars for *A. mackerrasae* refer equally to *A. cantonensis*. First stage larvae are about 0.3 mm long and 0.015 mm in width; second stage larvae are about 0.45 by 0.03 mm; third stage larvae are similar in size, though a little thinner; fourth stage larvae reach about 1.0 by 0.4 mm. The newly molted sub-adults are about 2 mm by 0.06 mm; they grow to about 12 mm (females) and 11 mm (males) before leaving the rat's brain and migrating to the pulmonary arteries (see the life cycle section, below), where they mature, reaching a size of up to about 35 by 0.6 mm (females) and 25 by 0.4 mm (males). A number of publications have provided good descriptions of *A. cantonensis*.[10,20,23]

Genera and subgenera of Angiostrongylidae can be distinguished based on the appearance of the adult male caudal bursa, the apparatus used to clasp the female during mating.[8] However, species of *Angiostrongylus* have rather few characters that serve to distinguish them and they are therefore difficult to identify. Nonetheless, adult males can be distinguished and identified based again on the appearance of the caudal bursa,[15] although there is some intraspecific geographic variation in this structure.[10] Third stage larvae can be distinguished from closely related species based on the appearance of the tip of the tail.[20,21,23]

Life Cycle

The most detailed accounts of the life cycle of an *Angiostrongylus* species are those of Mackerras & Sandars[18] for *A. mackerrasae* (which was not distinguished from *A. cantonensis* at the time[21,24]). The life cycle of *A. cantonensis* is essentially the same[2,3,5,21,24-26] and is summarized here (Figure 1). First stage larval worms are expelled in the feces of rats (the definitive host). Various species of rats can act as hosts.[2] These infected feces are ingested by snails or slugs (intermediate hosts), but it may also be possible that the larvae enter the snail by penetrating the body wall or via the respiratory pore.[2,3] Many species of snails and slugs can act as intermediate hosts.[2,27-29] The larvae develop to the third larval stage in the snails, remaining at that stage until either the snail is eaten or dies. Once snails carrying third stage larvae are eaten by a rat, they move through the rat gut to the small intestine. They then penetrate the walls of the intestine and enter the blood stream. They then travel passively in the blood stream, a proportion of them eventually entering the central nervous system and reaching the brain. Once in the brain the larvae develop to the sub-adult stage. Light infections appear to cause little damage to the brain and no obvious behavioral or other reaction, but heavy infections may cause more serious damage as well as behavioral symptoms.[18]

Having reached the sub-adult stage the worms leave the brain, passing into the venous circulatory system, and thence to the right ventricle of the heart and to the pulmonary arteries. Here the worms grow and mature, mate, and the females lay eggs. The eggs travel in the blood stream to the lungs (hence the name rat lungworm disease). The eggs hatch into first stage larvae in the tissue of the lungs. Depending on the level of infection, the rat may suffer significant damage to the arteries, caused by the bulk of the adult worms, and to the lungs, caused by inflammatory reactions to the larvae.[18] These first stage larvae then break through the walls of the bronchioles and alveoli, move up the trachea in respiratory secretions, and are swallowed, to be released in the feces. The cycle then repeats when snails ingest these infected feces. Assuming the rat eats the infected snail as soon as the larvae in the snail reach the third stage, the cycle takes about 45 days.

Human Infection

Humans (accidental host) become infected primarily in the same way that rats do, by ingesting snails or slugs infected with third stage larvae either deliberately or accidentally.[2,6] Larval development (Figure 2) follows the same course as in rats, up to the point at which the larvae reach the sub-adult stage in the person's brain. Most of these worms are then unable to re-enter the circulatory system and after moving around within brain tissue they die. Neurological damage appears to be caused both by the physical damage caused by the movement of the worms in the brain and by the inflammation caused by the immune reaction to the worms, which seems to be a more intense reaction to

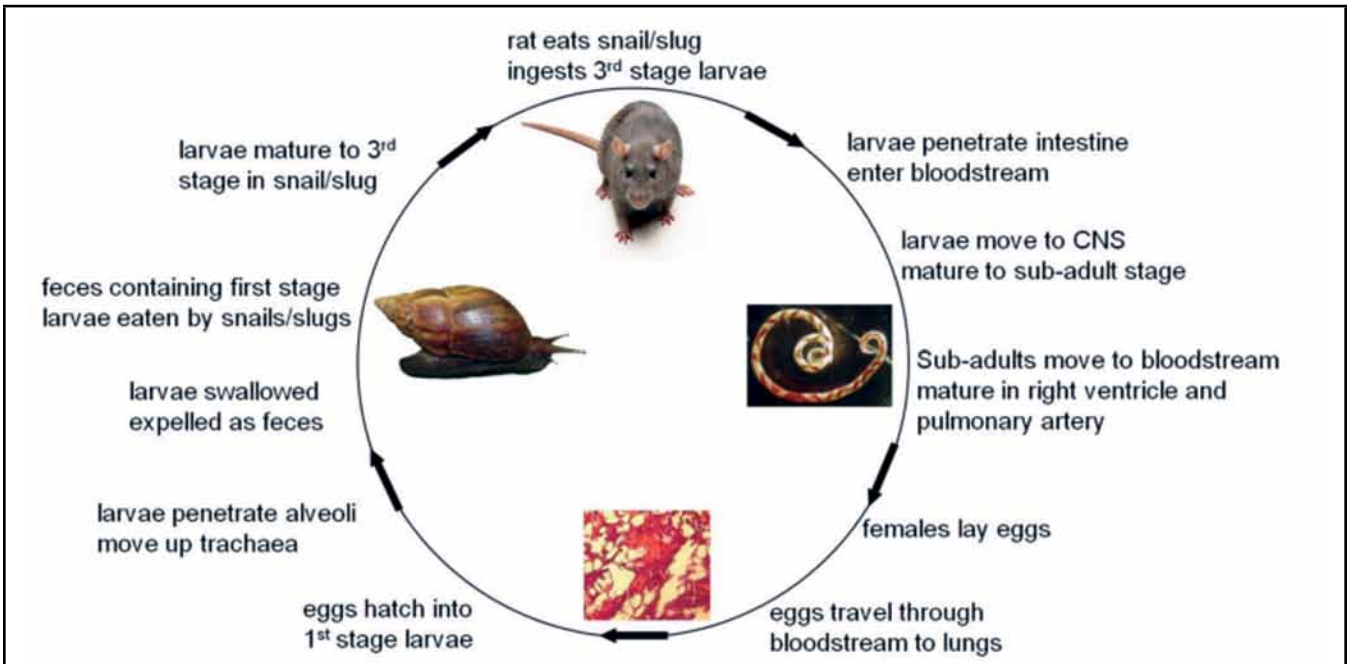

Figure 1. The natural life-cycle of *Angiostrongylus cantonensis*, the rat lungworm.

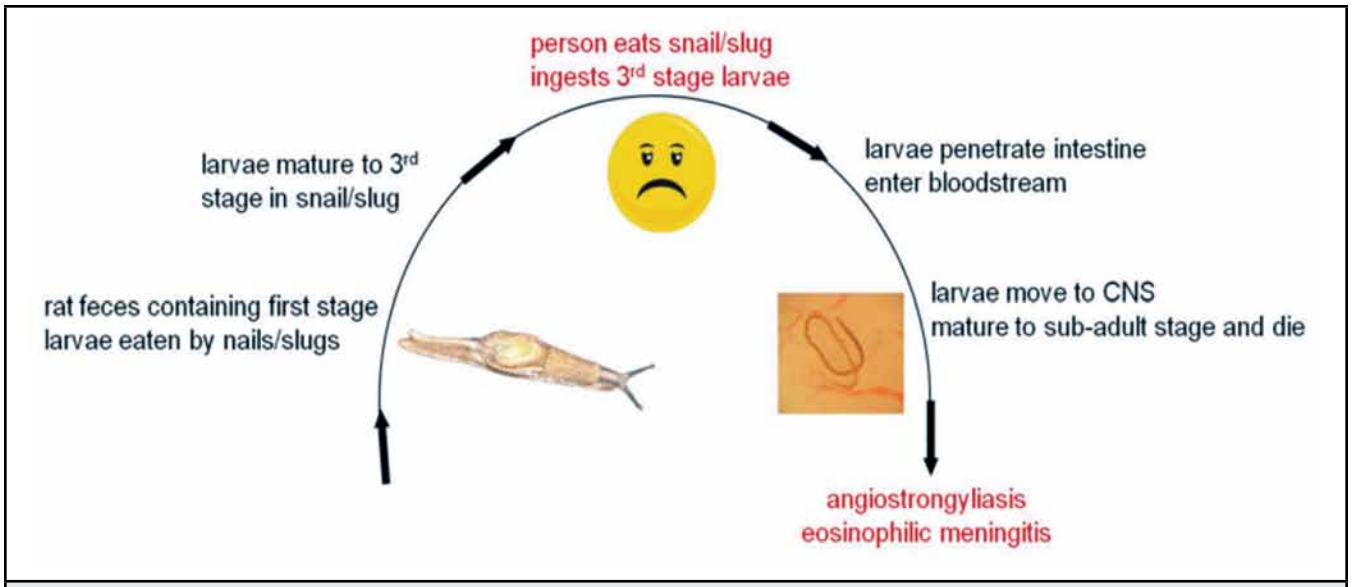

Figure 2. The truncated life-cycle of *Angiostrongylus cantonensis*, the rat lungworm, in an accidental (human) host.

the dead than to the live worms.[22,30,31] Some worms apparently do find their way to the pulmonary artery and lungs, but do not reproduce.[5,22,25,32] Worms have also appeared in the eyes.[2,33,34] as happens with infection by other neurotropic parasites.[22] Other animals have also been shown to be susceptible to infection as accidental hosts.[16,35-37]

Humans can also be infected by ingesting paratenic hosts, which are hosts in which *A. cantonensis* larvae cannot develop but remain alive for some time.[19,38,39] Paratenic hosts, which include a range of other animals (eg, freshwater shrimp, frogs, flatworms), become infected by eating infected intermediate hosts. If the intermediate hosts contain third stage larvae, these can then be passed to the person, in whom they develop and eventually die, as above.

Distribution of *Angiostrongylus cantonensis*

Angiostrongylus cantonensis was described from southern China in 1935.[12] It was reported from Taiwan in 1937 and subsequently from other parts of Southeast Asia (Thailand, Malaysia).[2] It probably originated somewhere in this region. Also by the 1960s, it had been reported from numerous Pacific islands,[2,40] including New Caledonia, Vanuatu, Fiji, Guam, Saipan, Chuuk, Pohnpei, Marshall Islands, Tahiti, Cook Islands, and Hawai'i. Reports of cases of eosinophilic meningitis in many of these islands probably reflected the spread of *A. cantonensis*.[1,2] It seemed to be confined to the tropics. Subsequently, it has been recorded widely,[41] including in Okinawa[42] and mainland Japan,[43] Papua New Guinea,[30] American Samoa,[4] Indonesia,[30] the Philippines,[30] Australia,[24] Sri Lanka,[33,30] India,[34,30] Réunion,[26] Mauritiuis,[30] Ivory Coast,[26] Egypt,[44] South Africa,[45] Madagascar,[30] Cuba,[46] Jamaica,[6] Puerto Rico,[30] Haiti,[47] Dominican Republic,[48] Ecuador,[49] Brazil,[10,23] the Canary Islands,[17] and the southeastern USA;[16,50] the list may not be comprehensive. It has the potential to expand its range beyond the tropics, facilitated by climate change, as shown in China.[28] There are increasing numbers of cases recorded in locations where *A. cantonensis* is not present (eg, various European countries and northern USA), mostly in people returning from regions where it is present.[6,41,51-53]

The rapid spread of the giant African land snail, *Achatina fulica* (sometimes referred to as *Lissachatina fulica*), has been suggested as a key factor in the spread of *A. cantonensis*, first across the Pacific and then to other regions of the world,[54] but this hypothesis is not well supported.[24,55] *Achatina fulica* is a good host of *A. cantonensis*,[27,28] and in areas where it has invaded it has become abundant in urban and other anthropogenic habitats where people readily come into contact with it.[56] But other invasive snail species are also good hosts,[27,28] for instance, the semi-slug *Parmarion martensi*, which is abundant in some residential areas on the island of Hawai'i.[57] However, the spread of rats may be more important in spreading *A. cantonensis*, especially to areas to which *A. fulica*, *P. martensi*, and other good intermediate hosts are not present, such as Jamaica and the Canary Islands,[24] and the diversity of possible intermediate snail and slug hosts facilitates this.[58] The pathways for the spread of *A. cantonensis* are complex.

Conclusion

While the systematics of Angiostrongylidae is in need of detailed study, the complex life cycle of *Angiostrongylus cantonensis* is relatively well understood. The parasite is spreading widely around the world, resulting in cases of angiostrongyliasis in places where it had not previously been recorded, facilitated by ease of global travel, globalization of commerce, and climate change.

Conflict of Interest

The author identifies no conflict of interest.

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Definitive, Intermediate, Paratenic, and Accidental Hosts of *Angiostrongylus cantonensis* and its Molluscan Intermediate Hosts in Hawai'i

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Abstract

Eosinophilic meningitis caused by infection with Angiostrongylus cantonensis*, a parasitic nematode, is an emerging infectious disease of humans and other animals, known as angiostrongyliasis or rat lungworm disease. Symptoms range from headache and muscle spasms in mild cases to coma and even death. Many human cases have been recorded around the world, with the majority in tropical and subtropical locations. The increase in numbers of human cases and the expansion of the geographic distribution of cases make this parasite and its hosts important research foci. Definitive hosts include various rat species such as* Rattus norvegicus, R. rattus*, and* R. exulans*, and a number of land and freshwater snails and slugs have previously been identified as intermediate hosts.[1] Both definitive and intermediate hosts are obligate to the life cycle of* A. cantonensis*. Paratenic hosts span a wide range of fauna and are not needed in the nematode's life cycle, but act as reservoirs in which different larval stages of the parasite can persist but not develop further; they include freshwater shrimp, flatworms, and frogs.[2-4] Accidental hosts, including humans and other mammals, as well as birds, permit development from the third larval stage to the subadult (fifth) stage but are then dead ends for the parasite.[5,6] These hosts are infected primarily through consumption of raw or undercooked intermediate or paratenic hosts, either intentionally or accidentally via contaminated produce.[7]*

In Hawai'i, there have been recent outbreaks with cases of infection on four of the main islands. Since there is currently a limited consensus on appropriate therapy, steps to prevent infection should be taken. The first step to facilitate this and to lay the groundwork for future management of the hosts is to identify the intermediate hosts of A. cantonensis *and to determine its geographic distribution within the Hawaiian Islands. To do this over 1000 specimens of 37 terrestrial and freshwater snail and slug species (30 introduced, 7 native) from the six largest Hawaiian Islands were screened using a molecular approach.[8] Total DNA was extracted from foot tissue of each specimen and was amplified using* Angiostrongylus*-specific primers.[8] Amplicons were visualized on agarose gels to determine if specimens were positive or negative for* A. cantonensis*. All of the positive specimens and a random sample of all other specimens tested were also reamplified using species-specific primers.[9] All positive samples were still positive with the newer primers. The parasite was present in 16 (14 alien, 2 native) of these species, from five of the six largest Hawaiian Islands. These species represent 10 phylogenetically diverse terrestrial pulmonate families and 2 more distantly related caenogastropod families (one terrestrial and one freshwater). This broad phylogenetic representation demonstrates that this parasite is not host specific, to the extent that perhaps even any snail or slug species could act as an intermediate host.*

Keywords

Angiostrongyliasis, Eosinophilic meningitis, Hawai'i, Nematodes, Rat lungworm disease, Slugs, Snails

Conflict of Interest

None of the authors identifies any conflict of interest.

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The Occurrence of the Rat Lungworm, *Angiostrongylus cantonensis*, in Nonindigenous Snails in the Gulf of Mexico Region of the United States

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Abstract

Nonindigenous apple snails, Pomacea maculata *(formerly* Pomacea insularum*), are currently spreading rapidly through the southeastern United States. This mollusk serves as an intermediate host of the rat lungworm parasite (*Angiostrongylus cantonensis*), which can cause eosinophilic meningitis in humans who consume infected mollusks. A PCR-based detection assay was used to test nonindigenous apple snails for the rat lungworm parasite in Louisiana, Texas, Mississippi, and Florida. Only apple snails obtained from the New Orleans, Louisiana, area tested positive for the parasite. These results provide the first evidence that* Angiostrongylus cantonensis *does occur in nonindigenous apple snails in the southeastern United States. Additionally,* Angiostrongylus cantonensis *was identified in the terrestrial species* Achatina fulica *in Miami, Florida, indicating that rat lungworm is now established in Florida as well as Louisiana. Although the study suggests that the rat lungworm is not widespread in the Gulf States region, the infected snail population could still pose a risk to human health and facilitate the spread of the parasite to new areas.*

Keywords

Angiostrongylus cantonensis*, Apple snail, Invasive species,* Parastrongylus cantonensis*,* Pomacea*, Rat lungworm*

Introduction

Pomacea maculata (formerly referred to as *Pomacea insularum*, the island apple snail, which is now a junior synonym)[1] is a large freshwater snail species native to South America that has been introduced to the southeastern United States. Established populations currently occur in Texas, Louisiana, Alabama, Florida, Georgia, South Carolina, and North Carolina.[2,3] This species can serve as a host for the rat lungworm, *Angiostrongylus cantonensis*,[4] a parasite that can cause disease in people who consume infected mollusks. *Angiostrongylus cantonensis* is established in Southeast Asia, Australia, the Pacific Islands, Caribbean, and more recently in the Americas, where it is the causative infective agent for eosinophilic meningitis, a serious parasitic infection of humans.[5-7] Previous studies detected the rat lungworm in rats in New Orleans[8] and in mammalian accidental hosts in the proximity of New Orleans.[9,10] In Florida, *A. cantonensis* was found in an infected gibbon at the Miami Metro Zoo.[11] These results suggest that *P. maculata* in these areas might serve as an intermediate host for *A. cantonensis* and facilitate the spread of the parasite as the snail's range continues to expand through the region.

Recently *P. maculata* have been observed in the canals of New Orleans where they are reportedly harvested for consumption by various ethnic groups, putting those groups at potential risk for infection with the parasite (John Palmisano, West Bank Drainage District, oral communication to John Teem, June 2007). The extent to which infections of rat lungworm occur in *P. maculata* populations in New Orleans remains unknown. In addition, there have been no studies to determine whether rat lungworm infections occur in *P. maculata* populations in Miami or in any of the other areas where *P. maculata* occurs in the states of the Gulf of Mexico region. It is further unknown whether other invasive snail species in the region are presently infected with *A. cantonensis*.

To determine the extent to which *P. maculata* populations within the region are infected with rat lungworm, samples were gathered from sites in Louisiana, Texas, Mississippi, and Florida and adult snail tissue analyzed for the presence of rat lungworm using PCR-based detection assays.[12] The extent to which *Achatina fulica*, a nonindigenous terrestrial snail species recently introduced to Florida, is infected with the parasite was also investigated.

Methods

From each location sampled (Table 1, Figure 1), nonindigenous apple snails were collected and frozen at -20°C. Using a sterile disposable scalpel and forceps, a section of the posterior portion of the foot muscle approximately 1 cm in length and 0.5 cm in diameter was cut from each snail and these samples were then frozen at -20 °C until processed for DNA extraction.

Total cellular DNA (tDNA) was extracted from approximately 0.1 g of foot tissue from each snail using the DNeasy blood and tissue kit (Qiagen) or Idaho Technologies 1-2-3 DNA purification kit following the manufacturers' protocols. The presence of *A. cantonensis* in the snail samples was detected by a real-time PCR assay.[12] Samples of *Achatina fulica* were also assayed by nematode extraction and identification methods that will be detailed elsewhere (Smith, unpublished). A subset of samples that tested positive was selected for confirmation of infection using a conventional PCR assay to amplify and sequence the 18S rRNA gene. Agarose gel electrophoresis was used to separate the resulting DNA fragments, which were sequenced to confirm the nematode species identity.[13]

Results

Pomacea maculata occurs in a large proportion of the watersheds in Florida, including the Miami area where a gibbon infected with *A. cantonensis* was identified previously at the Miami Metro Zoo.[11] The Miami Metro Zoo contains numerous canals and ponds that could potentially support *P. maculata*, yet no *P. maculata* were found in any of them. However, another nonindigenous apple snail, *Marisa cornuarietis* (the giant ramshorn snail) was found in a pond near the exhibit that once housed the gibbon infected with *A. cantonensis*.[11] *Angiostrongylus*

Table 1. Results of PCR-based detection of *A. cantonensis* in apple snail samples collected in the southeastern United States. PM - *Pomacea maculata*, MC - *Marisa cornuarietis*, AF - *Achatina fulica*.

Location			Lat./Long.	No. of samples	No. of positives	Species
1	Houston, Texas	Rice/crawfish farm	29 22 13.30 N 95 26 09.35 W	60	0	PM
2	Schriever, Louisiana	Swamp and bayou	29 43 45.51 N 90 51 21.25 W	40	0	PM
3	Gretna, Louisiana	Verret Canal	29 52 37.25 N 90 02 36.37 W	60	8	PM
4	Mandeville, Louisiana	Stormwater retention pond, drainage ditch	30 22 48.01 N 90 02 24.37 W	40	1	PM
5	Picayune, Mississippi	Residential community lake	30 33 48.35 N 89 38 52.40 W	42	0	PM
6	Everglades National Park, Florida	Tamiami Canal	25 45 43.91 N 80 45 59.10 W	52	0	PM
7	Miami, Florida	Miami Metro Zoo canal	25 36 33.47 N 80 23 52.37 W	52	0	MC
8	Miami, Florida	Miami residential areas	25 40 18.30 N 80 25 42.00 W	140	4	AF

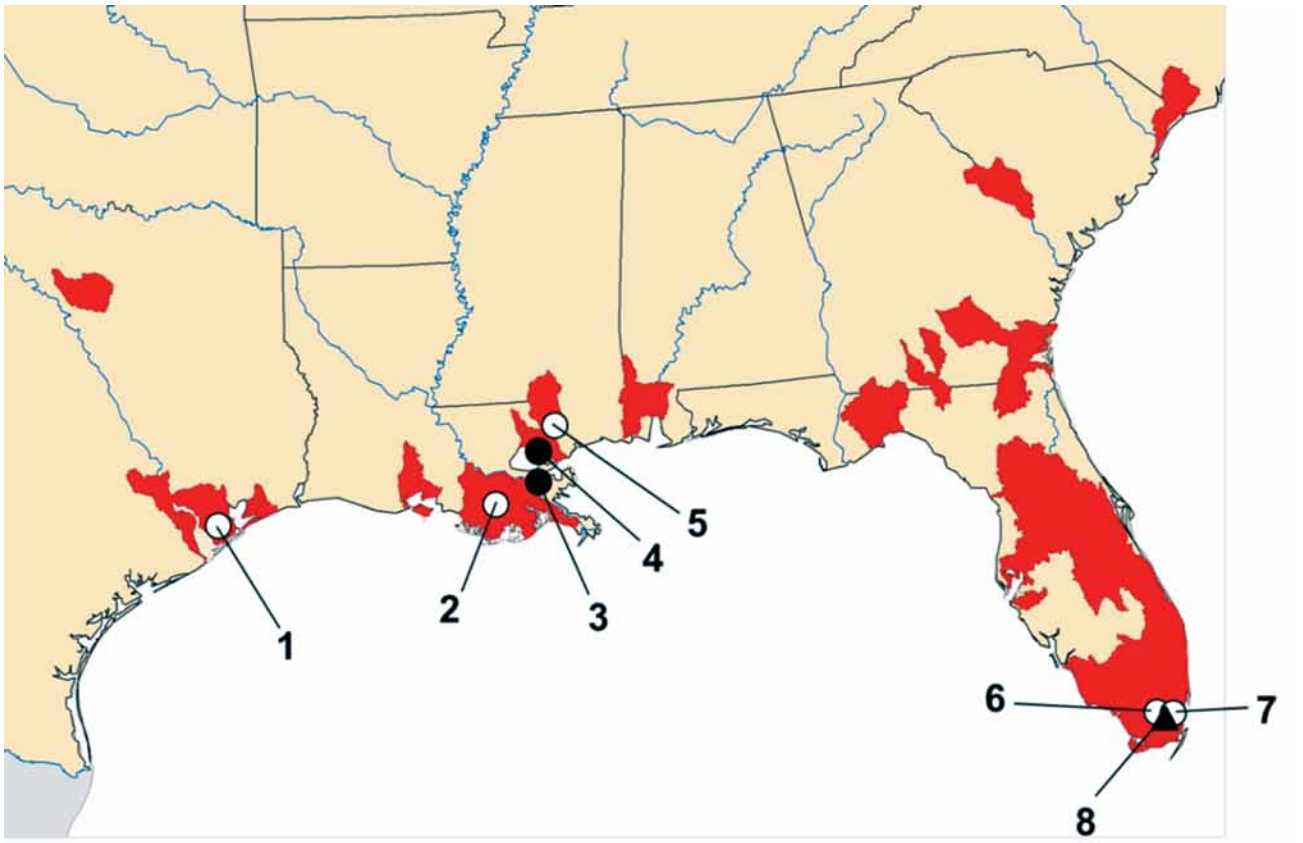

Figure 1. Geographic location of apple snail sampling sites (circles) in the southeastern United States in relation to watersheds where *P. maculata* populations have become established (shaded). Black circles and triangles indicate locations in which apple snails and giant African land snails (*Achatina fulica*), respectively, tested positive for rat lungworm. 1. Houston, Texas, 2. Schriever, Louisiana, 3. Gretna, Louisiana, 4. Mandeville, Louisiana, 5. Picayune, Mississippi, 6. Everglades National Park, Florida, 7. Miami Metro Zoo, Miami, Florida, 8. Residential Community, Miami, Florida. Map source USGS Nuisance Aquatic Species, http://nas3.er.usgs.gov/

cantonensis infects a broad range of mollusk species, including *M. cornuarietis*.[14] Accordingly, samples were taken from this *Marisa cornuarietis* population for testing. All 52 snails from the Miami Metro Zoo tested negative for the parasite using the PCR-based methods (Table 1), as did the *P. maculata* from a population within the nearby Tamiami Canal (north of Everglades National Park). These results indicate that rat lungworm is not widespread among apple snail populations in the area.

However, there is another invasive snail species recently detected in the Miami area, which could also serve as a new reservoir for the parasite. The giant African land snail, *Achatina fulica*, is a nonindigenous terrestrial snail discovered at multiple residential locations in the Miami area in September 2011. *Achatina fulica* serves as a reservoir for rat lungworm in other areas of the world where it has been introduced.[5,15] Therefore, samples of *Achatina fulica* were tested from 12 of the 19 core areas where *A. fulica* has been found in the Miami area. Four infected snails were detected in one of these areas, equivalent to a frequency of 7.4%. This area is approximately 8 km (5 miles) from the Miami Metro Zoo. The rat lungworm is therefore established not only in Louisiana but also in Florida.

Angiostrongylus cantonensis was also detected in *P. maculata* in two locations close to New Orleans. Infected snails occurred at a frequency of about 13% (8/60) in Gretna, Louisiana and 2.5% (1/40) in Mandeville, Louisiana. These two *P. maculata* populations are in separate drainage basins, in principally urban areas that are close to metropolitan New Orleans, 10 and 50 km (6 and 30 miles) away, respectively. A third sample of *P. maculata* taken from Schriever, Louisiana, tested negative. The Schriever population is in a drainage basin hydrologically distinct from both the Gretna and Mandeville populations, in a comparatively less urban area.

Although *A. cantonensis* is established in some parts of Louisiana, it is not known to what extent it has become established in Texas and Mississippi, the two Gulf states adjacent to Louisiana. Infection of a horse (accidental host) by *A. cantonensis* was reported in Picayune, Mississippi, in 1998.[9] In 2008, *P. maculata* became established in a residential lake in Picayune, suggesting that it could serve as a new reservoir for the parasite at this location. However, no *Pomacea maculata* from this lake tested positive for *A. cantonensis*. *Pomacea maculata* from the Houston, Texas, area also tested negative.

Discussion

This study has shown that *Pomacea maculata* populations infected with *A. cantonensis* are currently limited to Louisiana within the southeastern United States (Table 1, Figure 1). Detection of *A. cantonensis* in Gretna and Mandeville confirms the establishment of the parasite in the region and indicates that *P. maculata* has become a component of the mollusk reservoir for the parasite. A recent survey in China suggests that invasive apple snails (in this case *Pomacea canaliculata*) can facilitate the spread of *A. cantonensis* by providing an abundant mollusk reservoir to facilitate parasite reproduction.[15] The present study suggests that nonindigenous apple snails could similarly facilitate the spread of *A. cantonensis* in other regions and states in the southeastern United States as *P. maculata* further expands its range.

Although the present survey was limited in scope, there was no evidence to suggest widespread infection of *P. maculata* in states other than Louisiana. All samples tested in Texas, Mississippi, and Florida tested negative for the parasite. This may reflect an actual absence of *A. cantonensis* in these locations or the small number of sample locations tested. Despite *A. cantonensis* having been previously detected in a horse in Picayune, Mississippi,[9] it was not detected in *P. maculata* from this location. However, a similar finding occurred in Yunnan, China, where a recent survey revealed no infections of apple snails (in this case *P. canaliculata*) despite a previous history of *A. cantonensis* in the region.[15]

The negative results obtained with apple snails from Miami should also be considered tentative in view of other data suggesting *A. cantonensis* is established in the existing rat and terrestrial mollusk populations there. In addition to the infected gibbon at the Miami Metro Zoo, a black rat infected with *A. cantonensis* was detected in 2004 (Christine Miller, Miami Metro Zoo, oral communication to John Teem, February 2010), supporting the notion that *A. cantonensis* was present in the area at the time of the gibbon infection. Although it seems unlikely that *A. cantonensis* could be eliminated from a region once established, apple snail infection rates may fluctuate in response to unknown parameters such as the densities of local rat and mollusk hosts. Rat lungworm may therefore have been present in Miami prior to the appearance of the giant African land snail, but it may also have been brought in with this species at the time of its introduction.

The age of the *P. maculata* population may also be related to the level of infection and hence likelihood of detecting *A. cantonensis* in an area where it had previously been detected. For example, the *P. maculata* population in Schriever (in a drainage basin adjacent to the watershed in which New Orleans is located and where *A. cantonensis* has been present for at least 20 years), tested negative for *A. cantonensis*. However, this population has only been established since 2006, and the lack of infection may reflect a time lag for the newly established nonindigenous species to be incorporated into the pool of infected endemic mollusks.

The incidence of *P. maculata* infection in Louisiana was 6.4% (9/140), similar to the 6.8% for *P. canaliculata* in China.[15] However, it seems unlikely that the risk of human infection in Louisiana is similar to that in China, where the raw snails are considered a delicacy. Human infections of *A. cantonensis* in Louisiana have been attributed to eating uncooked infected mollusks or paratenic hosts,[16,17] but infections are rare because such behavior is uncommon. There is, however, a level of potential risk that cannot at present be quantified, but that will continue to exist since these snails are serving as a reservoir for the disease.

Another pathway for human infection by *A. cantonensis* has been described recently in Hawai‘i. Although Hawai‘i has

numerous nonindigenous snail species shown to be infected with *A. cantonensis*, including *P. canaliculata* and *A. fulica* (JR Kim, oral presentation, Rat Lungworm Disease Scientific Workshop, Honolulu, Hawai'i, August 2011), most of the recent cases of human infection were thought to be associated not with these species but with a recently introduced nonindigenous semi-slug, *Parmarion martensi*.[18] Human infections appear to result from accidental ingestion of small infected slugs or parts of slugs that are present on raw produce consumed from home gardens. *Parmarion martensi* is only one of many mollusk species in Hawai'i that can harbor *A. cantonensis*; however, the tendency of this species to associate with garden produce that is consumed uncooked may be an important factor in its role in human disease transmission. *Parmarion martensi* has not been detected in the southeastern United States but mollusk species (native or nonindigenous) with ecological traits and behavior like that of *P. martensi* may become infected with *A. cantonensis* and similarly increase the potential for human infections in the region. Apple snails and giant African land snails may thus contribute indirectly to human infections by providing a reservoir for *A. cantonensis* to infect other mollusk species in the same area that directly infect humans by their association with uncooked foods.

The detection of *A. cantonensis* in *P. maculata* in Louisiana and in *A. fulica* in Florida indicates that nonindigenous mollusks are presently serving as reservoirs to allow *A. cantonensis* to expand its range to other Gulf of Mexico states. Further research to define the human health risks associated with *A. cantonensis* as a result of nonindigenous mollusk introductions in the region should therefore include a regional survey of infection rates for both native and nonindigenous mollusk species in the southeastern United States.

Conflict of Interest

None of the authors identifies any conflict of interest.

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Genetic Diversity of the Rat Lungworm, *Angiostrongylus cantonensis*, the Major Cause of Eosinophilic Meningitis

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Abstract

Various aspects of the genetics of the rat lungworm, Angiostrongylus cantonensis*, are reviewed. This nematode has an XX/XO sex-determination mechanism, with the female having a 2n = 12 (XX) and the male 2n = 11 (XO) chromosome constitution. Allozymes (12 loci) exhibit a low proportion of polymorphic loci (P = .08) and low mean heterozygosity (H = 0.43) in specimens from Hawai'i, and no polymorphism or heterozygosity in specimens from Thailand. The phosphoglucomutase-2 (PGM-2) locus exhibits sex-limited expression, with no detectable enzyme activity in the male worms from either location. Based on the 12 allozyme loci, Nei's genetic distance between the Hawai'i and Thailand isolates is D = 0.03. The p-distance (proportion of nucleotide sites) based on cytochrome c oxidase subunit I (COI) is 3.61% between the Thailand and China isolates as well as between Thailand and Hawai'i isolates, and 0.83% between China and Hawai'i isolates. The partial DNA sequences of the 66 kDa protein gene show a great diversity of haplotypes, indicating both inter- and intra-population variation. Intra-specifc sequence variation is also found in the internal transcribed spacer regions. For the small-subunit ribosomal RNA (SSU rRNA) gene, two distinct genotypes have been recorded.*

Keywords

Allozymes, Angiostrongylus cantonensis*, Chromosome number, Emerging infectious disease, Eosinophilic meningitis, Genetics, Parasitology, Rat lungworm disease*

Introduction

Angiostrongylus cantonensis, a metastrongyloid nematode, is a primary cause of human eosinophilic meningitis or meningoencephalitis in many parts of the world.[1,2] Its life cycle involves a definitive rodent host and an intermediate molluscan host. The adult worms live in the pulmonary arteries of rats. Humans become infected by ingestion of the third stage larvae in molluscan intermediate hosts or paratenic hosts such as frogs, freshwater shrimp, crabs, and monitor lizards.[1-3]

Genetic diversity refers to both the vast numbers of different species as well as the diversity within a species, and any variation in the nucleotides, genes, chromosomes, or whole genomes of organisms. This short review summarizes what is known about genetic variation in *A. cantonensis*.

Chromosome Constitution

The two sexes of *Angiostrongylus cantonensis* differ in their diploid number because of an XX/XO sex-determination mechanism.[4] The male has 2n = 11 with an XO sex-chromosome constitution, while the female has 2n = 12 with an XX sex-chromosome constitution. To date the karyotype of *A. cantonensis* has only been reported for individuals from Japan,[5] Egypt,[6] mainland China,[7] Thailand,[4] and Hawai'i.[4] There appear to be no similar studies of the other *Angiostrongylus* species.

Allozymes

In the six allozyme systems studied in *A. cantonensis* from Hawai'i and Thailand (glucose phosphate isomerase, hexokinase, lactate dehydrogenase, malate dehydrogenase, malic enzyme, and phosphoglucomutase), each represented by two presumptive loci and hence comprising 12 loci, there was only one polymorphic locus, PGM-1 (phosphoglucomutase-1), which was present only in Hawai'i (Figure 1). Thus the proportion of polymorphic loci (P) was 0.08; mean heterozygosity (H) was 0.43.[8] It was represented by two alleles in the following propor-

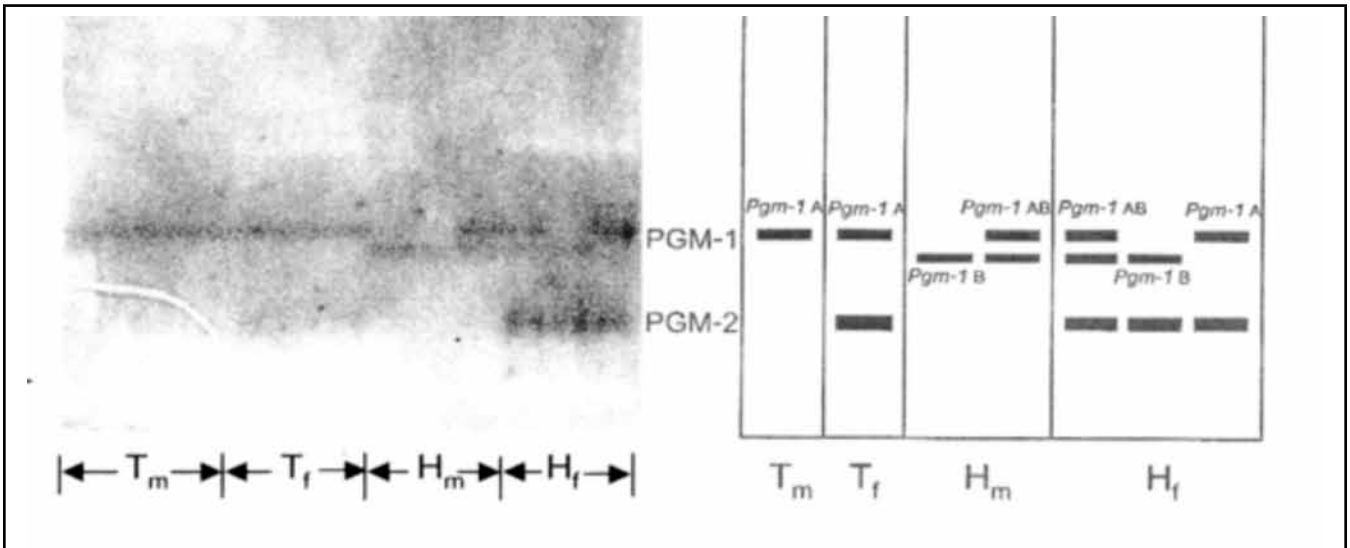

Figure 1. Phosphoglucomutase allozyme system of *Angiostrongylus cantonensis* from Hawai'i (H) and Thailand (T).

tions: PGM-1^{A}, 0.36 ± 0.03; PGM-1^{B}, 0.64 ± 0.03. Nei's genetic distance (D) between specimens from Hawai'i and Thailand is 0.03, indicating that they are genetically very similar. In contrast, the genetic distance between *A. cantonensis* and a congeneric species, *A. malaysiensis*, is 0.27.[8] The very low proportion of polymorphic loci in specimens from Hawai'i and the lack of polymorphism in those from Thailand may be attributed to the founder effect. The proportion of polymorphic loci in *A. cantonensis* in Japan has been reported to be 0.6.[9]

The PGM-2 locus is invariant, with a single band of enzyme activity in the female worms from both Thailand and Hawai'i. However, there is no detectable enzyme activity at this locus in male worms from either location (Figure 1). This non expression or 'null' PGM-2 phenotype in the male worms is presumed to be an example of sex-limited gene expression.[10]

Cytochrome *c* Oxidase Subunit I (COI)

Angiostrongylus cantonensis samples from Thailand, Hawai'i, and China form distinct clusters in maximum likelihood (ML), maximum parsimony (MP), neighbor-joining (NJ), and Bayesian inference (BI) trees.[11] The *p*-distance (proportion of nucleotide sites) between the Thailand and China samples is 3.61%, between Thailand and Hawai'i 3.61%, and between China and Hawai'i 0.83%.

Phylogenetic analysis of 18 geographical isolates of *A. cantonensis* from Japan, mainland China, Taiwan, and Thailand showed eight distinct COI haplotypes.[12] A single haplotype only was found in 16 of the 18 localities. However, two haplotypes coexisted in two localities. The low genetic variation of *A. cantonensis* in each location is attributed to founder effects.

The COI marker has proven useful for differentiating closely related species (eg, *A. cantonensis* and *A. malaysiensis*), differentiating geographical isolates of *A. cantonensis*, and determining the phylogenetic relationships of *A. cantonensis*, *A. costaricensis*, *A. malaysiensis*, and *A. vasorum*.[11] Based on COI sequences, *A. cantonensis* and *A. malaysiensis* cluster to form a clade, while *A. costaricensis* and *A. vasorum* cluster to form another clade. The *p*-distance between *A. cantonensis* and *A. malaysiensis* is 11.1-11.7%.[11]

Partial 66 kDa Protein Gene

The partial DNA sequences of the 66 kDa protein gene do not clearly distinguish *A. cantonensis* from Thailand, China, Japan, and Hawai'i.[13] They are represented by a great diversity of haplotypes, indicating both inter- and intra-population variation. The AC primers[13] successfully amplified genomic DNA of *A. cantonensis*, *A. costaricensis*, and *A. malaysiensis*. They did not amplify DNA of *Ascaris suum*, *Ascaris lumbricoides*, *Toxocara canis*, *Anisakis simplex*, *Trichinella spiralis*, *Ancylostoma caninum*, and *Strongyloides ratti*. Based on the 66 kDa gene, the genetic distance between *A. cantonensis* and *A. malaysiensis* is $P = 1.70\%$-4.08%, between *A. cantonensis* and *A. costaricensis* $P = 3.77\%$-5.77%, and between *A. malaysiensis* and *A. costaricensis* $P = 5.10\%$.[13] Partial DNA sequences indicate that *A. cantonensis* is sister to *A. malaysiensis*. The two species are clearly distinct but are more closely related to each other than to *A. costaricensis*.

Internal Transcribed Spacer Regions of Nuclear Ribosomal DNA

The DNA sequences of the internal transcribed spacer region-1 (ITS-1) in *A. cantonensis* from China, USA, and Brazil indicate intra-specifc sequence variation of 0.1-1.0% , and the sequence variation for ITS-2 is 0.0-1.3% in the China and Philippine isolates.[14] ITS-2 sequences yield poorly resolved phylogenetic relationships among *A. cantonensis*, *A. costaricensis* (from Brazil and Costa Rica), and *A. vasorum* (from Brazil and Europe) as well as *A. dujardini* (from Europe) (PR, PEL, HSY unpublished).

Small-subunit Ribosomal RNA (SSU rRNA) Gene

Two distinct SSU genotypes (G1 and G2) were identified among 17 *A. cantonensis*, one from each of 17 localities in Japan, mainland China, Taiwan, and Thailand.[12] Analysis of all available SSU rRNA sequences of *Angiostrongylus* species from GenBank indicates that *A. cantonensis* and *A. malaysiensis* form a cluster (clade) distinct from *A. costaricensis*, *A. dujardini*, and *A. vasorum* (PR, PEL, HSY unpublished).

Conflict of Interest

None of the authors identifies any conflict of interest.

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Angiostrongylus cantonensis and Rat Lungworm Disease in Brazil

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Abstract

The metastrongyloid nematode genus Angiostrongylus *includes 18 species, two of which are relevant from a medical standpoint,* Angiostrongylus costaricensis *and* Angiostrongylus cantonensis*. The first was described from Costa Rica in 1971 and causes abdominal angiostrongyliasis in the Americas, including in Brazil.* Angiostrongylus cantonensis*, first described in 1935 from Canton, China, is the causative agent of eosinophilic meningitis. The natural definitive hosts are rodents, and molluscs are the intermediate hosts. Paratenic or carrier hosts include crabs, freshwater shrimp, amphibians, flatworms, and fish. Humans become infected accidentally by ingestion of intermediate or paratenic hosts and the parasite does not complete the life cycle as it does in rats. Worms in the brain cause eosinophilic meningitis. This zoonosis, widespread in Southeast Asia and the Pacific islands, has now been reported from other regions. In the Americas there are records from the United States, Cuba, Jamaica, Brazil, Ecuador, and Haiti. In Brazil seven human cases have been reported since 2007 from the southeastern and northeastern regions. Epidemiological studies found infected specimens of* Rattus norvegicus *and* Rattus rattus *as well as many species of molluscs, including the giant African land snail,* Achatina fulica*, from various regions of Brazil. The spread of angiostrongyliasis is currently a matter of concern in Brazil.*

Keywords

Achatina fulica*, Angiostrongyliasis, Brazil, Eosinophilic meningitis,* Rattus norvegicus*,* Rattus rattus*, Snails*

Introduction

The metastrongyloid nematode *Angiostrongylus cantonensis* causes eosinophilic meningitis (and meningoencephalitis) in humans. This parasite, widespread in Southeast Asia and some Pacific islands, has now dispersed to other regions, including Latin America.[1] The spread of this emerging zoonosis is correlated with increased tourism, commerce, and the diversification of habits and customs in certain countries, factors that have facilitated the dispersal of the definitive and intermediate hosts of *A. cantonensis*.[2] In addition, the introduction of non-native molluscs plays an important role, as has been observed with the giant African snail, *Achatina fulica*, in Brazil[3-5] and the South American freshwater snail *Pomacea canaliculata* in China.[6]

Species of *Angiostrongylus* can infect domestic dogs and wild mammals,[7] as well as humans, as accidental hosts, causing parasitic diseases.[8] Besides *A. cantonensis*, another congeneric species, *A. costaricensis*, described from Costa Rica in 1971,[9] is important from a public health standpoint as the causative agent of abdominal angiostrongyliasis, a zoonosis recorded from the south of the United States to northern Argentina. In Costa Rica up to 500 human cases are reported annually.[10] In Brazil cases have been reported mainly in the southern States.[11] This paper focuses on *A. cantonensis* in Brazil.

Taxonomy

Attempts to organize the family Angiostrongylidae into genera and subgenera, based on the morphology of the rays of the caudal bursa and on the host species, have divided the scientific community, particularly in relation to the important human parasites, ie, *A. cantonensis* and *A. costaricensis*.

Dougherty[12] synonymized the following genera with *Angiostrongylus*: *Haemostrongylus*, *Parastrongylus*, *Pulmonema*, *Cardionema*, and *Rodentocaulus*. Subsequently, Skrjabin et al.[13] recognized four genera, accepting Dougherty's synonyms, with the exception of *Rodentocaulus*, which was retained as a valid genus along with two additional more recently described genera that were also considered valid, *Rattostrongylus* and *Angiocaulus*. Yamaguti[14] accepted the systematic arrangement proposed by Skrjabin et al.[13]

In 1970 Drozdz[5] proposed dividing the genus *Angiostrongylus* into two subgenera: *Angiostrongylus* (*Angiostrongylus*) with *Haemostrongylus*, *Cardionema*, and *Angiocaulus* as synonyms; and *Angiostrongylus* (*Parastrongylus*) with *Rodentocaulus*, *Pulmonema*, and *Rattostrongylus* as synonyms. Chabaud[15] abolished the subgenera, and recognized four genera: *Angiostrongylus* (with synonyms *Haemostrongylus*, *Cardionema*, and *Angiocaulus*), *Parastrongylus*, *Rodentocaulus*, and his newly created genus *Morerostrongylus*. However, Anderson[7] adopted the subgeneric classification of Drozdz, with *Morerostrongylus* as a synonym of *Parastrongylus*, which was treated as a subgenus of *Angiostrongylus*, but with the exception of recognizing *Rodentocaulus* as a distinct valid genus.

In 1986, Ubelaker[16] reorganized the family Angiostrongylidae, recognizing the genera *Angiostrongylus* (synonym *Haemostrongylus*), *Parastrongylus* (synonyms *Pulmonema*, *Rattostrongylus*, *Morerostrongylus*, *Chabaudistrongylus*), *Angiocaulus* (synonym *Cardionema*), *Rodentocaulus*, *Galegostrongylus* (synonym *Thaistrongylus*), and *Stefanskostrongylus*. Although this is the most recent taxonomic revision of the Angiostrongylidae, based on morphological similarity of the bursal rays and host animals, it has not been widely accepted, since, for instance, few people use *Parastrongylus* for *A. cantonensis*. Too few molecular data are available to help resolve the systematics of the family.

Based on the classification of Dougherty,[12] we recognize 18 species of *Angiostrongylus* from around the world (excluding those albeit nomenclaturally valid species that have been described on the basis only of female morphology). *Angiostrongylus vasorum*, *A. raillieti*, *A. gubernaculatus*, and *A. chabaudi* have carnivores as their definitive hosts. The remaining 14 species have rodents as definitive hosts: *A. taterone*, *A. cantonensis*, *A. sciuri*, *A. mackerrasae*, *A. sandarsae*, *A. petrowi*, *A. dujardini*, *A. schmidti*, *A. costaricensis*, *A. malaysiensis*, *A. ryjikovi*, *A. siamensis*, *A. morerai*, and *A. lenzii*.

Angiostrongylus cantonensis was originally described in the genus *Pulmonema* from specimens recovered from the lungs of naturally infected rats (*Rattus norvegicus* and *Rattus rattus*) from Canton, China.[17] The adult worms (Figure 1) are characterized by a filiform body in both sexes, tapering at the anterior end. Females are larger and more robust than males. Detailed morphological descriptions have been published elsewhere.[18,19]

Life Cycle and Hosts

The life cycle of *A. cantonensis* involves various species of terrestrial and freshwater gastropods as intermediate hosts and rats as definitive hosts.[20-23] As *A. cantonensis* occurs in the adult stage in the pulmonary arteries of the definitive hosts, commonly *Rattus rattus* and *R. norvegicus*, it is known as the rat lungworm. In experimental infection of *R. norvegicus*, the female worm lays eggs inside the pulmonary arteries, where they develop into the first-stage larvae (L_1), which then move to the interior of the alveoli. The larvae then migrate to the pharynx and are swallowed, pass through the gastrointestinal tract, and are eliminated in the feces.[21,24] Land or freshwater snails are the principal intermediate hosts, and become infected either by ingestion of L_1 in the rat feces or by penetration of these larvae through the body wall or respiratory pore.[25] In the mollusc tissue the L_1 molts twice (L_2 and L_3) and the period necessary for the development is around 20 days. Rats become infected mainly by the ingestion of intermediate hosts infected by L_3 larvae. These larvae then penetrate the intestinal wall and enter the bloodstream a few hours after being ingested. They reach the pulmonary circulation from the heart and are dispersed to various other organs by the arterial circulation. Many reach the brain and molt again, becoming L_4 larvae. The fifth molt into the subadult stage (L_5) occurs in the subarachnoid space, from where, after developing further, they migrate to the pulmonary arteries where they are found 25 days after infection. The worms then reach sexual maturity at around 35 days and the L_1 larvae can be found in the rodent's feces about 42 days after the exposure to the previous generation of L_1 larvae.

Infection in humans occurs when they eat raw or undercooked snails and slugs, or paratenic hosts, including land crabs, freshwater shrimp, fish, frogs, and planarians.[26] In humans, the young larvae reach the brain, where they die rather than migrating further and completing their development. This causes eosinophilic meningitis (or meningoencephalitis), which has neurological symptoms. Usually the infection does not kill the victim, except when there is massive exposure to infective L_3 larvae.[27]

The parasite displays broad specificity for intermediate hosts; many species of terrestrial and freshwater molluscs have been found naturally infected, including *Achatina fulica*, *Bradybaena similaris*, *Subulina octona*, *Pomacea canaliculata*, *Pomacea lineata*, other *Pomacea* species, *Deroceras laeve*, and species of *Pila*.[3,18,23,28] It has been found in various paratenic hosts, and although these are passive hosts in which the parasite does not undergo any development, they play an important role as they increase the opportunities for the parasite to infect definitive hosts.

In southern China, an apple snail species, *Pomacea canaliculata*, and the giant African snail, *Achatina fulica*, both non-native, are widespread and the number of cases of eosinophilic meningitis has been increasing, the transmission being linked to both species. Most recently, serious outbreaks have been reported, in most cases directly related to consumption of *P. canaliculata*.[6,29]

Angiostrongylus cantonensis and Angiostrongyliasis in Brazil

Sporadic outbreaks of eosinophilic meningitis caused by *A. cantonensis* were first reported in the Americas in the latter part of the twentieth century.[30] The zoonosis, or at least infected rodents, have now been reported in the Americas and islands of the Caribbean from the United States,[31] Cuba,[32,33] Jamaica,[34] Brazil,[3,4,26] Ecuador,[35] and Haiti.[36]

The first report of this zoonosis in Brazil was in the municipality of Cariacica, Espírito Santo State,[3] with subsequent reports from two municipalities (Olinda and Escada) in Pernambuco State,[18,26] and in the city of São Paulo, São Paulo State.[37] In the first two states naturally infected definitive and/or intermediate hosts had been discovered during the epidemiological investigation of the human cases. *Achatina fulica* was considered the vector in three of the four reported cases.[3,18,26] One of the cases in Pernambuco State was attributed to ingestion of undercooked apple snails (*Pomacea lineata*).[18] Specimens of *A. fulica* have been found infected with *A. cantonensis* larvae in south and southeastern Brazil since 2007 and more recently from Pará State in the Amazon region, northern Brazil.[38] A species of *Pomacea* was also associated with an outbreak of eosinophilic meningitis in Ecuador.[35]

Natural infection of definitive vertebrate hosts with *A. cantonensis* has also been reported in Brazil. Infected *Rattus rattus* and *R. norvegicus* have both been found in Pará State[38] and infected *R. norvegicus* have also been reported in the States of Rio de Janeiro (southeastern Brazil)[39] and Rio Grande do Sul (southern Brazil), according to Carlos Graeff-Teixeira (oral communication, June 2012). Some reports of infected rodents in urban areas were associated with epidemiological investigations following the occurrence of cases of eosinophilic meningitis.[39] The prevalence of *A. cantonensis* in rodents is highly variable[8] and does not suggest specificity among species in the genus *Rattus*.

How *A. cantonensis* arrived and became established in the Americas is not well understood, although Diaz[40] attributed its introduction to introduction of *Rattus norvegicus* in shipping containers. Its introduction to Brazil has been postulated as either with parasitized rats during the country's colonial period (1500-1822), when there was frequent contact with Africa and Asia[4] and/or by recent invasion of the giant African snail, *A. fulica*, which began in the 1980s.[41,42] As Brazil is currently experiencing the explosive phase of the invasion of *A. fulica*, which has now been recorded in 25 of the 26 states and in the Federal District (Figure 2), the emergence of eosinophilic meningitis is a matter of concern,[41,42] although many species of molluscs may act as intermediate hosts in Brazil.[43]

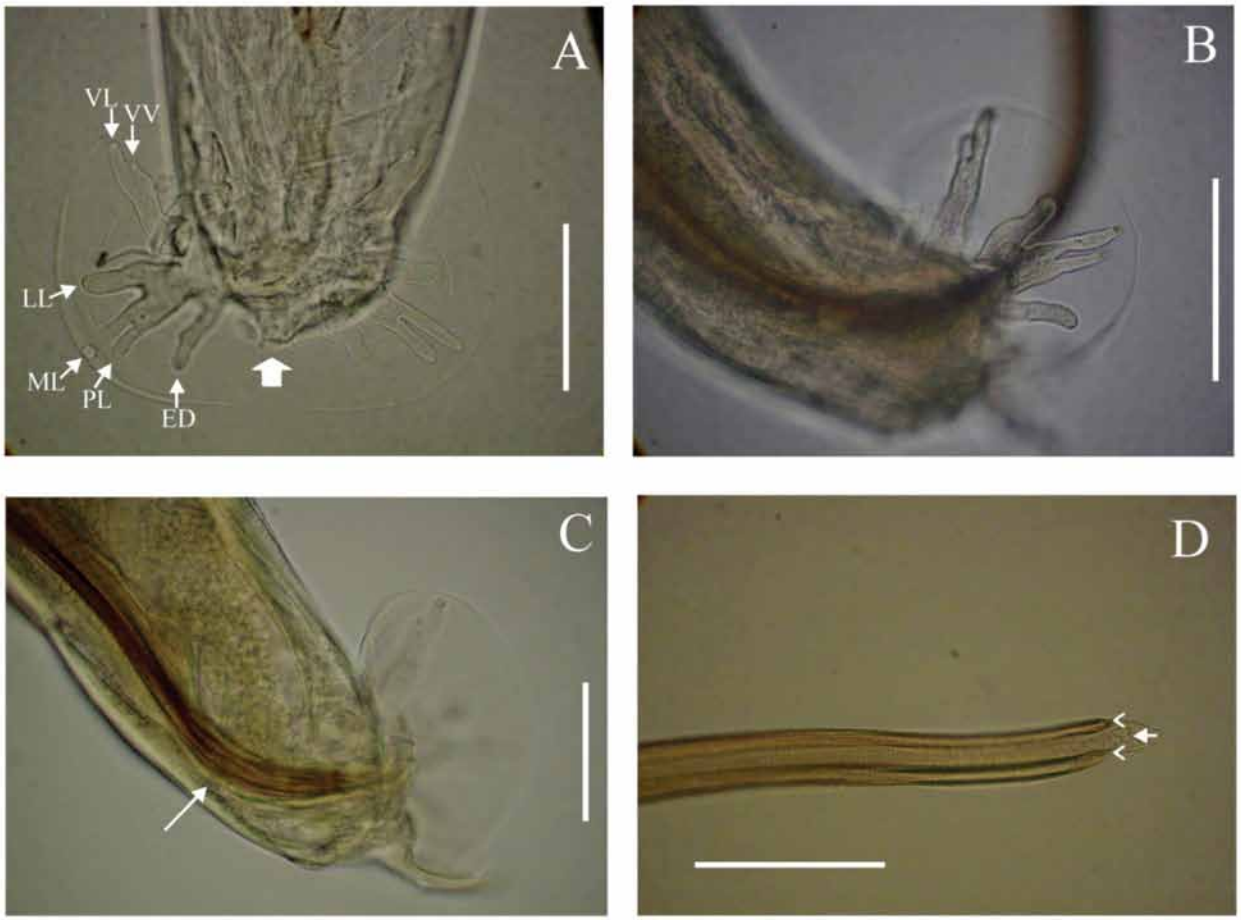

Figure 1. *Angiostrongylus cantonensis*; A. Male, dorsal view of caudal bursa, detail ventroventral (VV), ventrolateral (VL), laterolateral (LL), meiolateral (ML), posterolateral (PL), externodorsal (ED) and dorsal rays (large arrow); scale bar: 50 µm. B. Male, caudal bursa, lateral view; scale bar: 50 µm. C. Male, caudal bursa showing gubernaculum (white arrow), lateral view; scale bar: 50 µm. D. Male, detail of two spicules (arrowheads) joined by a sheath (arrow); scale bar: 100 µm.

Conclusion

The spread of *Angiostrongylus cantonensis* in Brazil is a matter of public health concern because of the widespread occurrence of infected rats and snails in peridomestic areas. There is a need for education of the population regarding disease transmission and prevention. Physicians should be made more aware of the possibility of *A. cantonensis* infection. And serological diagnosis of angiostrongyliasis should be available to facilitate appropriate medical treatment. Control and monitoring of intermediate and definitive hosts in areas of epidemiological relevance should be undertaken to limit the occurrence of new transmission foci.

Conflict of Interest

None of the authors identifies any conflict of interest.

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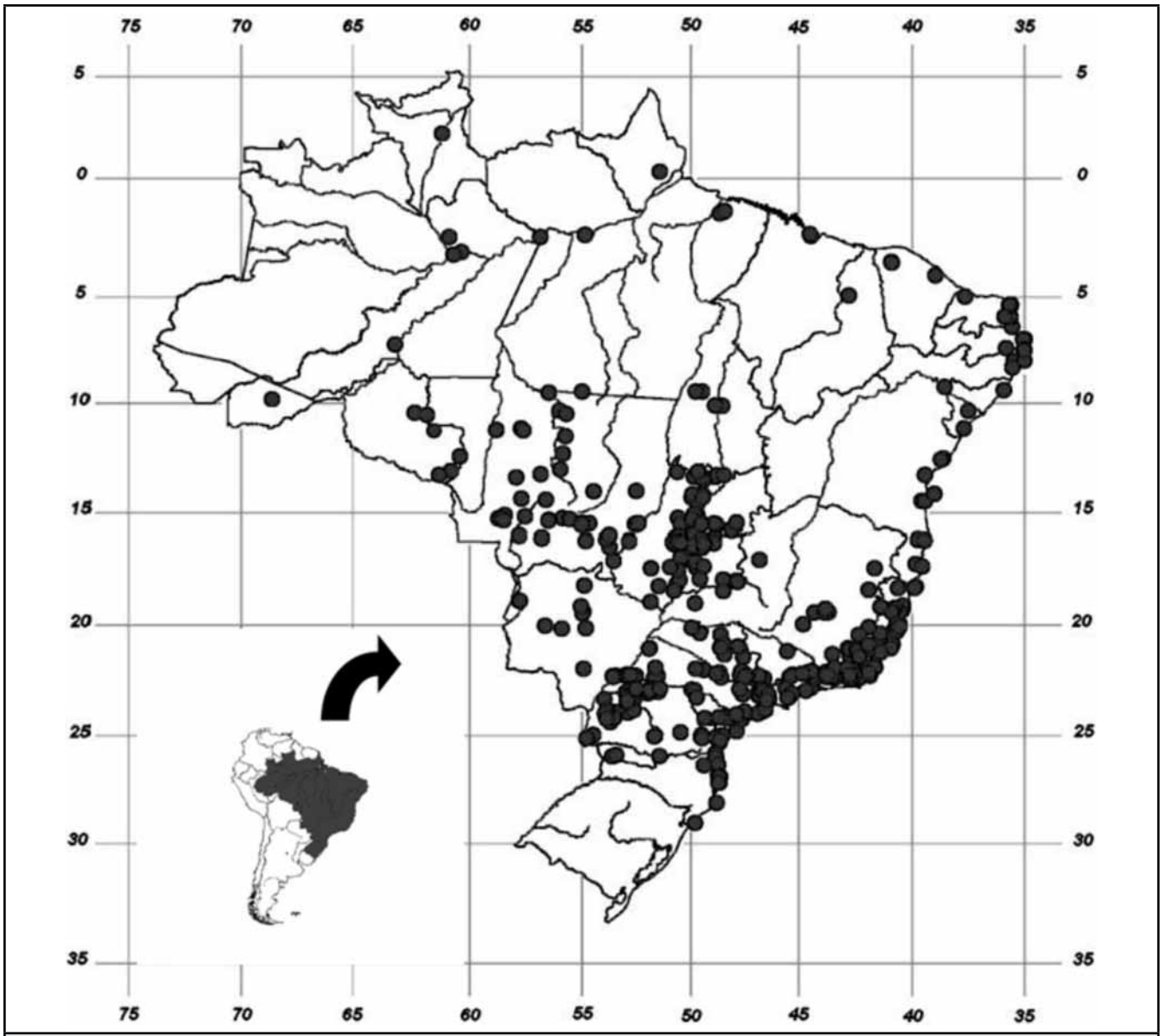

Figure 2. Current distribution of the giant African snail, *Achatina fulica*, in Brazil, updated from Thiengo, et al.[41]

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The Apple Snail *Pomacea canaliculata*, a Novel Vector of the Rat Lungworm, *Angiostrongylus cantonensis*: its Introduction, Spread, and Control in China

Ting-Bao Yang PhD; Zhong-Dao Wu MD, PhD; and Zhao-Rong Lun PhD

Abstract

The freshwater apple snail Pomacea canaliculata *was introduced to Taiwan then to mainland China in the early 1980s from Argentina, its native region, for the purpose of aquaculture. Because of the lack of natural enemies and its tolerance of a wide range of environmental conditions, both its abundance and distribution have dramatically increased and it has become a harmful species to local agriculture and other native species in many areas of China. Unfortunately, the snail also acts as an intermediate host of* Angiostrongylus cantonensis*, and has been implicated in transfer of the parasite to people, resulting in angiostrongyliasis manifested as eosinophilic meningitis. Efforts to prevent its further spread and population expansion were initiated many years ago, including the use of chemicals and biological control agents to control the snail.*

Keywords

Angiostrongyliasis, Apple snail, China, Control, Eosinophilic meningitis, Host, Introduced species, Nematode, Parasite, Pest, Pomacea canaliculata

Introduction to China

The native range of *Pomacea canaliculata*, one of two species commonly known in Asia as the golden apple snail, is Argentina and Uruguay. It is an invasive species and is now widespread in many countries of eastern and southern Asia, including the Philippines, Vietnam, Thailand, Japan, and Korea.[1] A cluster of eggs of *P. canaliculata* was brought to Taiwan at the end of the 1970s from Argentina by a Chinese person resident in Argentina. In 1981, *P. canaliculata* was intentionally introduced to Zhongshan, Guangdong province, China, as an aquaculture species and bred successfully.[2,3] Subsequently, during the 1980s, aquaculture of this snail rapidly expanded to many regions of 17 provinces, including Guangdong, Guangxi, Fujian, Sichuan, Shanghai, Hubei, Guizhou, Zhejiang, Jiangsu, Anhui, Beijing, and even to Gansu and Liaoning. During this time, many government sponsored technical training programs and publications greatly boosted the spread and culture of this snail in China. In the 1990s, the impetus for development of aquaculture gradually faded because of the distastefulness of the snail to many people. However, the species had established many natural populations in many areas where it had never been artificially bred and cultured, expansion of its distribution being facilitated by its wide tolerance of environmental conditions and broad food preferences. Gradually it became a devastating agricultural pest of wetland crops, most notably rice, in various Southeast Asian countries[2,4] and Japan.[5] *Pomacea canaliculata* was subsequently found to be an important intermediate host of *Angiostrongylus cantonensis*, the rat lungworm, which can infect humans, causing angiostrongyliasis manifested as eosinophilic meningitis.[6-7] In 2003, *P. canaliculata* was included in a blacklist of 16 invasive pests by the State Environmental Protection Administration (SEPA) of China.

Spread in Mainland China

There are no accurate data on the abundance and spread of *P. canaliculata* since its introduction to China in about 1980. However, based on reports from various parts of China, it has been suggested that the spread of *P. canaliculata* in mainland China took place in two main phases, a first phase of rapid spread during the 1980s resulting from domestic introduction for aquaculture, and a second phase of slow spread in the subsequent 20 years or so once major control measures against the snail were widely implemented (Figure 1).[8] *Pomacea canaliculata* is now well established in waters of southern China, in a band spanning from northeast to southwest. It has also been observed in mountainous areas at high elevations in Yunnan province. And it has crossed from the Pearl River valley into the Yangtze River valley, and now occurs in the southeast section of the latter.[9] In a separate area in the Sichuan basin, there is an independent population of *Pomacea canaliculata* that may be associated with the its earlier introduction for aquaculture.[9]

The most important factor driving the invasion of *P. canaliculata* is considered to be the environmental temperature, although many other variables, such as the level of dissolved oxygen, the pH of the water, and soil moisture during dormancy, are associated with overwintering success.[10] Experimental studies on *P. canaliculata* have found that the threshold temperature and effective accumulated temperature for its development were 11.4 °C and 1309 degree days respectively. Based on these results, it was predicted that this snail can overwinter in nearly all areas in China as long as they are in unfrozen water. In all provinces there are some natural waters that could permit it to complete one generation in two years, and in southern provinces

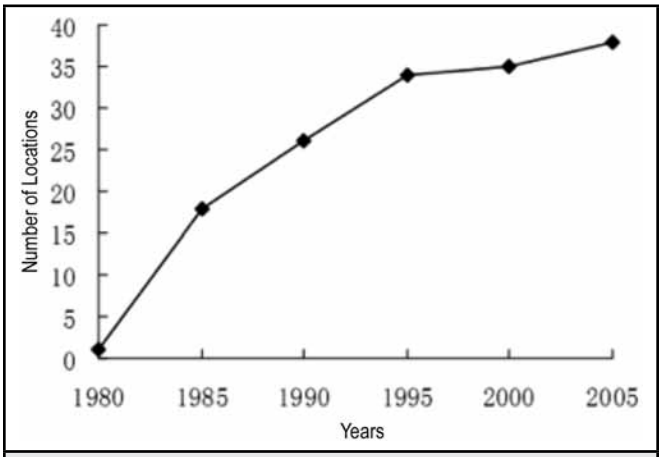

Figure 1. The number of locations in which *Pomacea canaliculata* was recorded in China from 1980 to 2005. Modified from Yang et al.[8]

such as Guangdong and Hainan that could allow it to complete three generations per year.[11] This potential is confirmed by the existence of the natural population in Sichuan province, despite many waters in this province being iced up in winter.[12] In Wenzhou, Zhejiang Province, the natural population of *P. canaliculata* moved northward about 10 km per year.[13] Based on its current distribution in China and the effective temperature accumulation for its development, it has been predicted that its range will expand into large areas further northwards by the 2030s, including the entire Chongqing municipality, Hunan, Hubei, Jiangxi, and Zhejiang provinces.[14] The predicted increase in the distribution between the 2020s and 2030s is 378,700 km^2. The region in which it becomes established will move further northeast, mainly including the Huaihe River valley in southern Henan and Anhui provinces, and central Jiangsu province.[14] Considering uncertainties regarding the rate of global climate change, the future distribution may be even greater than these predictions suggested.

Host of *Angiostrongylus cantonensis*

Angiostrongylus cantonensis, known as the rat lungworm, is a nematode parasite, with the adult worms being found in the pulmonary arteries of rats. Molluscs, ie, freshwater and terrestrial snails, serve as the intermediate hosts. By ingestion of uncooked or undercooked foods (eg snails) containing live third stage larvae of *A. cantonensis*, the parasite causes an infection in humans known as angiostrongyliasis, with the symptoms of eosinophilic meningitis, although humans are only accidental hosts of this species of nematode and in which it does not complete its life cycle. Eosinophilic meningitis caused by *Angiostrongylus cantonensis*, a potentially fatal disease, is considered an emerging infectious disease in mainland China.[12] Thirty-two species of molluscs in China have been screened for *A. cantonensis*; 22 of them (69%) harbored the parasite.[12] The highest rate of infection was recorded in the giant African snail, *Achatina fulica* (97%), followed by slugs (up to 100% in some species, ie, *Philomycus bilineatus* and *Vaginulus* spp.), and *Pomacea canaliculata* (69.4%). Although terrestrial snails and slugs showed higher rates and intensities of infections than freshwater molluscs overall, at least one freshwater snail, *P. canaliculata*, plays an important role in the epidemiology of angiostrongyliasis.

Extensive epidemiological evidence indicates that *P. canaliculata* is becoming the most important natural intermediate host for *A. cantonensis* in mainland China because of its high susceptibility to the parasite and its wide environmental tolerance. Third stage larvae of *A. cantonensis* were first detected in *P. canaliculata* in China in Wenzhou, Zhejiang province, from examination of 361 individuals of this snail species.[6] The prevalence (proportion of the sample that were infected) was 69.4% and mean intensity (number of larvae per snail) was about 32.6. Subsequently, there have been many other reports from other parts of China.[15-17] The broad and rapid expansion of the distribution of *P. canaliculata* due to its dispersal both on the land and in the water, the high prevalence and intensity of infection by *A. cantonensis*,[9] and the fact that it is still being collected as food by some people and offered in some restaurants, have contributed to it being the most important intermediate host not only in the regions where it now occurs, but also in provinces where it is not found, having been transported for food to such locations.[7] *Pomacea canaliculata* is a somewhat amphibious snail. Females emerge from the water to lay their eggs attached to emergent vegetation, rocks, logs, and other rigid surfaces. In the absence of water (eg, drained rice paddies) they survive by estivation in the mud. Thus, although the primary route of infection by *A. cantonensis* larvae is from ingestion of rat feces washed into the water, their ability to survive out of the water enhances their likely contact with rat feces and increases their chance of infection.

The biggest outbreak of eosinophilic meningitis caused by *A. cantonensis* in Beijing, where *P. canaliculata* does not occur, resulted from consuming undercooked *P. canaliculata* imported from more southerly regions where it does occur.[3] Based on reports of outbreaks of eosinophilic meningitis in China caused by *A. cantonensis*, 8 of 9 outbreaks resulted from consumption of undercooked *P. canaliculata* and only one from eating *Achatina fulica* (Table 1).[16] Thus, the emergence of angiostrongyliasis has largely been attributed to the spread of *P. canaliculata*.[14]

Table 1. Reported outbreaks of eosinophilic meningitis caused by *Angiostrongylus cantonensis* infection in mainland China (from Zhang, et al.)[16]

Year	Location (city, province)	Number of people infected	Source of infection	Reference
1997	Wenzhou, Zhejiang	65	*Pomacea canaliculata*	22
2002	Changle, Fujian	8	*Pomacea canaliculata*	15
2002	Fuzhou, Fujian	9	*Pomacea canaliculata*	23
2002	Fuzhou, Fujian	13	*Achatina fulica*	24
2004	Kunming, Yunnan	25	*Pomacea canaliculata*	25
2005	Kunming, Yunnan	9	*Pomacea canaliculata*	26
2006	Beijing	160	*Pomacea canaliculata*	27
2007	Zhaoqing, Guangdong	6	*Pomacea canaliculata*	28
2008	Dali, Yunnan	41	*Pomacea canaliculata*	29

Control of *P. canaliculata* in China

Since in China *Pomacea canaliculata* appears to be the main vector of angiostrongyliasis to humans, and because it is a major pest of crops in many regions of mainland China, widespread efforts have been implemented in attempts to control it and limit its further spread. These control measures can generally be divided into physical, chemical, biological, and agricultural methods.[18] Most simply, farmers hand-pick egg masses of the apple snails or collect the snail in the ditches and paddy fields.[19] Many commercial molluscicides, primarily niclosamide, crystal copper sulfate, sodium pentachlorophenate and fentin acetate, have proven to be effective, but with environmental side effects.[18] Many by-products and extracts of plants, such as tea seed cake, aqueous saponins, nicotine, and extract of *Eynedrella nodiflora* have been used to kill or control the snails.[20,21] Animals used for biological control of the snails mainly include ducks and snail eating fish such as black carp (*Mylopharyngodon piceus*). It has even been reported that ducks cultured in paddy fields could eliminate 99% of the adult snails and 92% of egg masses in rice fields.[18] Mechanized farming and rotation of aquatic and xeromorphic crops can kill some of the snails and prevent major outbreaks of the snail in crop fields.[10,19]

Conclusion

To reduce the chance of human infection by the parasite, one of the most effective methods would be interrupting its life cycle by controlling the intermediate hosts. The current wide distribution of *P. canaliculata* in China and the lack of a powerful, specific, and environmentally safe molluscicide make it extremely difficult to control populations of this snail in wild. Furthermore, the wide environmental tolerance exhibited by *P. canaliculata* facilitates its rapid spread, and its potential spread to all parts of China requires the highest attention. Global warming will assuredly facilitate its northward spread. Due to the close connection of this snail with outbreaks of eosinophilic meningitis, the abundance, distribution, and spread of this snail should be closely monitored. However, the most effective method to prevent human infection is to educate people to maintain good sanitation in food preparation areas, not to eat raw or undercooked snails, and to avoid eating raw vegetables that may harbor inconspicuous (eg juvenile) snails or slugs in regions where *A. cantonensis* is present.

Conflict of Interest

None of the authors identifies any conflict of interest.

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Human Parasitic Meningitis Caused by *Angiostrongylus cantonensis* Infection in Taiwan

Hung-Chin Tsai MD, PhD; Yao-Shen Chen MD; and Chuan-Min Yen PhD

Abstract

The major cause of eosinophilic meningitis in Taiwan is Angiostrongylus cantonensis. *Humans are infected by ingesting terrestrial and freshwater snails and slugs. In 1998 and 1999, two outbreaks of eosinophilic meningitis caused by* A. cantonensis *infection were reported among 17 adult male immigrant Thai laborers who had eaten raw golden apple snails (*Pomacea canaliculata*). Another outbreak associated with consuming a health drink consisting of raw vegetable juice was reported in 2001. These adult cases differed from reports in the 1970s and 1980s, in which most of the cases were in children. With improvements in public health and education of foreign laborers, there have since been only sporadic cases in Taiwan. Review of clinical research indicates inconsistent association of Magnetic Resonance Imaging (MRI) results with clinical features of eosinophilic meningitis. MRI features were nonspecific but there was an association between the presence of high brain MRI signal intensities and severity of peripheral and cerebrospinal fluid (CSF) eosinophilia. Inflammatory markers have been identified in the CSF of patients with eosinophlic meningitis caused by* A. cantonensis *infection, and vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and the matrix metalloproteinase system may be associated with blood-brain barrier disruption. Eosinophilic meningitis caused by* A. cantonensis *infection is not a reportable disease in Taiwan. It is important that a public advisory and education program be developed to reduce future accidental infection.*

Keywords

Angiostrongylus cantonensis, *Eosinophilic meningitis, Taiwan*

Epidemiology

The major cause of eosinophilic meningitis in the Pacific Islands and Taiwan is *Angiostrongylus cantonensis*, the rat lungworm.[1-4] Humans are infected with *A. cantonensis* by ingesting terrestrial and freshwater snails and slugs.[5-8] The major intermediate hosts for *A. cantonensis* in Taiwan are the giant African snail (*Achatina fulica*) and the golden apple snail (*Pomacea canaliculata*).[5-8] *Pomacea canaliculata* was introduced to Taiwan in 1979 as a food source. It spread widely in paddy fields and drainage ditches and has become an important cause of outbreaks of eosinophilic meningitis. Since the first human infection was reported in Taiwan in 1945,[1] many cases have been reported, mainly in children and most of them in the 1970s and 1980s.[9,10] A study of the epidemiologic characteristics of 125 cases of eosinophilic meningitis or meningoencephalitis that occurred in southern Taiwan in 1968 and 1969 revealed a close association of the disease with the rainy season.[4] Most of these cases were in children; most were among native Taiwanese people; and most patients had eaten a giant African snail prior to their illness. As Taiwanese people habitually do not eat snails uncooked, it was suspected that infection resulted from inadvertently ingesting *A. cantonensis* larvae liberated when the snails were prepared for cooking prior to consumption. In another study on the clinical characteristics of *A. cantonensis* infection among 82 children in Taiwan, 38 (46%) were male and 44 (54%) female, and 87% could be traced to a history of contact with the giant African snail.[10] The most common clinical symptom was fever (92%), followed by vomiting and headache. The sixth and seventh cranial nerves exhibited neuropathy in 20% and 11% of cases, respectively. Worms were recovered from cerebrospinal fluid (CSF) by lumbar puncture in 34 (42%) of the 82 cases. Albendazole and levamisole were used for clinical treatment and with good results.

The epidemiology of eosinophilic meningitis in Taiwan has changed since the 1990s, largely due to improvement in water quality and sanitation. Most of these more recent cases were in adults, especially foreign laborers.[11,12] Between 1991 and 2009, 37 cases were reported.[13] The median age in these cases was 32 years (range 2-80 years), with 35 (95%) being ≥18 years old. The median incubation period was 10.5 days. Most of the patients presented with headache (29, 78%), fever (25, 68%), and hyperesthesia (11, 30%). Among these patients, 22 (60%) were infected after eating raw snails, seven (19%) after drinking raw vegetable juice (probably contaminated with snails), and one after eating raw frog (paratenic host). Eight patients suffered from recurrence of headache after treatment and two patients died. Eosinophilic meningitis caused by *A. cantonensis* infection is not a reportable disease in Taiwan so the actual number of cases is probably underestimated. There have been only sporadic cases reported in recent years, mainly in foreign laborers.

Intermediate Hosts in Taiwan

The major intermediate hosts of *A. cantonensis* in Taiwan are the giant African snail (*Achatina fulica*) and the golden apple snail (*Pomacea canaliculata*),[5,8] both introduced species. In southern Taiwan one study found 14-31% of *P. canaliculata* with third stage larvae of *A. cantonensis*,[8] while a more recent study found infection rates in this species of 12-29% with an average of 36-65 motile larvae per infected snail.[14] The high infection rate of *P. canaliculata* emphasizes the risk of infection via eating raw snails. Regular surveillance of the infection rate of these intermediate hosts is recommended.

Magnetic Resonance Imaging (MRI)

MRI scans of the brains of 13 patients from the 1998 and 1999 outbreaks showed normal, high signal intensities over the globus pallidus and cerebral peduncle on T1-weighted imaging, leptomeningeal enhancement, ventriculomegaly, and punctate areas of abnormal enhancement within the cerebral and cerebellar hemisphere on gadolinium-enhancing T1 imaging, and a hyperintense signal on T2-weighted images.[15] There was a significant ($P < .05$) correlation of MRI signal intensity

in T1-weighted imaging with severity of headache, CSF pleocytosis, and CSF and blood eosinophilia.[15] However, in other cases, there was no association between intensities over the globus pallidus on T1-weighted imaging and *A. cantonensis* infection.[16] In another retrospective study,[17] the brain MRI findings were nonspecific, ie, normal (n = 1), leptomeningeal enhancement (n = 21), hyperintense signal lesions (n = 11) on T2-weighted MRI, and nodular enhancing lesions in gadolinium-enhanced T1-weighted imaging (n = 1). There were significant associations between high brain MRI signal intensities and peripheral eosinophilia ($P < .02$), CSF eosinophil count ≥ 10% ($P = .01$), and the presence of antibodies to *A. cantonensis* in the CSF ($P < .01$). The time from onset of symptoms to spinal tapping or brain MRI did not have an effect on the presence of MRI abnormalities. These brain MRI findings thus did not add any additional rigorous diagnostic evidence to the clinical evaluation of patients with eosinophilic meningitis. Brain MRI should therefore be used as a follow up modality rather than a diagnostic tool.

Inflammatory Markers in CSF of Patients with Eosinophilic Meningitis

Studies in Taiwan have shown that vascular endothelial growth factor (VEGF),[18] hepatocyte growth factor (HGF),[19] and the matrix metalloproteinase system may be associated with blood-brain barrier (BBB) disruption in patients with eosinophilic meningitis caused by *A. cantonensis* infection.[20] There was an association between CSF levels of VEGF, CSF protein, white cell counts, and eosinophil counts. However, the serum levels of VEGF fluctuated during the follow-up period.[18] The CSF/blood ratio of HGF, another neurotropic factor, was higher at presentation when compared with uninfected individuals, but the levels of HGF in CSF were not correlated with the amount of CSF cells or proteins.[19] In another study of 40 patients with eosinophilic meningitis caused by *A. cantonensis*, possible BBB dysfunction caused by matrix metalloproteinase-9 (MMP-9) and its regulation by tissue inhibitors of metalloproteinase (TIMPs) was evaluated.[20] The concentrations of MMP-2, MMP-9, TIMP-1, and CSF/serum albumin ratios (QAlb values) were significantly increased in patients compared with controls, but concentrations of TIMP-4 were significantly lower in patients. Gradual decreases in levels of QAlb, MMP-9, and TIMP-1, and increases in levels of TIMP-4 were observed in six of the patients during recovery.[20] These results suggest that the source of MMP-9 in the CSF of patients with eosinophilic meningitis is probably associated with leukocytes migrating from peripheral blood to the CSF.[20]

Conclusion

The epidemiology of eosinophilic meningitis in Taiwan has changed since the 1990s and now occurs mainly in adults, and in sporadic outbreaks. It is important that a public advisory describing the dangers of eating raw snails and drinking raw vegetable juices be developed to reduce future accidental infection.

Conflict of Interest

None of the authors identifies any conflict of interest.

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Angiostrongyliasis in Thailand: Epidemiology and Laboratory Investigations

Praphathip Eamsobhana PhD

Abstract

Cerebral angiostrongyliasis due to Angiostrongylus cantonensis *continues to affect human health and productivity in Thailand. The dietary habits of the populace have been an important contributing factor, particularly in the northeast of the country where the disease is endemic and the indigenous people enjoy a local undercooked snail dish called "koi-hoi". Hundreds of cases of disease continue to be reported annually. Because of the difficulty in obtaining a definitive diagnosis, immunological methods have played an important role in the confirmation of* A. cantonensis *infection. Although enzyme-linked immunosorbent assay (ELISA) and immunoblot are test formats that have been used over the past decade, modern molecular approaches, such as PCR-based diagnostic techniques, are being developed and assessed as additional tests for the diagnosis of cerebral angiostrongyliasis. This short review focuses on the history, incidence, and laboratory diagnosis of angiostrongyliasis in Thailand.*

Keywords

Angiostrongyliasis, Angiostrongylus cantonensis*, Diagnosis, Eosinophilic meningitis, Laboratory testing, Thailand*

Past and Present Angiostrongyliaisis in Thailand

The rat lungworm, *Angiostrongylus cantonensis*, is the primary cause of eosinophilic meningitis or eosinophilic meningoencephalitis. In Thailand, the disease was first recognized in 1955 and documented in 1957.[1] Many cases were recorded in the 1960s, starting in 1961 with two patients that developed eosinophilic meningitis after eating undercooked *Pila* snails.[2] The number of cases in the 1960s increased rapidly from those two cases to 572 cases in 1966. Of the 1164 cases from 1955 to 1966, 912 were from the northeastern provinces.[2-10]

Between 1965 and 1968, typical cases of eosinophilic meningitis were investigated throughout the country.[2] Among the 484 cases investigated, there was no apparent difference in the age distribution of patients from different geographic areas.[2] The youngest patient was 2 years old and the oldest 65 years old. Most of the patients belonged to the 20-39 age groups. Males were affected 2.6 times more frequently than females, with 348 males and 136 females. More than half of the patients were farmers. Laborers made up the second largest group of cases. Other cases included students, housewives, government officials, military men, and merchants.

During 1981-1984, 30 cases of eosinophilic meningitis in children were reported in the northeastern province of Khon Kaen.[11] The patients ranged from 6 to 14 years old, comprising 18 boys and 12 girls. The cases were reported throughout the year and there was no significant seasonal pattern in their occurrence. Two-thirds of the patients had a history of eating snails and raw food. One of them had ocular *A. cantonensis* infection. Twenty-nine of the children recovered completely but one died.

More recently, in 1991, an additional three cases of eosinophilic myelomeningoencephalitis were recorded.[12] During 1995-2005, 654 cases were treated at Srinagarind Hospital, Khon Kaen.[13]

Despite the difficulty of recovering *A. cantonensis* from infected patients, worms have been recovered from the cerebrospinal fluid (CSF) of a number of Thai patients,[10,14-16] and from the brain of at least ten fatal cases following brain biopsy.[9,11,17-19] Eleven living adult worms were recovered from the CSF of an 8 month old girl with a two week history of chronic fever and seizures.[15] In a fatal case at Siriraj Hospital, Bangkok, in 1990, many fifth stage larvae were detected in the brain.[17] The infection was attributed to eating raw or partially cooked livers from monitor lizards preceding the onset of symptoms. Unfortunately, no leftover liver was available to confirm the presence of infective larvae, but a subsequent study of 22 monitor lizards from five provinces in Thailand showed that 96% (21 out of 22) were infected with *A. cantonensis*, with most of the larvae in the liver.[20]

Fatal cases are relatively rare. Five fatal cases were recorded in Thailand in the 1960s,[9] four in the period 1974-1977,[19] one in 1981-1984,[11] and one in 1990.[17] Many live and dead fourth and fifth stage larvae were present in the meninges and brain tissue of these fatal cases.

Ocular angiostrongyliasis has been documented occasionally in Thailand. Three cases with young adult *A. cantonensis* recovered from the anterior chamber of the eye were recorded in the 1960s.[21-23] A case associated with eosinophilic meningitis was reported in 1971,[24] and a pediatric case in 1985.[11] Three cases of intravitreal infection have been reported.[25,26] In a woman who had eaten raw snails, the worm was located by the use of an intravitreal cryoprobe and was successfully removed via the pars plana with vitreous foreign body forceps.[26] Signs of meningitis were present in two men, in each of whom a small motile worm was found in the vitreous cavity.[25] In one case there was also a dead, disintegrated worm in the inferior portion of the vitreous cavity. Another seven cases (four men and three women) with intraocular angiostrongyliasis were documented in Srinagarind Hospital, Khon Kaen, between January 1995 and April 2005.[13] There is no evidence that surgical and laser interventions improve the course of the ocular disease. Visual outcome depends only on initial visual defects/acuity.

The first ever reported case of eosinophilic meningitis associated with sensorineural hearing loss involved a 59 year old woman who had chronic headache, neck stiffness, and left-side hearing loss.[27] Her condition, including hearing, improved after treatment with prednisolone.

Epidemiology in Thailand 2000-2009

According to recent statistics from the National Surveillance System, Department of Disease Control, Ministry of Public Health, Thailand,[28] for the period 2000 to October 2009, the rate of reported cases declined sharply from about 2.24 per 100,000 of the population (1,386 reported cases) in 2000 to 0.2-0.3 per 100,000 in 2005-2009, with 172 cases reported in 2009. The fatality rate varied among years. The rate was 0.07 per 100,000 of the population in 2000, 0.19 in 2002, and 0.78 in 2007. There were no fatal cases in 2001, 2003-2006, and 2008-2009 (Figure 1).

The reported cases over the years are in a broad range of age-categories, as reflected, for example, by the statistics for January-October 2009 (Figure 2). The highest rate of reported cases is in the 35-44 age group, with 0.41 per 100,000 of the population. This is followed by the 15-24 and 45-54 age groups with 0.39 and 0.37 per 100,000 of the population, respectively. Overall, there are more males than females among the reported cases, with a ratio of 1.77 to 1. Farmers constituted the majority of the reported cases, accounting for 70% (120/172). Thai farmers eat a local undercooked snail dish called 'koi-hoi', usually as an appetizer when drinking alcoholic beverages after their daily farming activities. Daily-paid workers constituted 15% (25/172) and pre-school children 7% (12/172) of the cases.

Cases of eosinophilic meningitis are reported year-round. However, during 2009, the number of cases rose steadily from

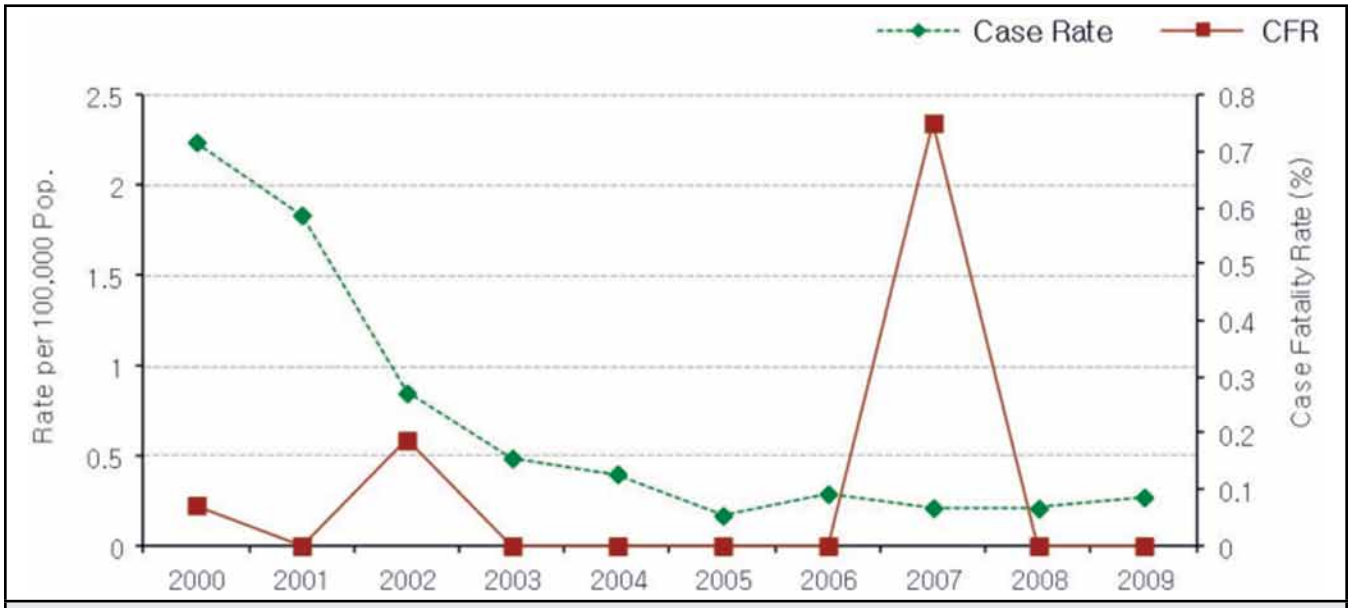

Figure 1. Total annual reported cases and fatal cases of eosinophilic meningitis per 100,000 of the population in Thailand, 2000-October 2009.[28]

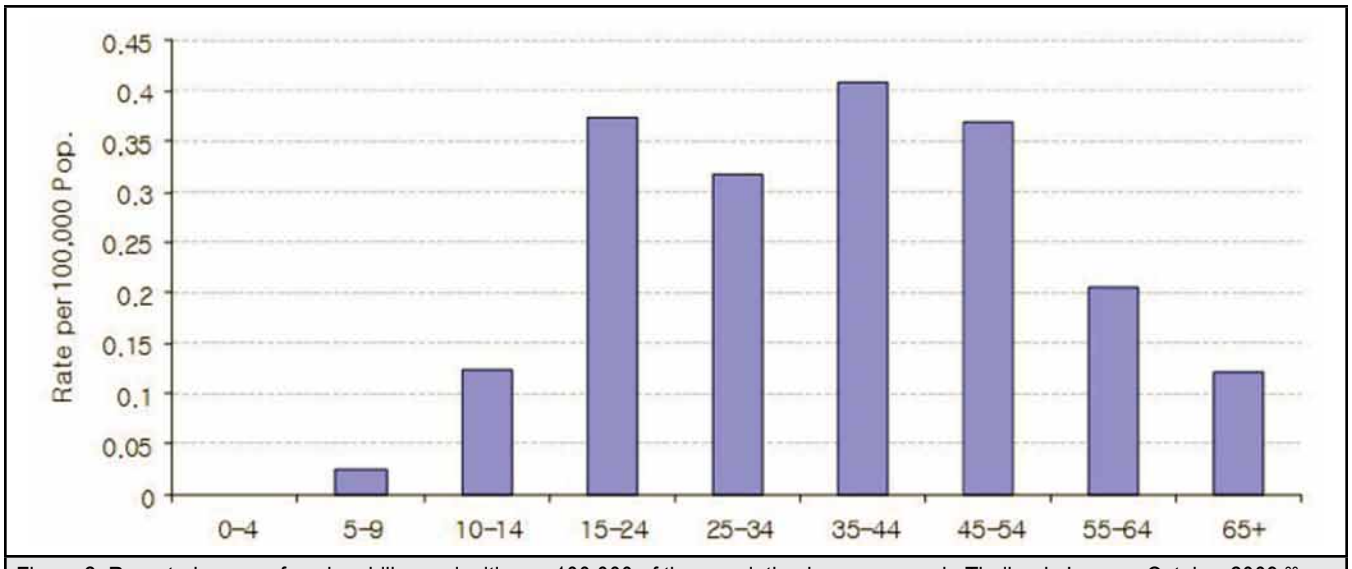

Figure 2. Reported cases of eosinophilic meningitis per 100,000 of the population by age-group in Thailand, January-October 2009.[28]

January to the end of the year (Figure 3), the reason for this remaining unknown.

The highest rate of annual reported cases was 23.08 per 100,000 in the northeast region, where Loei province accounted for more than half of the total reported cases in Thailand during January-October 2009 (Figure 4). Rates in all other provinces were much lower, with the second highest rate (1.23 per 100,000) in Phayao province and all other rates <1 per 100,000 (Figure 4).

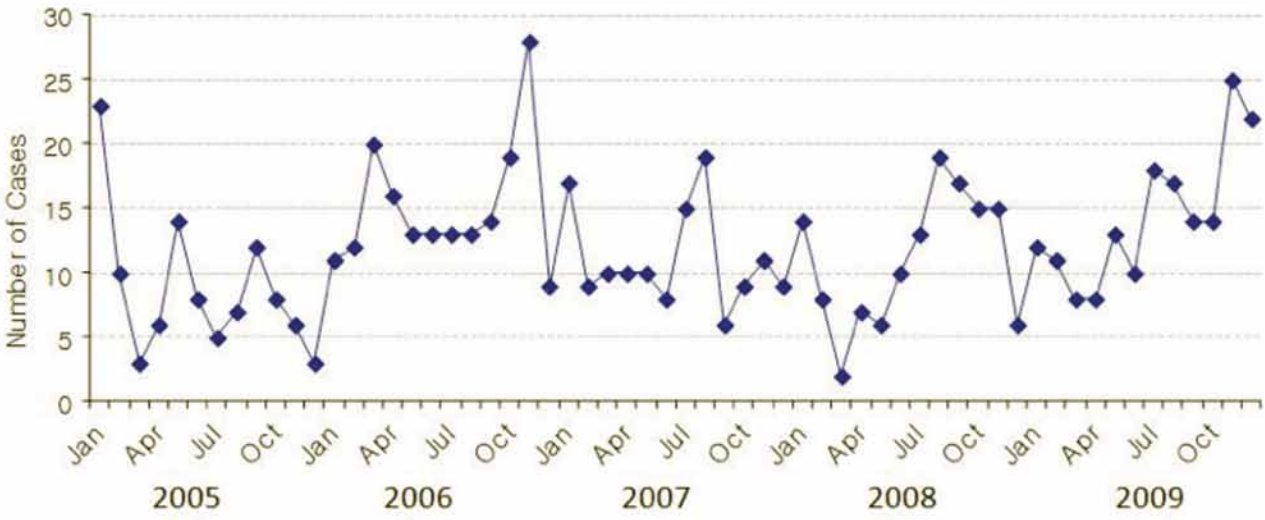

Figure 3. Monthly reported cases of eosinophilic meningitis in Thailand, 2005-2009.[28]

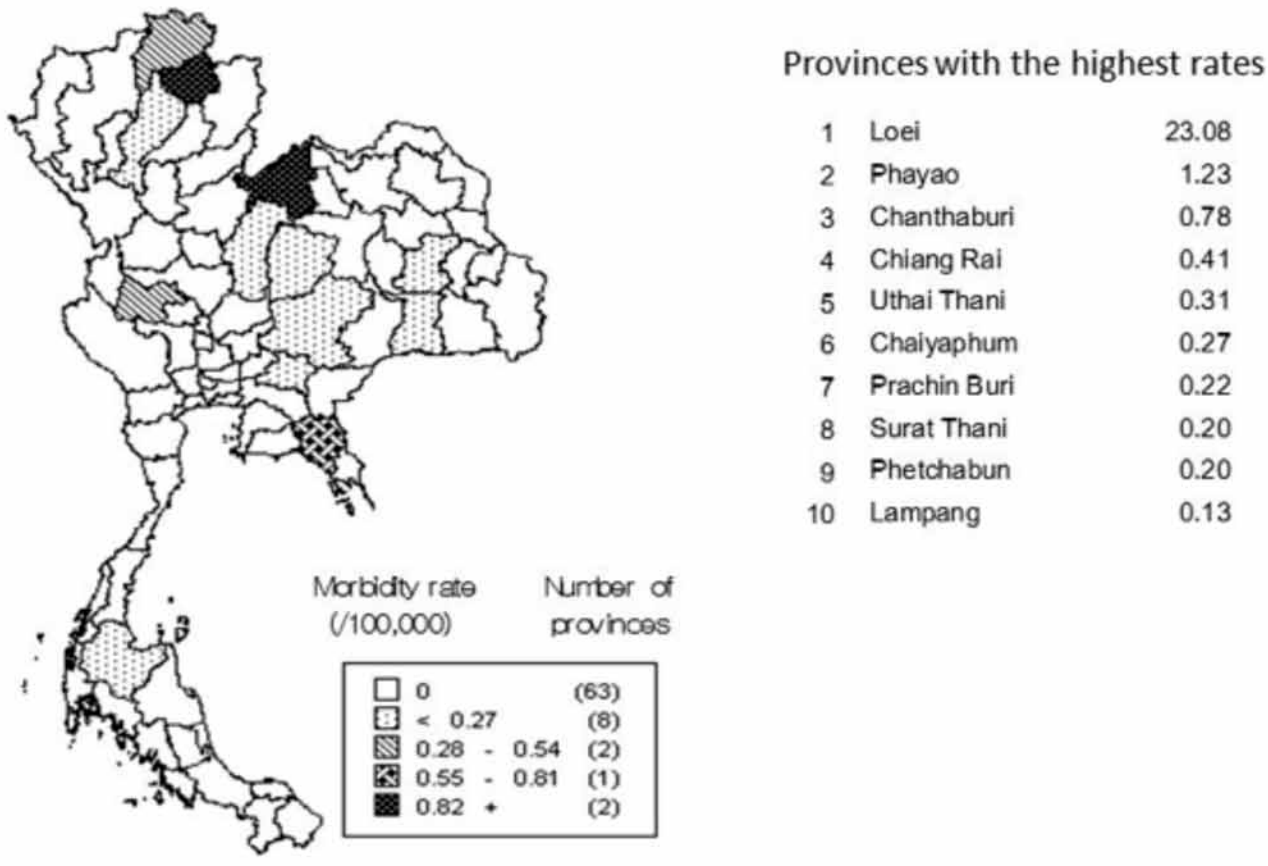

Figure 4. Reported cases of eosinophilic meningitis per 100,000 population in the ten provinces with the highest rates, Thailand, 2009.[28]

Laboratory Diagnosis

Eosinophilic meningitis caused by *A. cantonensis* is generally diagnosed based on clinical presentations and laboratory findings. Typically there is pleocytosis in the CSF with an eosinophil count of 26-75%, accompanied by a peripheral eosinophilia of 5-65%.[18] In Thai patients, presumptive preliminary diagnoses are usually made based on the person's specific eating habits, particularly among people living in the parasite endemic areas, the northeastern and central parts of the country.

Although eosinophilic meningitis is an indication of the infection, the various clinical presentations caused by *A. cantonensis* must be differentiated from those caused by other related helminths (ie, *Gnathostoma* spp., *Paragonimus* spp., and *Taenia solium* metacestodes) that are also endemic in Thailand.[29]

Immunological testing has been very helpful in supporting the clinical diagnosis. With the introduction of purified homologous antigens for detection of the parasitic infection, the problem of cross-reactivity that occurs in immunodiagnostic methods has been eliminated. A 31 kDa glycoprotein from *A. cantonensis* has been used as a highly specific antigen for immunodiagnosis.[30,31] In the Department of Parasitology at Siriraj Hospital, the immunoblot technique currently used for diagnosis of angiostrongyliasis has improved antibody detection. A standard ELISA using crude antigens is used for screening and all ELISA-positive samples are tested by immunoblot for routine confirmation. A serum reacting with a specific 31 kDa band is indicative of angiostrongyliasis.

Nevertheless, the current enzyme immunoassay format is time-consuming because of the need for multiple reagent additions and long washing and incubation steps. A more user-friendly, rapid, filtration-based immunogold assay is under evaluation. An initial non-enzymatic, dot immunogold filtration assay (DIGFA) with crude antigen preparation was used to detect specific immunoglobulin G (IgG) antibody against *A. cantonensis* in infected patients and was found to have a diagnostic sensitivity of 91% and specificity of 98% for human angiostrongyliasis.[32] In a subsequent collaborative study between Siriraj Hospital and the Institute of Parasitic Diseases, Zhejiang Academy of Medical Sciences, Hangzhou, People's Republic of China (with Dr. Xiao-Xian Gan), a modified rapid dot-immunogold test using purified 31 kDa antigen of *A. cantonensis* to enhance test sensitivity has performed well on clinical samples at Siriraj Hospital. This test is now being validated on serum samples collected from various areas in Thailand where *A. cantonensis* is endemic. As the test is rapid (3 min), easy to perform, and needs no special equipment, it is possible that a DIGFA test will soon replace the 2 hr immunoblot test for support of clinical diagnosis of human angiostrongyliasis. The approach is also promising in terms of future diagnostic test kits.

As an alternative to the antibody or antigen detection assay, a conventional PCR technique for the detection of *A. cantonensis* DNA in clinical CSF samples has been developed that unequivocally demonstrated the presence of parasites in patients.[33] Primers were designed based on a mRNA sequence encoding a 66 kDa native protein of the *A. cantonensis* adult worm present in CSF samples from Thai patients with serologically confirmed angiostrongyliasis. Primers produced an amplified fragment of approximately 300 base pairs in four out of ten patients studied. The nucleotide sequences shared 98.8-99.2% similarity with the reference sequence of *A. cantonensis*.[33] Although preliminary results are encouraging, more clinical samples from angiostrongyliasis patients and other clinically related parasitic infections with their full clinical information are still needed for evaluation of this PCR approach for diagnostic use, including its specificity and sensitivity.

With the rising need for more economical, reliable, and rapid diagnostic tools, the development of new diagnostic tests, such as those in a chromatographic test format, that can differentiate parasitic causes of eosinophilic meninigitis, including that caused by *G. spinigerum* and by *T. solium* metacestodes, are overdue. Since multiple parasite infections via food-borne pathways are common, particularly in endemic communities in northeastern Thailand, this need is even more urgent. Development of these tests will also enhance large-scale epidemiological studies, given the potential to screen multiple parasites in patient specimens at one time.

Conflict of Interest

The author identifies no conflict of interest.

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Rat Lungworm: An Emerging Zoonosis in Jamaica

Ralph D. Robinson PhD; Cecelia A. Waugh PhD; Cheridah D. Todd MPhil;
Jacob Lorenzo-Morales PhD; and John F. Lindo PhD

Abstract

Rat lungworm infection is caused by a metastrongylid nematode, Angiostrongylus cantonensis*. The parasite was first recorded in the pulmonary arteries and heart of domesticated rats in China in 1935: its medical importance, however, was established 10 years later when Nomura and Lin recovered* A. cantonensis *larvae from the cerebro-spinal fluid of a human teenager with meningitis in Taiwan in 1945.[1] Since then, the parasite has become recognised as a major cause of human meningoencephalitis worldwide.[2,3]*

In the Caribbean, A. cantonensis *has been documented in wild rats in Cuba,[4] Dominican Republic,[5] Grenada,[6] Haiti,[7] and Puerto Rico.[8] Human infections have been reported in Cuba[4] and Martinique.[9] However, the parasite appears to be absent from Barbados.[10] Land snails and slugs serve as intermediate hosts for the parasite, while a range of terrestrial and freshwater invertebrates may serve as paratenic hosts.*

Original investigations in Jamaica to assess the infection status of A. cantonensis *followed an outbreak of eosinophilic meningitis on the island.[11] They involved the collection of 437 wild rats (297 black rats,* Rattus rattus*, and 140 brown rats,* R. norvegicus*), and 777 terrestrial molluscs (representing 12 species of snails and slugs) from parishes making up the four Regional Health Authorities (RHAs) on the island.*

Adults of A. cantonensis*, measuring ~10 mm, were recovered from the cardiopulmonary system of 32.0% (n = 437) of the wild rats examined. The mean intensity of infection was 15.3 worms (n = 140). Multivariate analysis (binary logistic regression model) confirmed that* A. cantonensis *occurred significantly more frequently in* R. rattus *(odds ratio [OR] = 1.76); that* R. rattus *also harbored significantly more worms (mean = 16.8) than* R. norvegicus *(mean = 11.3 worms) (Student's t = -2.241; bootstrap [two-sided] P = .02); and that the majority of rodent infections occurred in the Northeast Regional Health Authority (OR = 11.66). The Northeast RHA includes a high proportion of wet, limestone forest.*

Of 777 snails and slugs examined, 12.5% harbored A. cantonensis*. These included* Thelidomus aspera *(18.7%, n = 369),* Pleurodonte *sp. (29%, n = 86),* Sagda *sp. (11%, n = 18),* Poteria *sp. (20%, n = 5), and veronicellid slugs (6%, n = 34). All four genera of snails represent new host records for* A. cantonensis*. The widespread occurrence of the parasite, combined with increasing (> 20) reports over the last 20 years of infections in humans who never travelled abroad,[12] indicates that autochthonous transmission is occurring, and that* A. cantonensis *represents an important emerging infection in Jamaica.*

Keywords

Angiostrongyliasis, Caribbean, Eosinophilic meningitis, Jamaica, Nematodes, Rat lungworm disease, Slugs, Snails

Conflict of Interest

None of the authors identifies any conflict of interest.

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Angiostrongylus cantonensis: Epidemiology in the Continental United States and Hawai'i

Sarah Y. Park MD and LeAnne M. Fox MD, MPH

Abstract

Autochthonous Angiostrongylus cantonensis *infection has been little recognized in the continental United States with the exception of Louisiana. In contrast, it was recognized in Hawai'i in the early 1960s, and the parasite has been considered endemic since. However, infections were rare until late 2004, when a case cluster was noted on the Island of Hawai'i. While still uncommon,* A. cantonensis *infection has continued to emerge throughout the state, especially on the Island of Hawai'i. Despite increased community awareness, the diagnosis is commonly missed, and the lack of diagnostic tests as well as the challenge of educating clinicians and the public are constant limitations to the prevention and control of this emerging infection.*

Keywords

Angiostrongyliasis, Emerging infectious disease, Rat lungworm disease

Conflict of Interest

Neither author identifies any conflict of interest.

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Clinical Aspects of Eosinophilic Meningitis and Meningoencephalitis caused by *Angiostrongylus cantonensis*, the Rat Lungworm

Gerald S. Murphy MD and Stuart Johnson MD

Abstract

Angiostrongylus Eosinophilic Meningitis is caused by human infection with larvae of the rat lungworm, Angiostrongylus cantonensis. *The clinical presentation includes a spectrum of disease, from meningitis through radiculitis, cranial nerve abnormalities, ataxia, encephalitis, coma, and rarely death. The condition is diagnosed by recognizing the triad of: the clinical syndrome, eosinophils in the cerebrospinal fluid or blood, and exposure history. A history of eating raw or poorly cooked snails is classic, but ingestion of other intermediate hosts or unwashed produce (such as lettuce) harboring hosts is not uncommon. Several serologic tests exist but none has yet been fully validated. There is good evidence that a 2 week course of high dose corticosteroids shortens the duration and severity of symptoms. There is somewhat weaker evidence that albendazole reduces symptoms. The combination of prednisolone and albendazole is being used more commonly for treatment. Some suggestions for future research are given.*

Keywords

Albendazole, Angiostrongylus cantonensis, *Anthelmintic, Corticosteroid, Diagnosis, Eosinophilic meningitis, Eosinophilic meningoencephalitis, Eosinophilic radiculomeningoencephalomyelitis, Human, Nematode, Prednisolone, Rat lungworm, Slug, Snail, Treatment*

Introduction

Human disease from infection by the rat lungworm, *Angiostrongylus cantonensis*, is primarily seen in the Central Nervous System (CNS). Ingested third stage larvae (L3) migrate to the brain and spinal cord where they molt to L4 larvae (day 6-7 post ingestion, in the rat) and then to L5 young adult worms (day 11-13).[1] During this development they wander through the brain, sometimes emerging in the subarachnoid space. In the definitive host, the rat, young adult worms migrate to the pulmonary arteries via the cerebral venous system, but in humans most worms presumably die in the CNS before reaching the lungs.[2-8] Their presence, movement, and death in the CNS, and the immune response provoked, probably all contribute to the symptoms and signs.

There is a spectrum of disease produced when *A. cantonensis* invades the human CNS.[3] Most patients present with a meningitis characterized by eosinophils in the CSF. But, heavy infestations can produce an encephalitis characterized by severe neurological symptoms, coma, and even death. Spinal cord involvement can produce radiculitis. This range of presentations has led to several variations in nomenclature. Two species of *Angiostrongylus* produce human infection; the other being *A. costaricensis*, which produces a gastrointestinal syndrome. So the term "Angiostrongyliasis cantonensis" was proposed by Alicata to specify the neurological disease.[9] "Neuroangiostrongyliasis" also has been used infrequently. Although *A. cantonensis* is the most common cause of eosinophilic meningitis, there are many other etiologies. We favor the term "Angiostrongylus Eosinophilic Meningitis" (AEM) to describe the entire spectrum of human infection of the CNS by *A. cantonensis*. We will include encephalitis, encephalomyelitis, and radiculitis under this term for simplicity. AEM specifies both the neurologic syndrome and the etiology in a way that succinctly describes the disease. Additionally, an ocular form of the disease is recognized.[10] We briefly summarize the diagnosis and treatment of AEM from the clinical point of view, and offer some suggestions for future research.

Diagnosis

Diagnosis of infection due to *A. cantonensis* (AEM) is based primarily on clinical criteria. The worm is infrequently found in patient specimens, and antibody responses to the parasite are most commonly demonstrated during convalescence. Therefore, recognition of the main clinical syndromes, elicitation of a specific food consumption history, and travel to, or residence in, endemic regions are critical to establishing a presumptive diagnosis and initiating therapy.

The most common clinical syndrome encountered by residents of and travelers to endemic regions is uncomplicated meningitis.[11] One of the present authors reported an outbreak of AEM among a group of medical students and friends who traveled to Jamaica.[12] While this outbreak may not have represented the most severe presentation of AEM, it was possible to carefully define the timing of symptom onset, range of symptoms, and laboratory findings present at the time of evaluation at the hospital. Symptoms began a median of 11 days (range 1 week to 1 month) after consumption of the implicated meal, with a trend toward earlier onset among those that were hospitalized compared to those not hospitalized. The main symptoms reported and their relative frequency included headache (100%), photophobia or visual disturbance (92%), neck stiffness (83%), fatigue (83%), hyperesthesias (75%), vomiting (67%), and paresthesias (50%). The headaches were described as progressive and severe, and the cutaneous sensory findings were randomly present on the extremities and/or the trunk and did not have a single dermatomal pattern of distribution. The only other focal neurological finding was a subtle resting tremor in one of the more severely affected students. Fever was uncommon. Formal ophthalmologic evaluations of 2 patients showed only mild papilledema in one patient. Cerebral spinal fluid (CSF) was examined microscopically in 7 patients without demonstration of larvae. These symptoms and clinical findings are similar to other reports of meningitis due to *A. cantonensis*.[13] In contrast, researchers in Taiwan described high recovery rates of worms from the CSF of affected children in which large volumes of CSF were obtained with a "pumping" technique.[14]

The hallmark of AEM is the presence of eosinophilia, either in the CSF or in the peripheral blood. While all patients in the outbreak discussed above had eosinophilia at some point in their clinical course, only half had CSF eosinophils at the time of their first lumbar puncture, and fewer than half had peripheral blood eosinophils on their initial blood draw.[12] The median CSF white blood cell count (375/mm^3), percent eosinophils (33%), protein (54 mg/dL) and glucose concentrations (59 mg/dL), and opening pressure (24 cm H_20) were similar to other reports.[13] The peripheral blood eosinophilia peaked 2 weeks after their acute presentation and resolved 1 month later.

Other clinical syndromes associated with *A. cantonensis* infection include encephalitis/encephalomyelitis and ocular angiostrongyliasis. Kliks, et al, gave a very detailed clinical description of an outbreak of radiculomyeloencephalitis among a group of Korean fisherman who shared a large meal of giant African snails (*Achatina fulica*) while in American Samoa.[5] The most prominent symptoms and findings in this outbreak involved sensory and motor disturbances of the legs with pain, weakness, absent reflexes, bowel/bladder dysfunction, and labile hypertension. In this outbreak and in other reports of severe infection, the neurologic symptoms were often preceded by a transient abdominal pain syndrome.[5,15] Three patients in this outbreak had particularly severe courses complicated by coma and quadriparesis. Although one patient died, the other patients recovered completely after several months. In contrast to the incubation period in our experience with uncomplicated meningitis (several weeks), the incubation in this outbreak was 1-6 days and suggested that severe disease due to *A. cantonensis* may be associated with ingestion of a large worm inoculum; potentially thousands of infected larvae are present in highly permissive intermediate hosts such as *A. fulica*.[16] Others have correlated the severity of AEM with the number of ingested snails.[17] An important consideration in the differential diagnosis of severe eosinophilic encephalitis with radicular symptoms is infection with *Gnathostoma* spp.[18] Other infectious (parasitic and occasionally non-parasitic) and non-infectious etiologies (eg, drugs, malignancies) may also occasionally manifest as eosinophilic meningitis (see Graeff-Teixeira, et al,[13] for a more complete differential diagnosis). Encephalitis and severe disease may also be more common in specific settings and age groups. A high percentage of cases reported from southern Taiwan involved children, where one third of the cases present with encephalitis, fever is common, and the overall mortality (4.9%) is considerably higher than is typically seen with uncomplicated meningitis in adults.[14] Finally, reports of ocular angiostrongyliasis describe patients who primarily complain of unilateral visual disturbance, sometimes with minimal systemic symptoms to suggest AEM.[10] A single worm is usually identified on fundoscopic exam of the affected eye.

A careful food intake and a travel history are also important in the diagnosis of AEM. One usually finds a history of ingestion of a raw or poorly cooked food source known to be an intermediate host (eg, snail, slug) or a paratenic host (eg, freshwater prawns, frogs, planaria, monitor lizards) for *A. cantonensis*. In other cases consumption of fresh produce is commonly noted, as with the romaine lettuce in the Caesar salad eaten by the travelers to Jamaica in the outbreak discussed above.[12] Sometimes mere contact with a snail during food preparation is all that is required for infection.[19] While most cases occur in Southeast Asia, the Pacific Basin, and nearby regions, cases have occurred in the Caribbean[12,20] and elsewhere,[13] and at least one autochthonous case has been reported in the continental US.[21] AEM continues to be seen at a low incidence in the Hawaiian Islands,[22] and one of the authors recently treated a patient there who had severe neurologic sequelae.[23]

Because of the severe symptoms present in AEM, brain imaging is often undertaken, but CT and MRI findings are relatively nonspecific and are supportive rather than diagnostic. CT abnormalities were noted in 6/19 (32%) of cases in one study and included leptomeningeal enhancement with contrast, mild ventricular dilation, and diffuse brain swelling.[24] There are no blinded studies of MRI findings, but several retrospective reviews have recently been published.[24-27] MRI appears to be abnormal in about 45%-69% of cases. Findings include leptomeningeal enhancement and increased signal intensity in the subcortical white matter of the cerebrum and cerebellum on T2 weighted and fluid attenuated inversion recovery (FLAIR) images. With gadolinium contrast, enhancing round, oval, or stick shaped lesions may be seen in the white matter measuring 3-14 mm in diameter on T1 weighted images. Lesions of the spinal cord, optic nerve, and lungs have been found infrequently. In one report a microcavity suggestive of a migratory tract was noted in the deep white matter.[24] In the report on the outbreak in travelers to Jamaica, head CT (n = 4) and MRI (n = 3) showed only non-specific leptomeningeal enhancement in one patient.

Specific evidence for AEM can be obtained serologically and several different ELISA and immunoblot assays have been studied.[28,29] The 31 and 29 kDa antigens prepared from adult female worms appear to have particular utility in assays for antibody detection,[30] but none of these assays is commercially available, standardized, and available for use outside of specific laboratories. Another limitation with serodiagnosis is that antibodies are not predictably present in the acute stage of infection. In the experience of the present authors, strong reactions to the 31 kDa antigen were present by Western blot in the convalescent sera of 11 of the 12 student travelers to Jamaica, but in the acute serum of only 1 patient.[12] Others have developed assays to detect circulating antigen.[31] A recently developed, species-specific, real-time PCR holds promise for the timely diagnosis of acute *A. cantonenis* infection that, if validated, may be more readily available to clinicians.[32]

Treatment

Treatment of Angiostrongylus eosinophilic meningitis (AEM) and meningoencephalitis is not well defined and remains controversial.[11,13,33-36] Mild cases resolve spontaneously without specific therapy. More serious cases can be improved with serial lumbar punctures and symptoms shortened with corticosteroid therapy. Severe cases can develop permanent, neurologic sequelae or

progress to coma and death, so specific treatments to reduce morbidity and mortality would be welcome. Yet, there are very few convincing studies of therapy for AEM, in part because it is a rare disease and tends to occur in more rural areas. Studies from different populations demonstrate different severities of illness, possibly related to the size of the inoculum of *A. cantonensis* L3 larvae ingested.[3] For example, adult Thais who consume *Pila* or *Pomacea* snails, with a relatively low larval burden, tend to have milder disease,[3,17,37] whereas ingestion of the giant African snail (*Achatina fulica*) with its high inoculum,[16] such as happened with adults in Samoa[5] and among children in Taiwan,[3,14] can lead to severe or fatal disease. Children also tend to have more severe disease than adults.[3,17]

In discussing treatment, we are confronted by the lack of knowledge regarding the pathophysiology of the disease. Part of the pathology of AEM appears related to increased intracranial pressure, and many reports have noted the immediate, although usually temporary, relief of headache afforded by a spinal tap.[3,11,12,37] This increased intracranial pressure (ICP) may be related to vasodilation of both arteries and veins seen in the subarachnoid space and brain parenchyma, decreased absorption of CSF, or brain edema.[2,5] One autopsy study suggested death may result from tentorial herniation due to increased ICP.[5] Autopsies of fatal cases of AEM have shown that numerous live worms (L4 larvae and L5 young adult worms) are present at the time of death, and they leave migration tracks with visible, axonal damage in both grey and white matter.[2,5,6] In addition, a robust, Th2 type inflammatory response, characterized by eosinophils, develops in the CNS and subarachnoid space.[2,38] The timing of this response may be most pronounced as the larvae molt from L4 to young adult stage and begin to emerge from the brain parenchyma into the meningeal vessels.[1,2,38] It is not clear which of these processes is most responsible for the pathology. To complicate matters further, anthelmintic drugs appear to be more effective at killing larval forms than adult worms,[39,40] yet patients generally seek medical care about the time when the L4 to young adult molt is just taking place.[1]

Most experts recommend high volume spinal taps to relieve headache and prevent the pathology associated with increased intracranial pressure.[3,11,14,37] The frequency is dictated by the patient's clinical course, with worsening headache and neurological status suggesting the need for a repeat tap. Acetaminophen and non-steroidal anti-inflammatory agents (NSAIDs) do not seem to offer much relief.[11,37]

Steroids have been postulated to work by reducing intracranial pressure and by blunting the inflammatory reaction to dying worms.[11,12,41] There is one double blind, placebo controlled, randomized trial to test corticosteroids in the treatment of AEM.[42] Chotmongkol, et al, enrolled 129 Thai adults with AEM but without altered consciousness; 63 subjects received prednisolone 20 mg orally, thrice daily, for 2 weeks; 66 received placebo. Patients received acetaminophen for headaches but no anthelmintics. The number of subjects who still had headache after 14 days was 5/55 (9.1%) in the prednisolone group versus 25/55 (45%) in the placebo group ($P < .001$). The median days to resolution of symptoms was 5 versus 13 in the treatment and control groups, respectively ($P < .001$). No relapses or serious side effects were noted. Despite the rather high drop out rate (8 in the treatment and 11 in the control group), this well designed study provided the first convincing evidence that high dose steroids could be beneficial in treating AEM. These researchers conducted an uncontrolled study using the same dose of prednisolone for only 1 week and found that 47/52 (90%) had recovered by day 7, but 8 (15%) relapsed, suggesting that a 1 week course was too short.[43] They also reported 11 comatose cases separately, seven of whom received high dose corticosteroids for variable periods of 2-15 days and 4 of whom did not.[44] None of the patients in either group improved and 10/11 died, suggesting that steroids alone may not be helpful once the patient is in coma. An earlier study had seen no benefit from a 5 day course of 30-60 mg/d prednisone, but the report compared results from several hospitals and details were not given.[37] A more recent report described a small outbreak in which 5 patients were given dexamethasone for 1 week followed by prednisone for 1 week.[45] All 5 initially improved; 3 relapsed, but then responded to repeat spinal tap and more steroids.

The use of anthelmintics to kill worms in the CNS is controversial, because it was postulated that an immune reaction to rapidly dying worms would be worse than allowing them to die or migrate out of the CNS naturally.[37,46] Animal studies with a mouse model of AEM have not demonstrated this, but rather have shown that treatment with flubendazole, mebendazole, or albendazole reduces both worm burden and inflammatory response.[39,47-50] Thiabendazole was not effective in mice.[47] Only a study using a rabbit model has shown an decrease in inflammation due to albendazole therapy.[51] There is only one published double blind, placebo controlled, randomized anthelmintic trial without corticosteroids in human AEM.[52] Jitpimolmard, et al, enrolled 71 Thai adults with AEM but without altered consciousness; 36 randomized to albendazole 7.5 mg/kg, orally, twice daily, after meals, for 2 weeks, and 35 to placebo. Patients received acetaminophen for headaches but no corticosteroids. The number of patients who still had headache after 14 days was 7/34 (21%) in the treatment group versus 13/32 (41%) in the placebo group ($P = .08$). The mean days to resolution of symptoms was 8.9 in the albendazole group versus 16.2 in the controls ($P = .05$). Acetaminophen use was 24.2 doses in the albendazole group versus 38.1 in the controls ($P < .01$). No serious side effects were noted. Thus albendazole alone may be effective in decreasing duration and severity of symptoms, but this has not been definitely proven. There is a report of two patients with AEM in the New Hebrides (now Vanuatu) who were thought to have gotten markedly worse during treatment with thiabendazole.[4] During a particularly severe outbreak in American Samoa, thiabendazole was used in 9 of 16 patients, and no appreciable salutary or deleterious clinical responses were noted.[5] Anecdotal reports of mebendazole, albendazole, and ivermectin have shown mixed results, but it is difficult from these uncontrolled reports to infer cause and effect.[15,53-55] Flubendazole has also been tested in animal studies, but is not

licensed for human use in most locales.[47-49] Of the benzimidazoles, albendazole has the highest bioavailability in the central nervous system, and may be the anthelmintic of choice.[56] Absorption of albendazole is better after a fatty meal, and CSF levels are increased when steroids are given concomitantly.[57]

Recently, a combination of corticosteroids and anthelmintics has been tested, but there are no blinded, placebo controlled trials. Chotmongkol, et al, conducted an open labeled trial of albendazole in combination with prednisolone versus prednisolone alone, at the previously reported doses, in 110 Thai adults with uncomplicated meningitis.[58] No significant differences were noted between the study groups, but the study did not have a sufficient number of subjects to prove no difference statistically. However, no harmful effects were seen in adding albendazole to prednisolone. These researchers also conducted an uncontrolled study demonstrating that combination therapy with mebendazole 5 mg/kg orally, twice daily, plus prednisolone 20 mg orally, thrice daily, for 2 weeks, produced a similar cure rate to that seen previously with the albendazole plus prednisolone combination.[59] Tsai et al compared two outbreaks a year apart in Taiwan.[17,60] In the first outbreak, 8 patients received mebendazole 100 mg twice daily for 4-11 days, and 7 of these patients also received dexamethasone alone or dexamethazone followed by prednisolone for 7-25 days. During the subsequent outbreak, 9 patients treated only with acetaminophen and naproxen served as historical controls. Median duration of illness was 13 days in the mebendazole plus prednisolone group versus 27 days in the controls. Animal studies in mice treated with the combination of albendazole and prednisolone have shown that the mice treated with albendazole alone or the combination tend to have a milder immune response than untreated controls or those treated with prednisolone alone, suggesting that the decreased worm burden resulting from anthelmintic therapy led to a less inflammatory immune response while also reducing worm migration.[40,61]

Suggestions for Further Research

Further research into the pathophysiology of this disease is needed, including autopsies of fatal human cases and experiments using animal models. The relative contribution of the several possible causes of neurological injury should be determined to help direct therapy.

Corticosteroids, which ameliorate intracranial pressure and blunt the immune response, are unlikely to prevent direct axonal damage from migrating worms, so there is a theoretical benefit to therapy with anthelmintics to kill the worms. Albendazole therapy has not proven harmful, despite expectations of some earlier researchers, although the question of anthelmintic treatment is still in some doubt because there was borderline significant efficacy seen with albendazole alone in the only double blind, placebo controlled trial.[52] Because earlier treatment with albendazole was more effective in animal studies,[39] additional human trials could be envisioned, with subjects stratified by duration from ingestion of intermediate host, or onset of symptoms, until initiation of therapy. Patients with higher worm burdens may respond differently to therapy, so studies could also be stratified by severity of illness. Other anthelmintics could be explored. Ivermectin reaches low concentrations in the mouse brain[62] and human CSF,[63] but it is effective against some other tissue nematodes at very low concentrations.[64] The effectiveness of ivermectin against *A. cantonensis* could be tested in the mouse model.

Anthelmintic and corticosteroid combinations have been used successfully in several studies, but this combination therapy has not been tested in a double blind, placebo controlled trial. In the studies to date, both groups had resolution of symptoms on day 3 or 4 of therapy,[58,59] suggesting that either the disease was relatively mild or the effect of steroid therapy was so potent that no benefit from anthelmintic could be seen. The possible benefit of anthelmintic treatment may be easier to demonstrate in patients with more severe disease. The challenge is to design a trial that enrolls subjects with sufficient disease severity and measures sensitive enough outcome variables to convincingly assess the presence or absence of differences between treatment groups.

Studies of severe cases of AEM with encephalitis, manifested as neurological signs and altered consciousness, are needed. A standard method for quantitating the severity of illness would facilitate comparison of studies from different regions. Measurements of intracranial pressure should be recorded, when practical. A standardized method of laboratory confirmation of infection with *A. cantonensis* is needed. Where practical, studies should include both adults and children. Because most areas have relatively low rates of infection and see small, sporadic outbreaks, multicenter trials with standardized, pre-approved protocols could be considered.

Conclusions

A presumptive clinical diagnosis of AEM can usually be made in a patient with consistent clinical symptoms and findings along with appropriate travel or residence and food consumption history. Severe headache with cutaneous paresthesias or hyperesthesias and evidence of eosinophilia on CSF or peripheral blood analysis are the usual symptoms and laboratory findings obtained, although eosinophilia may not be present on initial evaluation. Ingestion of a snail or fresh produce in an endemic region is the most frequent history given, but a history of ingestion of other intermediate or paratenic hosts may also be elicited. In addition, the endemic regions appear to be expanding. Serology is helpful in establishing the specific diagnosis, but is often not helpful with the initial clinical management of AEM. Serial lumbar punctures are effective in reducing headache, probably by temporarily relieving increased intracranial pressure. There is one pivotal study showing that a two week course of high dose corticosteroids is beneficial and safe in AEM without altered consciousness.[42] Based on this study, prednisolone 20 mg orally thrice daily or prednisone 60 mg orally daily may be considered. Prednisolone can be tapered after the two week course, as symptoms allow, but should be given for at least 2 weeks. Acetaminophen may be used as adjunctive therapy, but NSAIDs should be avoided if corticosteroids are employed,

because of increased risk of gastrointestinal bleeding. Although current evidence in support of anthelmintics is not as strong, there is modest evidence of improvement with albendazole alone in one well designed human trial,[52] and several studies have found no major evidence of harm, particularly if given with corticosteroids. Earlier concerns of worsened symptoms using anthelmintics have not been demonstrated in trials with albendazole, and until better data are available, it would seem prudent to consider albendazole treatment in combination with steroids for AEM, and in severe AEM in particular. Available animal data suggest that albendazole should be given for 2 weeks, and that it is more effective when given earlier in the course of illness.[39,40]

Conflict of Interest

Neither author identifies any conflict of interest.

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A Severe Case of Angiostrongylus Eosinophilic Meningitis with Encephalitis and Neurologic Sequelae in Hawai'i

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Abstract

Angiostrongylus eosinophilic meningitis is caused by infection with larvae of the rat lungworm, Angiostrongylus cantonensis*. We report the case of an adult who ingested a raw, giant African snail (*Achatina fulica*) on the island of O'ahu in Hawai'i and developed an eosinophilic meningoencephalitis with severe headache, confusion, sixth cranial nerve palsy, ataxia, limb weakness, and paresthesia. He was treated with lumbar punctures to relieve pressure, high dose corticosteroids, and 14 days of albendazole. He had a prolonged convalescence, requiring 3 months of prednisone, and still had evidence of motor nerve weakness 4 months after exposure. A field investigation at the site of exposure yielded 5 of 9* Achatina fulica *snails with evidence of* A. cantonensis *DNA by PCR. Cerebrospinal fluid samples from the patient were negative acutely but positive on day 15 of symptoms, using an investigational, real-time PCR assay. We discuss clinical management of this case in light of the current medical literature.*

Keywords

Achatina fulica*, Albendazole,* Angiostrongylus cantonensis*, Angiostrongylus eosinophilic meningitis, Case report, Cerebral angiostrongyliasis, Corticosteroid, Eosinophilic meningitis, Hawai'i, Human, Meningoencephalitis, Neuroangiostrongyliasis, Radiculomeningoencephalitis, Snail*

Introduction

Rat lungworm infection of the human central nervous system can have devastating consequences. Although often described as a self-limited meningitis requiring no treatment, there is a spectrum of disease, and severely affected patients may suffer from encephalitis, radiculomyelitis, permanent neurologic injury, or even death.[1] The etiologic nematode, *Angiostrongylus cantonensis*, persists in the pulmonary veins of the rat, its primary host, and is perpetuated by snails, its intermediate hosts.[2] Humans are accidental hosts and acquire the infection through eating raw or poorly cooked snails and slugs, or by eating paratenic (transport) hosts such as freshwater shrimp, frogs, monitor lizards, and flatworms (Planaria),[3] or inadvertently through eating vegetables contaminated with these hosts.[4-7] Infectious larvae migrate to the central nervous system where they undergo two molts, emerging as young adult worms in the subarachnoid space. Because humans are not definitive hosts, most worms die before reaching the pulmonary arteries. Symptoms are caused by larval migration through the central nervous system and the resulting axonal damage, inflammatory reaction, and increased intracranial pressure.[8-10]

The condition is often termed "angiostrongyliasis," but a related nematode, *Angiostrongylus costaricensis*, causes a distinct gastrointestinal syndrome, so throughout this text we will use the term Angiostrongylus Eosinophilic Meningitis (AEM) for the spectrum of neurologic disease caused by *A. cantonensis*, from meningitis through severe encephalitis. The disease appears to have expanded from Southeast Asia and Taiwan to Australia and across the Pacific Basin during the last century.[11] Over 2800 cases in 30 countries have been reported worldwide, including recently in the Caribbean, China, and Latin America.[12] Several cases have been reported in travelers to endemic areas[5,13] and in military personnel.[14,15] *Angiostrongylus cantonensis* is present in the Hawaiian Islands and researchers in Hawai'i were instrumental in proving that the nematode was a cause of eosinophilic meningitis.[16-19] Over the past decade this infection has been increasingly recognized in Hawai'i,[7] and the State made angiostrongyliasis a reportable disease in 2005, after five cases of AEM were reported within a four-month period.[20]

The diagnosis of AEM is generally made when eosinophilia is noted in the cerebrospinal fluid (CSF) in the setting of appropriate neurologic symptoms and a history of accidental or intentional consumption of molluscs or other hosts.[20-23] Specific diagnostic testing for *A. cantonensis* is only available from certain research laboratories, and results are usually not available during the acute phase of the illness. The appropriate treatment remains controversial,[12,22-25] although several recent studies from Thailand have given some guidance.[26-29] Intentional consumption of hosts might be reduced if more people were informed of the devastating results that can occur with this infection. To provide a clear picture of this rare but serious disease, we present a case of AEM with severe symptoms and prolonged neurologic sequelae in a military member serving on the island of O'ahu. We discuss the case in light of the current medical literature.

Case Report

During the autumn of 2010, a previously healthy 22-year-old service member presented to his local clinic with a 4-day history of arthralgias and 2 days of profuse night sweats and generalized myalgia involving his trunk and limbs. He had a leukocytosis of 17,500/μl with an absolute eosinophil count of 2100/μl, and was treated symptomatically with analgesics. Two days later he returned with new onset headache, was noted to be confused, and was referred to the regional medical center.

In the emergency department, the patient denied any recent trauma or exposure to toxins, illicit substances, sick people, arthropods, or rodents. Two weeks prior to presentation, he had participated in a field training exercise on O'ahu, but was unaware of anyone else from his unit who was ill. His past

medical history was otherwise noncontributory; he was taking no other medications, and had no known drug allergies.

Physical examination revealed a 22-year-old, Caucasian male, alert but in moderate distress from generalized pain. Blood pressure was 152/78 mmHg, pulse 93 bpm, respirations 17/min, and oral temperature 98.2 °F (36.8°C) , with an oxygen saturation of 98% on room air. There were no meningeal signs and the rest of his physical examination was unremarkable. Hematology revealed a leukocyte count of 11,700/μL with 14% eosinophils (absolute eosinophil count 1670/μL). Hematocrit, electrolytes, liver function tests, and urinalysis findings were within normal limits. A non-contrast, head computed tomography (CT) was negative. A lumbar puncture (LP) was performed, and CSF analysis revealed 338 WBC/μL, with a differential of 68% lymphocytes, 20% mononuclear cells, 15% eosinophils, and 0% neutrophils. The CSF protein was 117 mg/dL and glucose was 51 mg/dL; Gram stain was negative for bacteria; fungal stain was negative for *Cryptococcus*, and no worms were seen. The CSF opening pressure was not recorded. A diagnosis of eosinophilic meningitis was made.

The patient was admitted to hospital and started on 60 mg daily intravenous prednisolone for possible AEM. CSF cultures from admission were sterile. Magnetic resonance imaging (MRI) on hospital day (HD) 3 revealed subtle, leptomeningeal enhancement consistent with meningitis. The patient improved and was discharged next day with a prescription for oral prednisone.

Five days after discharge, which was day 14 post onset of symptoms (POS), the patient returned to the emergency department with complaints of feeling strange and a worsening headache. He had become confused, agitated, anxious, and not oriented to place or time. His blood pressure was 158/95 mmHg and his temperature was 100.6 °F. Physical examination was unremarkable, but WBC was 18,000/μl (absolute eosinophil count 900/μl). A CT scan without contrast was again unremarkable. Upon LP, intracranial pressure was > 55 cm H_2O, and CSF analysis yielded 1248 WBC/μL with 57% lymphocytes, 27% eosinophils, 15% mononuclear cells, and 1% neutrophils. He was re-admitted to hospital, prednisone was increased to 80mg/d, and albendazole 600 mg orally, twice daily, was added.

The morning after readmission (day 15 POS) the patient was noted to have a left sixth cranial nerve (abducens) palsy with diplopia. Over the next 2 days his mental status worsened; he underwent a therapeutic LP and improved. Over the next several days, his confusion slowly continued to improve, but he developed ataxia, difficulty coordinating complex movements, limb paresthesias, and weakness in the intrinsic muscles of both hands and in his lower legs. On HD 7 (day 20 POS), magnetic resonance angiography demonstrated leptomeningeal enhancement with punctuate foci of restricted diffusion within the right sylvian fissure and scattered white matter lesions with increased signal on T2-weighted and fluid attenuated inversion recovery images, consistent with inflammatory or ischemic change.

Symptoms and signs peaked on HD 9 (day 22 POS) after which the patient gradually improved, with clearing sensorium and increasing strength. He was discharged on HD 12, walking with assistance, with diplopia because of persistent abducens palsy, 4/5 right triceps strength, weakness in the intrinsic muscles of both hands, and lower extremity ataxia.

Two days after discharge, he completed 14 days of albendazole, twice daily, and a rapid prednisone taper was begun. Ten days after discharge (day 35 equivalent) the patient reported worsening of headache; so, a more gradual taper was used. By 5 weeks after discharge, the patient had greatly improved, but some motor weakness persisted. At three months, his deficits had decreased to diplopia at far leftward gaze and mild grip weakness. His gait had normalized, and while he continued to undergo physical and occupational therapy, he was able to carry on normal activities including running, although at a reduced pace. His clinical course is summarized in Table 1. The final diagnosis was AEM with encephalitis and radiculitis, probably secondary to *A. cantonensis* infection following ingestion of a raw snail.

Although asked at the first emergency department visit, a history of snail ingestion was not obtained until HD 6 of the second hospitalization. His symptoms had begun approximately 9 days after ingestion of the snail. During follow up the patient related that, during the field exercise on O'ahu prior to his illness, he had swallowed an entire raw snail, un-chewed, as part of a wager.

Field Investigation

The case was reported to the local military public health officer. A field team, including a Hawai'i Department of Health epidemiologic investigator and an entomologist from U.S. Army Garrison Hawaii-Schofield Barracks, conducted an environmental investigation in the area where the patient's exposure occurred. Molluscs were readily observed, and 9 giant African snails (*Achatina fulica*) and one Cuban slug (*Veronicella cubensis*) were collected.

Laboratory Testing

Clinical and environmental samples were sent by Hawai'i Department of Health as part of an ongoing research collaboration with the Division of Parasitic Diseases and Malaria, Centers for Disease Control and Prevention (CDC), for testing using real-time polymerase chain reaction (PCR).[30] The testing protocol was originally developed for host tissue testing (rats and molluscs);[31] validation for testing clinical specimens is ongoing. Five (56%) of 9 *A. fulica* snails and the one slug were found to harbor *A. cantonensis* DNA. The patient's CSF specimen collected 6 days POS (acute sample) was negative, whereas the sample collected day 15 POS (mid-course sample) was positive for *A. cantonensis* DNA by real-time PCR.

Discussion

We report a severe case of AEM with encephalitis and prolonged neurologic sequelae in an adult. The infection was acquired on the island of O'ahu in Hawai'i, by ingesting a raw giant African snail (*Achatina fulica*). Although the likely diagnosis was considered at admission to hospital, some doubt remained because the patient did not admit until 19 days after onset of illness to

Table 1. Clinical Events and Laboratory Results in a 22-Year-Old Male with Angiostrongylus Eosinophilic Meningitis.							
dpi[a]	**dpos[b]**	**Event/Status**	**Treatment**	**Blood: wbc × 10^3/µl / eosinophils**	**Blood: absolute eosinophil count (cells/µl)**	**CSF: wbc/µl / eosinophils**	**PCR for *A. cantonesis***
0		Approximate time of snail ingestion					
9	0	Myalgias begin					
13	4	Seen in outpatient clinic	Analgesics	17.5/12%	2100		
15	6	First hospital admission	Prednisolone 60mg/d	11.7/14%	1670	338/15%	Negative
17	8	Lumbar puncture; MRI		13.2			
18	9	First discharge	Prednisone 20mg/d	16.6/12%			
20	11	Treated in ED		21.9/9%			
23	14	Second hospital admission	Prednisone 80mg/d; begin albendazole	18.0/5%	860	1248/27%	
24	15	Abduscens palsy					Positive
26[c]	17	Mental status worse	Prednisone 80mg/d + albendazole continue; therapeutic lumbar puncture				
28	19	Staff learns of snail ingestion					
29	20	MR angiogram					
30	21	Ataxia, paresthesias					
32	23	First signs of clinical improvement					
34	25	Second discharge					
37	28	First outpatient follow up visit	Finish albendazole; begin prednisone taper				
44	35	Headache worse	Prednisone 80mg/d				
57	48	PCR data received	Tapering prednisone more slowly				
85	76	Diplopia resolved					
99	90	Mild abduscens and limb weakness persists	Off prednisone				

[a]days post ingestion of snail. [b]days post onset of symptoms. [c]no further laboratory data available

eating the snail. *Achatina fulica* from the area of ingestion were later confirmed to carry *A. cantonensis*. A real-time PCR assay, currently under validation for diagnosis of clinical samples, amplified *A. cantonensis* DNA from the patient's mid-course, but not acute CSF. Therapeutic LPs, corticosteroids, and anthelmintics were all used for treatment. The patient required a month of high dose steroids plus a 2-month taper and still had residual weakness 4 months post exposure.

In making the diagnosis of AEM, a history of potential or actual ingestion of raw or poorly cooked hosts is important, but the history elicited from a patient may be unreliable, because the patient has no memory of eating the usual hosts, the host was chopped up in contaminated raw food such as inadequately washed salad, or the patient is too embarrassed to admit it. Our patient revealed eating the snail only after his condition had severely worsened. However, we knew he had touched the snails, and that *A. cantonensis* is present in the Hawaiian Islands.[20] The Hawai'i Department of Health has received reports of 38 cases of autochthonous AEM from 2005 through 2011 (SY Park, 2011, unpublished). In addition, AEM has been seen previously in military personnel serving in Hawai'i.[15] Neurologic symptoms and signs at presentation are generally non-specific, but more characteristic signs, such as cranial nerve palsies, limb weakness, paresthesias, and ataxia may occur.[1] This patient had headache, myalgias, and confusion on day 6 POS, but developed a characteristic abduscens nerve palsy on day 15 POS, and then paresthesias, limb weakness, and ataxia evolved over the next week. CT usually does not show abnormalities in patients with AEM, but MRI may show subtle findings.[32] In our case, the MRI on day 8 POS had only subtle leptomeningeal enhancement, but magnetic resonance angiography on day 20 POS showed somewhat more characteristic, although non-specific, changes. Diagnosis and treatment of AEM are more thoroughly discussed elsewhere in this issue.[33]

Because of the nonspecific initial presentation and the rather wide differential for eosinophilic meningitis, there is a need for specific laboratory testing to confirm the diagnosis of AEM. Dot blot ELISA and Western Blot assays based on a 31 kDa protein from adult *A. cantonensis* have proven useful to detect antibodies against the organism in infected patients[34,35] but are not widely available. PCR has recently been developed as an alternative to morphological identification of infectious larvae in mollusc tissue,[30,31] and this real-time PCR assay was used in our field study. The 56% parasitism rate of *A. cantonensis* in molluscs collected from the site where the patient ingested the snail is in agreement with recent estimates of infectivity rates among molluscs from Hawai'i.[36] PCR has recently been adapted for human testing in abdominal angiostrongyliasis caused by *A. costaricensis*.[37] Acute and mid-course CSF specimens from this case were tested with the same real-time PCR assay used to

detect *A. cantonensis* in molluscs. *Angiostrongylus cantonensis* DNA was not amplified from the day 6 POS (day 15 post ingestion) sample but was amplified from the day 15 POS sample. Although this test has not been standardized for diagnostic purposes, it was reassuring to the treating physicians that there was evidence of *A. cantonensis* DNA in the patient's CSF. By using PCR to amplify even small quantities of parasite DNA in patient samples, the potential exists to detect organisms and thus confirm the diagnosis sooner compared with antibody tests.

There is currently no clear consensus on the best therapy for AEM.[12,22-25] Several investigators have reported that high volume LPs improve headaches temporarily, probably by reducing intracranial pressure.[1,5,21,24,38] Therapeutic LPs were helpful in our patient for symptomatic relief. Steroids have been used to help decrease intracranial pressure and blunt the immune response.[5,24,38] One double-blind, placebo-controlled, randomized trial demonstrated that high dose corticosteroids, given for 2 weeks, reduced duration of illness in Thai adults with AEM.[26] These patients, however, appear to have had milder illness than the present patient, because most patients treated with prednisone had resolution of symptoms by day 5. Therapy of severe disease has not been studied systematically, and further studies are needed to determine the best treatment for severe disease. Our patient was treated with high dose corticosteroid, and initially improved, then worsened temporarily before recovering.

It has been suggested that anthelmintic therapy might cause harm by the rapid killing of worms and a subsequently worsened immune response,[38,39] but this has not been seen in mouse studies.[40,41] Unlike the case in neurocysticercoisis in which worm cysts are immobile, in AEM there is a theoretical advantage to killing worms because they migrate through the CNS and cause axonal damage according to autopsy studies.[8-10] There is one double-blind, placebo-controlled, randomized trial of albendazole given without steroids in the treatment of Thai adults with AEM.[28] It showed a reduction in days of symptoms that just reached statistical significance, and there were no adverse effects. However, most clinicians would give corticosteroids concomitantly with an anthelmintic such as albendazole in treating neuroparasitoses. Recently several investigators have reported using a combination of corticosteroid, and albendazole to treat AEM,[29,42,43] but no double-blind, placebo-controlled, randomized trial has been reported. In general, unblinded studies have shown cure rates in patients receiving both prednisolone and albendazole similar to those with prednisolone alone. It is possible that the effects of albendazole may be masked due to the efficacy of the steroids. When our patient was re-admitted, albendazole was added to prednisone based on the rationale that the anthelmintic might prevent further axonal damage from live worm migration, although the clinical evidence to support this intervention is not strong. After this, his condition continued to worsen for 7 days before significant improvement was noted. It is not clear whether the worsening was due to addition of albendazole or to the further progression of the disease. Animal studies have shown that albendazole is most effective when given early in the disease, before the larvae molt to young adults.[40,44] This last molt occurs approximately 11-13 days post ingestion in the animal model,[2] which would have been 2-4 days POS in the present patient. Thus, it is possible that the albendazole the patient received on days 14-28 pos may have been more effective if given earlier in the patient's course.

AEM is often described as a mild, self-limited condition.[26,38,43] However, severe cases including fatalities have been reported.[1,9,45,46] The present patient had an unusually long persistence of neurologic symptoms and clearly had encephalitis, but was never comatose. Other severe cases have been associated with *A. fulica* ingestion.[1,9,45] Pediatric patients also tend to present with worse disease.[1,43] It has been postulated that the severity of infection may be related to the size of the inoculum of infective larvae relative to the mass of the patient.[1,43] Although the human infective dose is not known, 50 infective larvae have been used to infect each rodent in experiments.[40,44] *Achatina fulica* were found to harbor a median of 5200 larvae in a study from American Samoa, and one snail contained 90,800 larvae.[47] A quantitative assessment of larval burdens in the *A. fulica* snails collected from the area of ingestion in this case is presented elsewhere in this issue.[48] The present patient swallowed a large, raw *A. fulica*; yet his squad mate chewed one and spit it out without becoming ill. Although the inoculum in the specific snail the patient swallowed is unknown, it is possible that the severity of his illness may have been related to the ingestion of a high number of infective *A. cantonesis* larvae.

The host range of *A. cantonensis* has been expanding, and intermediate hosts such as the giant African snail are becoming invasive in new areas. Travelers to areas where the rat lungworm is present can also be exposed. Clinicians should have a high index of suspicion for AEM with any case of eosinophilic meningitis. For treatment, high volume LPs as needed, and prednisolone or prednisone 60mg daily for 2 weeks are now accepted therapy for adults. After 14 days, corticosteroids can be tapered gradually as symptoms allow. One might consider adding albendazole 7.5 mg/kg twice daily for 2 weeks in addition to corticosteroids, although the evidence for albendazole benefit is not as strong as that for steroids, and further trials are needed. Parents, teachers, and military leaders should be warned that molluscs and other potential hosts and raw vegetables that might have been contaminated by molluscs and that are not fully cooked can cause *A. cantonensis* infection.

Conflict of Interest

None of the authors identifies any conflict of interest.

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A Severe Case of Rat Lungworm Disease in Hawai'i

Kathleen Howe BA

Abstract

A 23-year-old man living on the island of Hawai'i developed a life threatening case of eosinophilic meningitis caused by infection with Angiostrongylus cantonensis *(rat lungworm disease: RLWD). He was comatose for 3 months, incurring brain and nerve damage sufficiently extensive that he was not expected to recover. The case was complicated by secondary infections of methicillin-resistant* Staphylococcus aureus*,* Clostridium difficile*, and pneumonia, which resulted in an empyema requiring a thoracoscopy and decortication. He was treated with prednisone, mebendozal, and pain medication for RLWD, and antibiotics and antifungal medications for the secondary infections. The administration of herbal supplements was requested by the family and approved, and these were administered through a gastric tube. Less than a month after being declared in a persistent vegetative state the man was able to talk, eat, and had regained some muscle functions. After release from the hospital he continued the use of supplements and received treatments of intravenous vitamin therapy. Four years after onset of the illness he is able to ride a bicycle, is a part time student, plays guitar, and is fluent in two foreign languages. RLWD is an emerging tropical disease of growing importance in Hawai'i.*

Keywords

Angiostrongyliasis, Angiostrongylus cantonensis*, Case report, Chinese medicine, Coma, Eosinophilic meningitis, Herbal supplements, Vitamin therapy*

Introduction

Rat lungworm disease (RLWD) is a global, emerging tropical disease of growing importance in the Hawaiian Islands, particularly on Hawai'i Island itself, where the majority of the cases have occurred. The disease is caused by a nematode, *Angiostrongylus cantonensis*, first discovered in China in 1935[1] but now reported from other parts of Asia, Australia, the Caribbean, Pacific islands, Brazil, and Ecuador.[2] Since the first case of eosinophilic meningitis putatively caused by *A. cantonensis* was reported in 1945, more than 2,800 cases of RLWD have been reported in approximately 30 countries.[2] Snails and slugs are the intermediate hosts and the semi-slug *Parmarion martensi*, a recent invasive arrival in the Hawaiian Islands,[3] is thought to be primarily responsible for recent outbreaks of RLWD on the Island of Hawai'i.[4] In the area where the disease outbreak occurred, the Puna district of Hawai'i island, *P. martensi* are very numerous and in surveys 77.5% of specimens were infected with the infective third larval stage of *A. cantonensis*.[4] Recent qPCR techniques[5] have shown infection levels to be as high as almost 7000 parasites in a single semi-slug.[6]

RLWD cases in Hawai'i since 2004 have resulted in permanent disability, coma, and death. In a 50 month study period (January 2001-February 2005) 24 of 84 cases of eosinophilic meningitis were attributed to infection by *Angiostrongylus cantonensis*.[7] Cases occurred primarily in two clusters: November 2004 - January 2005 and December 2008 - February 2009. An informal report sent to the College of Pharmacy, University of Hawai'i at Hilo, from the State of Hawai'i Department of Health (November 2011) reported 35 documented cases of RLWD in Hawai'i since January 2001. Because diagnosis is difficult, especially in mild cases, case numbers may well be higher. The case described in this paper was one of two, non-connected cases of RLWD that resulted in coma around the same time (December 2008/ January 2009). Both victims were initially diagnosed with flu and not admitted to the Hilo Medical Center even after multiple visits to the emergency room. When finally admitted, doctors at the center were unfamiliar with the disease and appropriate treatment. In the particular case documented in this paper, the family and a community member presented physicians with peer reviewed publications from Southeast Asia that outlined the use of steroids and anthelmintics in cases of RLWD and requested these treatments be administered.

Early treatment could be a significant factor in improved recovery, making the awareness of this disease and its treatment important in Hawai'i. The need for studies on long-term recovery is equally important, for, while most victims have not succumbed to the disease, neither have those most seriously affected recovered fully. Pain medication is usually prescribed for the ongoing symptoms many victims suffer, and many of these have permanent disabilities. No studies have been done in Hawai'i on long-term recovery from RLWD.

Case Report

A 23-year-old man from the Kapoho area of the Puna District on Hawai'i island was hospitalized at the Hilo Medical Center on December 26, 2008. Before becoming ill he was healthy and active. Prior to hospitalization he suffered from severe headache, stomach pain, and muscle and joint pain. He was taken to the emergency room twice, treated with morphine for pain, and released. He was admitted on his third visit when he was unable to urinate. Shortly after his admission he developed skin sensitivity such that even a light touch caused great distress. He was eventually diagnosed with eosinophilic meningitis resulting from parasitic infection by *Angiostrongylus cantonensis*, probably caused by accidental ingestion of an infected slug host. He was prescribed prednisone to suppress the immune system, as well as morphine and oxycodene for pain. On January 2, treatment with the anthelmintic mebendozal was begun (10 mg/kg for 14 days).

The patient began to show improvement. However 16 days after hospital admission (January 11) he began to experience double vision, increasing exhaustion, and stabbing head pain. The following day his manual dexterity decreased significantly and within 24 h he was comatose. An MRI showed inflammation involving white fiber tracts and bilateral basal ganglia, as well as questionable hemorrhagic products scattered throughout white matter tracts of both hemispheres and within the cerebellum. He was intubated, ventilated, and flown to the Queen's Medical Center, Honolulu, for additional diagnosis.

Magnetic Resonance Imaging (MRI) of the brain showed what looked to be worm tracks in his brain. Medical records show his condition reported as "grave with a dismal prognosis and no hope of much recovery." Evaluation at the Queen's

Medical Center showed a left side empyema, hydrocephalus, post encephalitis brain atrophy, and history of eosinophilic meningitis complicated with severe demyelinating encephalitis, and cachexia. Two days later he opened his eyes and was occasionally able to respond to a request to squeeze the hand of a family member or nurse. Eleven days after the patient became comatose a tracheotomy was performed and a gastric tube was inserted. The family then requested the administration of supplements and Chinese herbs. A physician suggested using a Chinese medication (Xing Nao Wan) that had been used in a similar case of *A. cantontensis* infection of a 23-year-old man in China, which had resulted in coma; however, after four months of treatment that patient recovered and was discharged from the hospital with mild memory loss.[8] Other supplements requested by the family were used as well; these were the blue-green algae spirulina (*Arthrospira platensis*) and chlorella (*Chlorella vulgaris*), as well as noni (*Morinda citrifolia*).

On January 27, after the tracheal and gastrointestinal tubes were in place, the patient was flown back to the Hilo Medical Center. He was taken off the ventilator, as his breathing was not grossly impaired. However, the tracheal tube remained in place to ensure a clear airway. He remained in ICU for 4 days and was then moved to a nursing unit. On January 31, the supplements used in Honolulu were approved for use at the Hilo Medical Center. These supplements were administered three times a day through the gastrointestinal tube. Curcumin (*Curcuma longa*) was added to the regime as well as acupuncture treatments. Over a 3-month period, family members assisted with administration of supplements and Chinese medicine, and with medical necessities such as suction of the lungs, tracheal care, and gastrointestinal tube feeding. The patient also underwent daily massage and range of motion exercises.

Shortly after the patient became comatose (January 12) he developed complications including pneumonia, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Clostridium difficile* infections. Antibiotics and anti-fungal medications were prescribed for these. The patient was inert as of the time he entered a coma and therefore was manually turned every 2 hr. In early February, his left eye began to drift inward, a result of nerve damage caused by the parasite. In mid February his blood pressure and heart rate began to rise and he was put on blood pressure medication. Soon after it was determined he had an enlarged heart. A brain MRI taken on February 19 reconfirmed the Queen's Medical Center evaluation. A lumbar puncture performed on February 23 had an opening pressure of 25 cm H_20, WBC 203, 21% neutrophils, 40% lymphocytes, 33% monocytes, protein 102, and leukocytes 32. A shunt to relieve fluid in the brain was discussed, but ongoing infections precluded the operation. The patient ran a persistent fever and was put on antibiotics. His fever did not respond to treatment, and a CAT scan revealed a pleural effusion. A percutaneous thoracentesis was performed on March 3 but was unsuccessful. He was flown to the Queen's Medical Center, Honolulu, for a video assisted thoracoscopy including decortication for persistent empyema, and chest tubes were inserted on March 9. An MRI taken at Queen's determined he had communicating hydrocephalus and he was declared to be in a persistent vegetative state. He was returned to Hilo Medical Center 5 days later.

From the end of January and into February, although clinically comatose, the patient showed some signs of cognizance, responding appropriately to some conversation by what was interpreted by nurses, nurse aides, and family members as laughter or sadness (crying). In mid February the hospital speech therapist began working with the patient on swallowing reflexes, with the desired goal of removing the tracheal tube. By the end of February the patient was able to chew and swallow ice chips, nod yes and no, laugh, and grip hands.

Progress continued into March. On March 3 the tracheal cuff was deflated for a short period of time, allowing the patient to breathe through normal airways. At this time the patient was beginning to move his hands. Progress was impeded by the need for a lung operation and insertion of chest tubes. Chest tubes were removed on March 22, and on March 23 the tracheal cuff was deflated for the entire day and the patient was able to eat yogurt. Physical therapy was also begun on the same day. On March 27 the tracheal tube was changed to a fenestrated tracheal tube, and on March 29 the patient's status was upgraded and he was discharged to the long-term care ward at the Hilo Medical Center.

On March 30 a Passy-Muir Valve was provided to allow for speech therapy, and on the same day a standing table was used by physical therapists to help the patient stand for the first time. By this time the patient was regaining use of his forearms. On April 1 the patient spoke for the first time and was able to converse in short sentences. The pain in his legs that was present near the beginning of his illness had generally subsided, replaced by a feeling of cold and numbness on his left leg and arm and areas of his face, and he had lost use of two fingers on his left hand. His short-term memory was greatly impaired but his long-term memory was intact. His gaze was disconjugate with some nytagmus present, and he had severe ataxia, some hallucinations, and insomnia. Over the course of the month his swallowing reflexes improved, and he was able to eat and drink. By the end of April, while still extremely weak, he was able to walk a short distance (6-8 m) with a walker and two assistants. He was released from the Hilo Medical Center on April 30, 2009.

Use of Supplements

There are many claims for supplements that have not yet been validated scientifically. However, plant-based medicines have been used by various cultures for hundreds, if not thousands, of years. In this case, doctors had no hope for recovery. Therefore various supplemental treatments (Table 1) were administered through the stomach tube on a daily basis as it was agreed that they may provide some benefits and would probably not cause harm.

Four months after release from hospital, the patient began vitamin therapy. The treatment and doses were prescribed by a licensed doctor and administered by a registered nurse. The therapy consisted of two intravenous nutrient infusions administered at alternating sessions as a drip. One was a

Table 1. Supplements and vitamin treatments administered.			
Supplement	**Brand**	**Dosage**	**Other information**
In hospital			
Spirulina (*Arthrospira platensis*)	Hawaiian Pacifica	0.5 tsp 3x/day	Phytonutrient, blue-green alga, superior source of digestible protein, source of omega-3 fatty acids and gamma-linolenic acid
Chlorella (*Chlorella vulgaris*)	NOW Foods	0.25 tsp 3x/day	Phytonutrient, blue-green alga, source of chlorophyll
Noni (*Morninda citrifolia*)	Eclectic Institute	2 capsules 3x/day	Freeze dried fruit
Curcumin (*Curcuma longa*)	Pure Encapsulations	2 capsules 3x/day	Anti inflammatory
Bu Nao Wan	Plum Flower	4-6 tablets 3x/day	Replacement for Xing Nao Wan, contains *Schisandra chinensis*
Additional after release from hospital			
EPA/DHA (eicosapentaenoic acid/ docosahexaenoic acid) from fish oil	Pure Encapsulations	2 capsules (1,000 mg per capsule) 3x/day	Omega-3 fatty acids for cognitive function, promotes oxygen and nutrient delivery to the brain, supports cardiovascular health, important for optimal joint function
Acetyl-l-carnitine	Pure Encapsulations	500 mg 2x/day	Enhances cellular energy and neurotransmitter metabolism
Vitamins B1 (benfotiamine), B12 (methylcobalamin)	Nerve Support Formula	4 capsules 3x/day	Decreases symptoms of peripheral neuropathy
5-HTP (100 mg)	Pure Encapsulations	1 capsule 3x/day	Supports serotonin levels that can lead to positive effects on emotional well-being and wake/sleep cycles

phosphatidylcholine/glutathione mixture; the other consisted of high doses of vitamin C plus B vitamins and trace minerals, sometimes referred to as a Myers Cocktail after its creator, Dr. John Myers, of Johns Hopkins University.

The intravenous treatments were administered 3 times per week for 4 months. Within 14 days of beginning treatments the patient's left eye began to straighten and he remarked on improved vision and mental clarity. Additionally, both of his physical therapists commented that the ataxia and his ability to tell left from right had improved. Within 3 weeks of beginning the intravenous injections his sleeping and bowel and bladder symptoms showed signs of improvement, he was able to begin walking without two canes for support, and he was able to walk longer distances (~100 m) without having to rest.

Discussion

After discharge from hospital the patient still suffered from the severe and debilitating effects of the disease. His recovery, while painful and slow, has been steady. Four years after the onset of the disease he continues to take supplements. Those he consistently takes are fish oil, curcumin, vitamins B-1 and B-12, acetyl l-carnitine and 5HTP. His use of prescription medications ceased within 6 months of being released from the hospital. He still experiences problems with balance and vision, and he still suffers from bouts of insomnia. Lack of energy and vigor were extreme issues for 3 years. However in the fourth year after the onset of the illness he has shown marked improvement. He is currently enrolled as a part-time student at a local community college. His vision is somewhat corrected with glasses, which allow him to read and write. His balance has improved with physical therapy and an exercise regime that focuses on core strength. He is able to ride a bicycle to and from classes and for other purposes. He plays guitar and is fluent in two foreign languages. In light of the dim prognosis given at onset of the disease, the recovery is considerable, indeed, quite remarkable. The protocol at Hilo Medical Center has now improved for victims presenting with symptoms of RLWD. Cases of RLWD on Hawai'i island have resulted in permanent disability, making this serious disease worthy of further research to develop effective treatment, particularly for the long-term neurological effects.

Conflict of Interest

The author identifies no conflict of interest.

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The Role of Eosinophils in Angiostrongyliasis: Multiple Roles for a Versatile Cell?

William L. Gosnell PhD and Kenton J. Kramer PhD

Abstract

Human infection with the rat lungworm, Angiostrongylus cantonensis*, is characterized by a vigorous eosinophil response that gives the disease its name, eosinophilic meningitis. The actual role eosinophils play, both protective and destructive, in this infectious process is still largely a mystery. Research since 2002 has indicated that eosinophils are a multifaceted granulocyte that contributes to a wide range of physiological and pathological processes depending on their location and activation status. This article suggests an expanded role for eosinophils as both classic antiparasitic effector cells and as immune regulatory cells in eosinophilic meningitis caused by* Angiostronglyus cantonensis.

Keywords

Angiostrongylus cantonensis*, Cerebrospinal fluid, Eosinophilia, Food, Immunology, Meningitis, Parasitology*

Background

Dr Paul Ehrlich, using a newly discovered dye, eosin, discovered eosinophils in the blood of humans and other animals in 1879. The cells were named eosinophilic granulocytes because of their reddish orange staining properties.[1] Eosin binds highly basic proteins that constitute the granules of these cells. These granules can be classified into four different populations: crystalloid granules, primary granules, small granules, and secretory vesicles. The largest are the crystalloid granules that store the majority of granule proteins in eosinophils. These granules contain an array of cationic proteins, designated major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN), and are arranged as a crystalloid core consisting of MBP-1 (and MBP-2) and a matrix of the ECP, EDN, and EPO proteins. Differential release of these proteins is mediated by directed exocytosis and degranulation.[2,3]

Search Strategy and Selection Criteria

For this short review, PubMed was searched for reports published in English between May 2002 and May 2012 with the search terms "eosinophil," "eosinophilic meningitis," and "*Angiostronglyus cantonensis*." Although publications from the past ten years were mainly selected, commonly referenced and highly regarded previous publications were not excluded. Reference lists of articles identified by this search strategy were also searched and relevant reports selected.

Function

Most eosinophils in the human body are distributed in the mucosal tissues and the classical view of eosinophil function is that they are terminally differentiated granulocytes primarily involved with either the destruction of helminthic pathogens, such as intestinal and tissue nematodes and schistosomes, or associated with the disease process of allergic diseases, such as asthma.[3] Since 2002 this narrow view of eosinophil function has given way to a broader concept of the role eosinophils play in the maintenance of immune homeostasis, in part because of the production of a wide range of diverse proinflammatory and regulatory cytokines and chemokines and functions such as antigen presentation.[4,5] This evolving role for eosinophils in the host immune response has these cells capable of both an effector function as well as participating in tissue repair and immunoregulation.[6]

Disease Process

Human disease with the rat lungworm, *Angiostrongylus cantonensis*, is associated with three main clinical entities, meningitis, encephalitis, and ocular involvement because of the presence of the organisms in these tissues.[7-9] The initial infectious process begins with ingestion of infective third stage larvae (L3) that penetrate the gut lumen. This allows the parasites to enter the circulatory system where they eventually gain access to the central nervous system (CNS).[9] The presence of the parasites in the CNS results in a clinical syndrome characterized by eosinophils in the cerebrospinal fluid (CSF).[10] While there are a number of different etiologies for eosinophilic meningitis, the most common infectious cause is *A. cantonensis*.[11] The predominant clinical feature of eosinophilic meningitis due to *A. cantonensis* is headache followed by development of a variety of meningeal signs and symptoms that include neck pain/stiffness, fever, nausea, and vomiting.[12,13] Imaging studies, such as magnetic resonance imaging (MRI), of patients with eosinophilic meningitis have demonstrated abnormalities in many but not all patients. These abnormalities, while not pathognomonic for this infection, tended to present as single or multiple enhancing lesions of the leptomeninges and parenchyma of the brain.[14,15] These changes have been attributed to the migration of the parasite, inflammation, or granuloma formation from dead or dying worms.[12,13,15,16]

Eosinophilic meningitis is generally a self-limiting disease[12] with mortality rates reported to be less than 1%[11,17] so necropsy studies of human angiostrongyliasis are limited. Published autopsy and subsequent pathologic studies of fatal cases generally show the following features: (1) meningeal infiltration by eosinophils, macrophages, and lymphocytes, (2) distinct tracks within the brain parenchyma associated with cell debris, (3) microthrombi and inflammatory cells that are assumed to be due to the migration of the parasite, and (4) the presence of classic granulomatas surrounding dead worms. Histologically a large numbers of eosinophils have been associated with meningeal infiltration and the granuloma formation but perhaps surpris-

ingly not in close association with viable or well preserved larval worms as would be expected for a direct role in parasite killing.[18-20]

Eosinophilic Response

Understanding of the role eosinophils play in the human immune response to helminthic pathogens is far from complete.[21] Work since 2002 to further elucidate the immunobiology of eosinophils has led to consideration of a broader role for these cells in immunity to helminthic infections.[22,23] The classical view of eosinophils as solely effector cells for parasite killing has given way to the idea that these cells play important roles in regulation of cellular immune responses.[24] Eosinophils could be envisioned to have a number of different functions in response to *A. cantonensis* infections. An example of this diverse functionality can be illustrated in the formation of granulomas around dead or dying *A. cantonensis* larvae. Similar to granulomas observed with schistosomiasis, granulomas in angiostrongyliasis contain large numbers of activated eosinophils that produce T_H2-associated cytokines that drive granuloma development.[25] *In vitro* work involving interleukin 5 (IL-5) knockout mice suggested that eosinophils are involved in the direct killing of the intracranial worms.[26] While a recent mouse study found that blocking the CCR3 chemokine receptor, which is abundant on the surface of eosinophils and is responsible for their activation and chemotaxis, did not affect worm burden as a result of decreasing the number of intracranial eosinophils.[27] Whether these findings can be translated to humans remains to be elucidated,[6] as it is crucial to keep in mind the immunological differences between species and to consider such when applying mouse models to the mechanism of human disease. Presently there is sparse evidence in the literature to directly support the idea of eosinophils participating in the direct killing of *A. cantonensis* larvae in the CNS. Therefore it is equally likely that the recruitment of eosinophils into the brain parenchyma may occur secondarily as a result of parasite death.

Expanded Role for Eosinophils in Eosinophilic Meningitis

It is possible to speculate that the central function of eosinophils may be in tissue remodeling, repair, and, more importantly, immuno-regulation. It is interesting to note that eosinophils express two cytokines: interleukin 12 (IL-12) and gamma interferon (IFN-γ), both of which serve to down regulate T_H2 type inflammatory responses. Indeed, IL-12 has been shown to inhibit allergen-induced T_H2 cytokine responses and eosinophil degranulation.[3] This suggests that eosinophils may have the ability to release cytokines that regulate eosinophil modulated T_H2 type tissue inflammation resulting from migrating parasites.[4] Eosinophils (as well as other immune cells such as T-cells) have also been shown to store, produce, and release neurotrophins such as brain-derived neurotrophic factor (BDNF).[28,29] It has been suggested that locally produced BDNF in the CNS mitigates inflammation-dependent neuronal damage.[30] Furthermore it could be hypothesized that the pathology of human angiostrongyliasis may be due in part to the inability of eosinophils to modulate T_H2 type effector functions such as granuloma formation and inflammation initiated by the migration, death, and disintegration of the *A. cantonensis* larvae. This inability of the eosinophil population to modulate the host inflammatory response may be due to host factors such as polymorphisms in eosinophil cytokine receptor expression, parasite factors such as strain variations in *A. cantonensis*, or a combination of both. Further studies are required to truly appreciate the role eosinophils play in disease associated with human *Angiostrongylus cantonensis* infection.

Conclusion

Understanding of the function of eosinophils has significantly increased over the past few years. However, more research is needed to define their role in *Angiostrongylus cantonensis* infections. Only by understanding this intricate biological role that eosinophils play in eosinophilic meningitis can rational treatment protocols be proposed and tested.

Conflict of Interest

Neither author identifies any conflict of interest.

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Differential Diagnosis of CNS Angiostrongyliasis: A Short Review

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Abstract

The diagnostic criterion for eosinophilic meningitis (EOM) is the identification of an absolute count of 10 eosinophils per ml or more than 10% of the total white blood cells in the cerebrospinal fluid (CSF) in the proper clinical context. The most common cause of EOM is Angiostrongylus cantonensis *infection, termed meningitic angiostrongyliasis (MA). Neurognathostomiasis (NG) is the main parasitic disease in the differential diagnosis of meningitic angiostrongyliasis. This short review is based on articles published on Medline between 2000 and 2012 related to EOM. There are three main approaches that can be used to differentiate between MA and NG, involving clinical factors, history of larval exposure, and serological tests. MA patients presented with acute severe headache but without neurological deficit, combined with a history of eating uncooked snails or slugs. NG patients always presented with motor weakness, migratory swelling, radicular pain and had history of eating uncooked poultry or fish. Specific antigenic bands in immunoblot tests are helpful tools to differentiate the two diseases. Other causes of eosinophilic meningitis are neurocysticercosis, cerebral paragonimiasis,* Toxoplasma canis, Baylisascaris*, tuberculous meningitis, and cryptococcal meningitis.*

Keywords

Angiostrongyliasis, Differential diagnosis, Eosinophilic meningitis, Gnathostomiasis, Rat lungworm disease

Introduction

Angiostrongyliasis, caused by the nematode *Angiostrongylus cantonensis*, is an emerging disease and has been reported worldwide particularly in tropical countries.[1] There are three major forms of human angiostrongyliasis: meningitic, encephalitic, and ocular.[2] In addition, there are several case reports with gastrointestinal, spinal, or cochlear involvement.[3-5]

The majority of angiostrongyliasis patients exhibit the meningitic form that leads to eosinophilic meningitis. The presence of an absolute count of 10 eosinophils per ml or more than 10% of the total white blood cells in the cerebrospinal fluid (CSF) meets the definition of eosinophilic meningitis and is one of the clinical clues for meningitic angiostrongyliasis.[2]

Although the causes of eosinophils in the CSF need to be explored further, the most common cause of eosinophilic meningitis is infection by *A. cantonensis*. Other causes include other parasitic infections , tuberculosis, cryptococcal meningitis, use of nonsteroidal anti-inflammatory drugs (NSAIDs), or malignancy. *Gnathostoma spinigerum* is the most important other cause of meningitic angiostrongyliasis. Even though eosinophilic meningitis can be diagnosed clinically, there are some situations in which its causes cannot be identified. This short review aims to differentiate the causes of CNS eosinophilia, focusing primarily on meningitic angiostrongyliasis.

This short review is based on articles published on Medline between 2000 and 2012. There are three main factors that can be used to differentiate between meningitic angiostrongyliasis and CNS gnathostomiasis, including clinical factors, history of larval exposure, and serological tests (Table 1).

Clinical Factors

Acute severe headache is the most common presenting symptom of meningitic angiostrongyliasis,[6] while CNS gnathostomiasis causes intracerebral hemorrhage, subarachnoid hemorrhage, myelitis, or radiculitis.[7] Symptoms of CNS gnathostomiasis depend on the location to which the worm migrates. Hemiparesis or hemiplegia, acute severe headache with neck stiffness, paraparesis, or radicular pain are presenting symptoms of the syndromes mentioned above. CSF eosinophils may be found particularly associated with subarachnoid hemorrhage, myelitis, or radicultis.

In addition to the presenting symptoms, some clinical features are suggestive of CNS gnathostomiasis. These include migratory swelling and radicular pain. Migratory swelling is defined as swollen soft tissue along the extremities that lasts for a few days before moving to adjacent areas. The swollen area can be painful and exhibit redness. Acute severe pain or radicular pain usually along the distribution of the cervical nerve roots may be found before developing CNS lesions.[8] Radicular pain from gnathostomiasis is different clinically from the pain experienced by patients with angiostrongyliasis. In angiostrongyliasis, about 5% of patients may complain of severe pain similar to

Table 1. Clinical features to differentiate the two main causes of eosinophilic meningitis.

Factors	Angiostrongyliasis	Gnathostomiasis
Presenting symptom	Acute severe headache	Motor weakness
Migratory swelling	None	Yes
Pain	With focal numbness	Along nerve root
Peripheral eosinophilia	Yes	Yes
CSF[a] appearance	Coconut juice	Non-traumatic bloody
Brain imaging	No pathognomonic sign	SAH[a] or unusual site ICH[a]
History of larval exposure	Uncooked snails or slugs	Uncooked poultry, fish
Diagnostic immunoblot band	29 or 31 kDa	21 or 24 kDa

[a]CSF: cerebrospinal fluid; SAH: subarachnoid hemorrhage; ICH: intracerebral hemorrhage

that experienced by patients with gnathostomiasis. However, the pain in angiostrongyliasis will not be along the nerve root distribution, as it is in gnathostomiasis. Also, the pain is very sensitive to touch and is accompanied with focal numbness. If patients with eosinophilic meningitis have focal numbness, it is very likely that *A. cantonensis* is a causative agent.[6] Both symptoms (migratory swelling and radicular pain) may occur with or without CNS gnathostomiasis.

Peripheral eosinophilia is common in both angiostrongyliasis and CNS gnathostomiasis, exhibited by up to 80% of patients.[6,7] However, it cannot be used to differentiate between angiostrongyliasis and gnathostomiasis. Nonetheless, it can be helpful in conjunction with a known history of larval exposure. For example, peripheral eosinophilia with a history of eating raw snails, combined with CSF eosinophils, may indicate angiostrongyliasis. The number of CSF white blood cells and percent of CSF eosinophils cannot be used to differentiate between the two diseases. However, gross appearance of the CSF may be different. In meningitic angiostrongyliasis, the CSF may appear like coconut juice. *Gnathostoma spinigerum* larvae may commonly cause hemorrhage in the CNS. As a result, in gnathostomiasis the CSF may have non-traumatic bleeding or contain unclotted blood.

Brain imaging may be helpful to differentiate the two diseases. Even though most meningitic angiostrongyliasis cases do not exhibit pathognomonic brain imaging signs, certain abnormal signs that are not specific for angiostrongyliasis may be found, such as periventricular linear hypersignal lesions, small nodules, or small hemorrhagic tracts.[9,10] In CNS gnathostomiasis, abnormal brain lesions are quite pathognomonic and specific. Unusual intracerebral hemorrhage, tract-like intracerebral hemorrhage, non-traumatic subdural hematoma, or unexplained subarachnoid hemorrhage are reported.[8,11,12] Common sites for intracerebral or hypertensive hemorrhages include basal ganglia, pons, thalamus, and cerebellum. For spinal gnathostomiasis, there is no specific lesion. The increased signal in the spinal cord was the only common finding in myelitis caused by *G. spinigerum*.

History of Larval Exposure

History of larval exposure as a risk factor for parasitic infection is the crucial factor for differentiating causative parasites. Many species of snails, including the giant African snail (*Achatina fulica*) and apple snails (*Pila* and *Pomacea* species), and slugs act as intermediate hosts of *A. cantonensis*, and paratenic hosts include freshwater shrimp, frogs, and monitor lizards.[13] Eating these hosts uncooked is a risk for developing meningitic angiostrongyliasis and the incubation period ranges from 1-90 days.[6,14-17] Freshwater fish and shrimp, poultry, snakes, and frogs are risk factors for gnathostomiasis. Note that freshwater shrimp and frogs can carry both parasites. Unlike angiostrongyliasis, gnathostomiasis can be silent in humans for years.

Serological Tests

Definitive diagnosis of any parasitic disease can be made based on the presence of young or mature parasites. In angiostrongyliasis or gnathostomiasis, it is rare to find the larvae in human cases.[1,18] Serological diagnosis is a helpful diagnostic tool. The problem with serological tests, however, is that they are not readily available. Polymerase chain reaction and immunoblot technique have high sensitivity and specificity. Regarding immunoblot techniques, the antigenic bands for angiostrongyliasis are 29and 31 kDa bands,[19,20] while for gnathostomiasis they are 21 and 24 kDa bands.[7] The specificity of the 29 kDa band for eosinophilic meningitis caused by *A. cantonensis* is 100%, while that of the 21 and 24 kDa bands for CNS gnathostomiasis is 95.5%.[21] The sensitivity of the diagnostic immunoblot band method is 50-100% for angiostrongyliasis and 80-90% for gnathostomiasis.[7]

Other Causes of Eosinophilic Meningitis

Other causes of eosinophilic meningitis are neurocysticercosis, cerebral paragonimiasis, *Toxicaria canis*, *Baylisascaris*, tuberculous meningitis, and cryptococcal meningitis.[22] There are several forms of neurocysticercosis. Not all forms cause eosinophilic meningitis. Subarachnoid cysticercosis is the major form of neurocysticercosis that results in raised levels of CSF eosinophils. Patients usually present with subacute to chronic headache. Intramuscular cysticercus may be a supportive evidence for cysticercosis. Magnetic resonance imaging of the brain showing multiple cystic lesions, and serological tests for neurocysticercosis are helpful.

Two less common parasitic causes of encephalitis in children with CSF eosinophils are toxocariasis and baylisascariasis.[22] Exposure to dog or raccoon feces is a risk factor for toxocariasis and baylisascarisis, respectively. A history of ingestion of freshwater crabs in patients with cavitary lung lesions or characteristic brain calicifications is suggestive of paragonimiasis. The clinical setting is an important clue for tuberculous or cryptococcal meningitis. Most patients have lymphocytic CSF, but only few have eosinophilic meningitis. An Indian ink exam of the CSF is a worthwhile test for cryptococcal infection.

Conflict of Interest

None of the authors identifies any conflict of interest.

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The Current Status of Laboratory Diagnosis of *Angiostrongylus cantonensis* Infections in Humans Using Serologic and Molecular Methods

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Abstract

Laboratory diagnosis of angiostrongyliasis relies on serological techniques, since definitive diagnosis is insensitive. Modern antibody detection methods focus on antibodies to the 29 and 31 kDa proteins of the parasite. Antigen detection may ultimately prove to be more reliable than antibody detection but no method has been adopted for clinical diagnostic use. Diagnosis using PCR amplification of DNA sequences specific to Angiostrongylus cantonensis *have been developed but have not yet been validated for clinical use. Diagnostic tests have not been developed commercially and in the United States tests developed experimentally by non-commercial laboratories have to be approved by the Food and Drug Administration before they can be sold to other laboratories for diagnostic purposes.*

Keywords

Angiostrongyliasis, Diagnosis, Eosinophilic meningitis, Molecular methods, Serological methods

Introduction

Angiostrongylus cantonensis is a leading cause of eosinophilic meningoencephalitis. Angiostrongyliasis is often suspected when eosinophilic pleocytosis is observed in cases of acute meningitis, particularly if accompanying risk factors, such as residence or travel to an area known to be endemic for the parasite, are also present.[1,2] A more conclusive diagnosis can be established by direct observation of the parasite in cerebrospinal fluid (CSF) or by using laboratory tests specific for *Angiostrongylus*-specific antibodies or parasite DNA.

Direct observation of *A. cantonensis* in the CSF is not common.[3,4] Third stage larvae may be present in the brain and CSF one to three weeks after infection. Third stage larvae are slender, measuring 460-520 μm long by 22-27 μm wide, with a rhabditoid esophagus and a tail that constricts before tapering to a blunt end.[5] Juvenile worms, which may be seen in brain biopsy and also in the vitreous of the eye, can be morphologically distinguished from other nematodes, such as *Gnathostoma spinigerum*, that might be found in the CSF, based on size. The parasite continues to grow in the human host, and may rarely approach the size of adult worms found in rats.[6]

Antibody and Antigen Detection

Because *A. cantonensis* is not reliably observed in the CSF of patients with angiostrongyliasis, laboratory diagnosis has historically relied on immunodiagnostic methods to detect parasite specific antibodies. Immunodiagnostic methods for angiostrongyliasis were employed in the 1960s soon after *A. cantonensis* was determined to be the probable etiologic agent of eosinophilic meningitis in Asia and the Pacific. An early diagnostic test was an intradermal test based on a skin reaction to adult *A. cantonensis* extracts.[3] Results were indicative of angiostrongyliasis if the reaction to adult *A. cantonensis* extracts was three times greater than the reactions to a phosphate buffer control and to *Gnathostoma spinigerum*, *Paragonimus westermani*, *Dirofilaria immitis*, and *Toxocara canis*. Positive reactions were frequently elicited in asymptomatic individuals or patients with other parasitic infections.[7]

Various laboratory methods that focused on detection of *Angiostrongylus*-specific antibodies were developed in the 1970s, including indirect haemagglutination, complement fixation, indirect fluorescent antibody staining of frozen worm sections, lipid extracts of adult *A. cantonensis*, and latex agglutinations tests.[8] Enzyme linked immunosorbent assay (ELISA) methods were developed in the late 1970s and used crude antigen extracts prepared from young adult *A. cantonensis*.[9] Yen and Chen described ELISAs using partially purified *A. cantonensis* extracts prepared from either juvenile or adult *A. cantonensis*.[10] To reduce non-specific reactions, immunoadsorption was used to remove cross-reacting antigens of *Toxocara canis*, *Ascaris lumbricoides*, and *Clonorchis sinensis*. Both the juvenile worm and the adult worm preparations performed similarly in these experiments; neither preparation performed with significantly greater sensitivity or specificity than the other.[10]

Specificity continued to plague antibody detection methods so scientists sought to identify individual protein antigens that might be more specific than total worm extracts. Immunoblot studies demonstrated that serum antibodies from most patients with angiostrongyliasis specifically recognized the 29 kDa and 31 kDa proteins that are present in adult worm preparations.[11-15] In one report, the 31 kDa protein was more specific than the 29 kDa protein, with minimal cross reactivity from antibodies generated by other commonly encountered tissue-invading helminths.[13] Specific antibodies are present in serum and CSF so either specimen can be used for immunodiagnosis; however, detection of serum antibodies to the 31 kDa protein was reportedly more sensitive than CSF antibodies in one study.[10]

Purification of the 31 kDa protein by electroelution from SDS-polyacrylamide gels resulted in a highly sensitive and specific ELISA.[16] The purified 31 kDa protein was used to develop a dot blot assay for use in regional hospitals in Thailand.[17] An inter-laboratory evaluation of the dot blot assay proved that the method was easy to perform and results were reliable and reproducible across nine regional hospital laboratories.[17] The purified 31 kDa antigen has subsequently been incorporated into a multiplex assay for diagnosis of angiostrongyliasis with success.[18]

Although most studies have focused on the 29 kDa and 31 kDa antigens, detection of antibodies to other *A. cantonensis* proteins may also prove to be equally sensitive. Monoclonal antibodies have been used to purify a 204 kDa protein from subadults (stage 5) that was 91% sensitive and 98% specific in patients with eosinophilic meningitis.[19]

Several studies have evaluated specific immunoglobulin subclass responses, either to *A. cantonensis* crude somatic extracts or, in one study, to the 29 kDa protein specifically.[20,21] Specific IgG1 was the most sensitive class of immunoglobulin for immunodiagnosis of angiostrongyliasis using an *A. cantonensis* somatic antigen preparation.[20] In another study, detection of IgG4 specific for the 29 kDa *A. cantonensis* antigen was the most reliable subclass to measure, with a sensitivity of 75% and a specificity of 95%. Detection of *A. cantonensis* IgA and IgM were not useful.[10,20]

One important limitation of antibody detection for diagnosis is that serum antibody production follows acute symptom onset, sometimes significantly. In an outbreak in Jamaica in 2000, only 8% of acute phase serum specimens (collected 5-18 days after symptom onset) were positive, whereas 83% of the convalescent phase sera, (collected 31-45 days after symptom onset) were positive.[22]

Antigen detection in serum or CSF may ultimately prove to be more reliable than antibody detection for diagnosis of angiostrongyliasis. Several antigen detection methods have been reported in the literature, but none has been adopted for clinical diagnostic use. Monoclonal antibodies generated against adult *A. cantonensis* were used in an assay to detect *A. cantonensis* antigens in serum; this method was highly specific, but only 50% sensitive.[23] Another method was developed that detected a 204 kDa antigen that was present in both CSF and serum; detection in CSF was reported to be more sensitive than in serum.[24]

Immunodiagnostic tests for the detection of antibodies or antigens of *A. cantonensis* are not commercially available. Detection of antibodies to the 31 kDa protein is currently the assay of choice for immunodiagnosis of angiostrongyliasis but testing is available only at Mahidol University in Thailand at present. Proteomics approaches to purify the 31 kDa protein to generate recombinant forms of the protein are underway.[25] Availability of a recombinant form of the 31 kDa or other diagnostic proteins will make immunodiagnosis more widely available. Serologic testing for angiostrongyliasis is also available at the Khon Kaen University in Thailand, using assays based on detection of antibodies to the 29 kDa protein of *A. cantonensis*.

Molecular Detection

Recently a conventional nucleic acid amplification test (NAAT) that amplified a 1,134 bp fragment from the *A. cantonensis* 18S rRNA gene and a real-time PCR assay (TaqMan) targeting the internal transcribed spacer-1 (ITS-1) were developed for detection of *A. cantonensis* in invertebrate hosts.[26-28] The TaqMan assay has also been used to support the diagnosis of angiostrongyliasis in eosinophilic meningitis cases by detecting *A. cantonensis* DNA in CSF.[29,30] NAAT assays are not fully validated for clinical use at this time; therefore serological testing is still recommended to support NAAT results. NAAT testing for angiostrongyliasis is available at the Centers for Disease Control and Prevention (http://www.dpd.cdc.gov/DPDx/HTML/Contactus.htm).

Regulatory Requirements

A discussion of diagnostic testing without some mention of the regulatory framework in the United States would be incomplete. Neglected diseases such as angiostrongyliasis do not attract sufficient resources from commercial test developers because costs are not rewarded with justifiable sales, so laboratories must develop their own tests. Performing laboratory developed tests (LDT) can be billed to clients, but the tests cannot be sold to other laboratories for diagnostic purposes (ie, as a kit) without U.S. Food and Drug Administration (FDA) approval.[31] Historically, regulation of LDTs has been within the Clinical Laboratory Improvement Amendments (CLIA) framework. Laboratories performing diagnostic testing must be certified by the Centers for Medicare and Medicaid Services (CMS) or a recognized organization such as a state health department or the College of American Pathologists (CAP), to be in compliance with CLIA. As such, developing laboratories must conduct formal verification to establish performance characteristics before results can be used for decisions regarding patient care. These performance characteristics include accuracy, precision, sensitivity, and specificity. Once analytical characteristics are determined, the clinical sensitivity and specificity must be verified in actual specimens. There is, however, no clear distinction between CMS and FDA jurisdiction. In fact, the FDA has asserted its authority over LDTs, categorizing them as medical devices in draft guidance documents as early as 1998.[31] The present FDA policy of exercising enforcement discretion by not regulating these tests as medical devices is uncertain, and may not be clarified until final guidance documents are published.

Conflict of Interest

None of the authors identifies any conflict of interest.

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Expression of Recombinant Antigenic Proteins from *Angiostrongylus cantonensis*: A Brief Report

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Abstract

Cerebral angiostrongyliasis is an acute inflammation caused by the infection of the nematode Angiostrongylus cantonensis *that results in eosinophilic meningitis. The current immunological assay of choice is an immunoblot that detects antibodies to a 31 kDa protein present in crude extracts of the female worm. Recently we have identified diagnostic targets from excretion and secretion products and determined the composition of the 31 kDa antigen after 2-D gel electrophoresis and mass spectrometry. Here we cloned and expressed five proteins in prokaryotic and eukaryotic systems. Recombinant proteins were purified and analysed by Western blot assays and among them 14-3-3, Lec5 and ES7 were recognized by* Angiostrongylus*-specific serum, although the signal was weak.*

Keywords

31 kDa antigen, Angiostrongylus*, Eosinophilic meningitis, Recombinant protein*

Introduction

In the genus *Angiostrongylus* two species are of medical importance: *A. costaricensis*, which lives in the mesenteric arteries of wild mice and causes abdominal angiostrongyliasis in humans, and *A. cantonensis*, which lives in the pulmonary arteries of rats and may cause eosinophilic meningitis or meningoencephalitis (EM) in humans. EM is an acute disease caused most often by the presence of *A. cantonensis* larvae in the meninges. The diagnosis is based on parasitological or molecular findings of either parasites or their DNA in cerebro-spinal fluid (CSF) or specific antibodies in the CSF or serum. Several studies have investigated the utility of various antigens, either crude or partially purified proteins, for sensitive and specific detection of *A. cantonensis* specific antibodies.[1] The current immunological assay of choice is an immunoblot that detects antibodies to a 31 kDa protein present in crude extracts of the nematode.[2] The 31 kDa antigen has been purified and used also in dot blot tests in regional hospitals in Thailand,[3,4] but this process is laborious and results in a low yield of material, making standardization and distribution to other diagnostic centers difficult.

Therefore, with the ultimate goal of generating a recombinant protein antigen or antigens for angiostrongyliasis diagnosis, a proteomics approach was used to obtain amino acid sequence from the 31 kDa protein and from other potential diagnostic targets present in the excretory/secretory fraction of cultured *A. cantonensis* adults. The composition of the 31 kDa antigen was determined after 2-D gel electrophoresis and mass spectrometry. Amino acid sequence data were obtained from at least 3 different proteins: the 14-3-3 phosphoserine-binding protein (14-3-3), a protein containing a nascent polypeptide-associated complex domain (NAC), and the putative epsilon subunit of coatomer protein complex isoform 2 (Ep31). It was shown that the antigenicity of the native 31 kDa protein is dependent on the presence of carbohydrate moieties.[5] Another study identified 17 proteins that may prove to be useful diagnostic targets for EM, including proteins with amino acid sequence homology with galectin-5 (Lec5), peroxiredoxin, hemoglobinases, heat shock proteins, protease inhibitors, and a putative protein (Es-7).[6] Recombinant proteins from the 31 kDa protein or excretion and secretion products (ES) were expressed in prokaryotic and eukaryotic systems and evaluated as potential diagnostic targets.

Material and Methods

In this study five proteins were selected for recombinant expression: three identified from 31 kDa antigen (14-3-3, NAC, Ep31) and two identified from ES (Lec-5, Es-7). Genomic sequences were obtained by parallel tag sequencing using a Roche 454 GS FLX sequencer and the corresponding cDNAs were amplified by PCR (polymerase chain reaction) using Platinum Taq DNA polymerase. The amplification reaction was: DNA polymerase Pfx 1u, DNTPs 5 mM, buffer 1x, primers 10 pmol each, $MgSO_4$ 1.5 mM, cDNA 50 ng. PCR cycling conditions were: 95 ºC for 5 min, 30 cycles of 95 ºC for 30 s, the specific primer annealing temperature (Table 1) for 30 s, 72 ºC for 1 min, followed by a final extension at 72 ºC for 7 min.

Recombinant proteins were expressed using either the 6xHis-tag Champion Pet200® prokaryotic expression system (Invitrogen, Life Technologies,Grand Island, NY) or pFastBac vector (Bac-To-Bac® Invitrogen) baculovirus expression system.

For prokaryotic expression *Escherichia coli* DE3 BL21 cells were used. Cells were grown in Luria-Bertani (LB) broth supplemented with kanamycin (100 mg/mL) with shaking (250 rpm) at 37 °C. At log phase IPTG (Isopropyl-β-D-thiogalactoside) (1 mM) was added and expression was performed for three hours. Cells were then centrifuged for 10 min at 3000 *g* and the pellet was suspended in lysis buffer (PBS, Pefabloc SC 1:100, leupeptin 1:1000, and pepstatin A 1:100, 0.1% of Triton X-100, pH 7.4). The lysed cells were sonicated for three pulses of 30 s each at 15% of amplitude. Soluble proteins were harvested by centrifugation at 20,000 g for 1 h. Recombinants were purified by affinity chromatography to nickel and eluted with imidazole (250 mM). Protein quantification was performed by the Bradford method.[7]

As post-translational modifications in eukaryotes are different from those in prokaryotes the recombinant proteins were then expressed in insect cells using a baculovirus expression system to produce recombinant forms of the proteins with

Table 1. The number of DNA base pairs sequenced for each protein coding locus, primer sequences, GenBank accession numbers of DNA sequences from which primers were designed, and annealing temperatures.

Protein	Number of base pairs	Primers[a]	GenBank accession number	Annealing temperature
Ep31	877	F: CACCATGTCTGGGGTTGATCGTTTGT R: TTAAGCGGCAACAAGTTCATCA	FM207709.1	66 ºC
ES7	648	F: CACCATGCGGTCAATTCTGATCTATT R: TTAACTGACGTAGAGCCAGTGA	FM207698.1	61 ºC
Lec-5	969	F:CACCATGAGGATGAAGGTGTTGCT R:CTTCATACTCCTGGAGCATCGTTG	JN133965.1	59 ºC
14-3-3	925	F:CACCATGACGGACAACAGGGGCGA R:TCAGTTGGCACCCTCTCCTTGTTC	JN133967.1	71 ºC
NAC	583	F:CACCATGGTTGCCGCGGTGGAAGT R:TTAACAAATAACTGAGAATCAA	JN133968.1	55 ºC

[a]F - foward, R - reverse

more extensive post-translational modifications. Recombinant baculoviruses were constructed by homologous recombination using site specific transposition into DH10Bac *E. coli* cells. DNA from the constructed Bacmids was purified and used to transfect *Sf*-9 insect cells for recombinant virus production. Cells were cultivated in serum free medium and infected with the virus. Viability and diameter were monitored every day using a Vi-Cell XR cytometer. After cells reached about 17 μm and less than 50% viability they were harvested by centrifugation (1,500 g) and suspended in 50 mM Tris-HCl, pH 7.4, 1% Tween-20. Lysed cells were centrifuged at 20,000 g to pellet insoluble proteins. The recombinant proteins were purified on a cobalt column and eluted with an imidazole gradient.

SDS-PAGE using 12% polyacrylamide gels and Western blot analyses were used to confirm expression of the recombinant proteins. After electrophoresis the proteins were electro-transferred onto nitrocellulose membranes and blocked with 5% skim milk in PBS with 0.05% Tween-20 for 1 h at room temperature. Membranes were then incubated for 2 h with a pool of sera (1:200 dilution) prepared from either *Angiostrongylus*-specific control, serum from uninfected controls, and/or anti-6xHis monoclonal antibody (Invitrogen). After three washes, membranes were probed with a secondary peroxidase-conjugated anti-human IgG (Sigma, St. Louis, MO) (diluted 1:8000) or anti-mouse IgG (Sigma) for 1 h at room temperature. Diaminobenzidine (DAB) (Sigma) (0.05% DAB, 0.015% H_2O_2 in PBS, pH 7.4) was added as developer reagent. For specific *Angiostrongylus* control, a pool of 20 serum samples prepared from 20 patients histopathologically diagnosed with abdominal angiostrongyliasis (caused by *Angiostrongyulus costaricensis*) was used. Heterologous antigens have been used in the diagnosis of angiostrongyliasis since both *A. cantonensis* and *A. costaricensis* possess cross-reactive antigens that can be used to diagnose infections with either pathogen.[8,9] Five serum samples collected from people with no travel history outside the United States were pooled and used as the 'normal' control serum pool.

PROSITE (prosite.expasy.org) was used to predict post-translational modifications such as glycosylation and lipidation.

Results and Discussion

A recombinant protein containing homology with the putative epsilon subunit of coatomer protein complex isoform 2 (Ep31) was expressed in the prokaryotic system and purified by nickel affinity chromatography. Western blot analysis using the anti-6His monoclonal antibody showed that Ep31 was expressed at the expected molecular mass, which was 36 kDa (33 kDa + an extra 3 kDa encoded by the plasmid) (Figure 1, A). The yield of the recombinant Ep31 was about 4 mg/ml. Purified Ep31, collected after the first round of imidazole elution, (Figure 2, lane 8) was used in a preliminary evaluation for antigenicity using the *Angiostrongylus*-specific serum pool. The recombinant Ep31 was recognized by both the normal and the *Angiostrongylus*-specific serum pools (Figure 1, B and C), which indicated a lack of specificity as a diagnostic antigen.
Similarly, a recombinant form of ES-7 was expressed in the prokaryotic system and purified by nickel affinity chromatography. The molecular mass of the ES-7 protein was 26 kDa (23 kDa + 3 kDa encoded by the plasmid) (Figure 3). The yield of the recombinant ES-7 was about 0.8 mg/ml. Two purified ES-7 fractions were evaluated. Fractions 1 and 2 from the imidazole elution were analysed by Western blot (Figure 3, C and D). The *Angiostrongylus*-specific serum pool produced a weak reaction, in the absence of reactivity with the normal serum pool. This result indicated that ES-7 may have some diagnostic value; however titration of the antigen and tests for cross reactivity to other helminth infections are necessary.

The recombinant proteins 14-3-3 and Lec-5 were expressed in *E. coli* and evaluated in the same manner. Each had the expected masses, 31 kDa (28 kDa + 3 kDa encoded by the plasmid) and 36 kDa (33 kDa + 3 kDa encoded by the plasmid), respectively (Figure 4). The recombinant proteins were assayed for antigenicity using Western blot (Figure 4). 14-3-3 was not preferentially recognized by the *Angiostrongylus*-specific serum pool, while Lec-5 was differentially recognized, although the specific reaction was weak (Figure 4, B, lane 8).
Four proteins were expressed in the prokaryotic system. Two identified from the ES sample, Lec-5, and ES-7, showed reactivity with the *Angiostrongylus*-specific serum pool, while

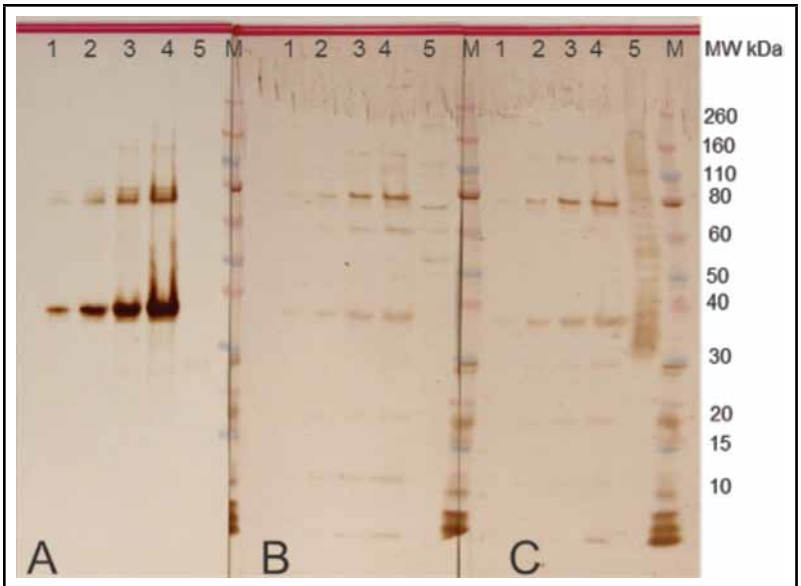

Figure 1. Expression of Ep31. Putative epsilon subunit of coatomer protein complex isoform 2 (Ep31) of *Angiostrongylus cantonens* expressed in *E. coli*. Western blot analyses were performed using monoclonal anti-His tag (A), normal human (B) and *Angiostrongylus*-specific (C) serum. Lane 1 - Ep31 0.5 µg /lane; lane 2 - Ep31 0.3 µg/lane; lane 3 - Ep31 0.2 µg/lane; lane 4 - Ep31 0.1 µg /lane; lane 5 - crude extract of *A. cantonensis* 0.8 µg/lane. M - molecular weight in kDa.

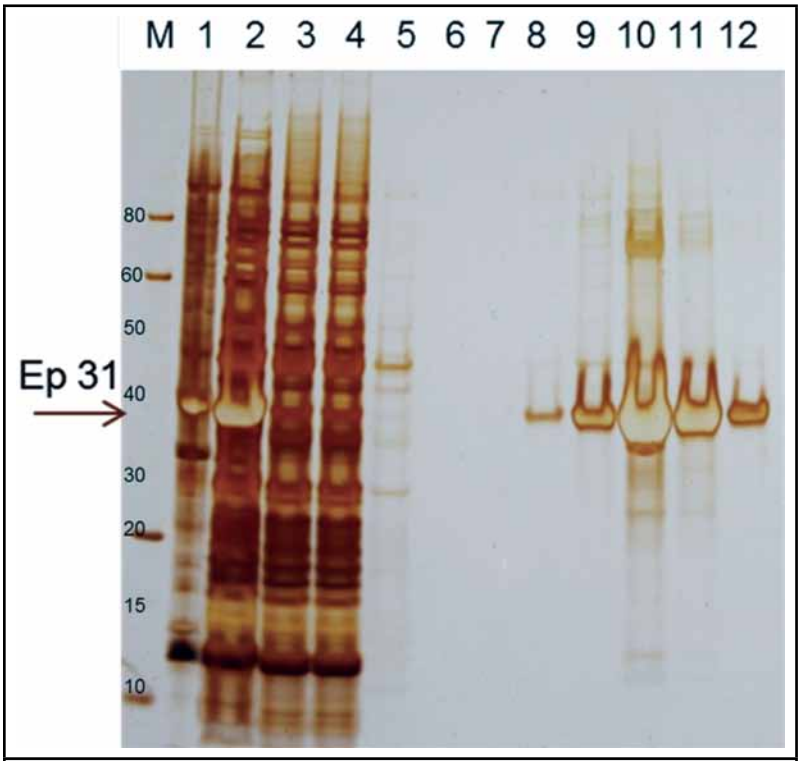

Figure 2. Purification of Ep31. The recombinant protein Ep31 purified by affinity chromatography to nickel. Lane 1 - Ep31 cell lysed insoluble proteins; lane 2 - Ep31 cell lysed soluble proteins; lane 3 - column unbound proteins; lanes 4 and 5 - column washes; lane 6 - Ep31 elution 1; lane 7 - Ep31 elution 2; lane 8 - Ep31 elution 3; lane 9 - Ep31 elution 4; lane 10 - Ep31 elution 5; lane 11 - Ep31 elution 6; lane 12 - Ep31 elution 7; M - molecular weight in kDa. The arrow indicates the Ep31.

14-3-3 and Ep31 showed reactivity with both the *Angiostrongylus*-specific and the 'normal' serum pools. Analyses by the PROSITE program revealed sites for many post-translational modifications, including glycosylation and lipidation. These modifications have been implicated in the antigenicity of *A cantonensis* proteins as previous studies showed that oxidation of the carbohydrates on the native 31 kDa antigen reduced antibody reactivity,[5] suggesting that sugar moieties might be needed for antibody recognition.

14-3-3 proteins are dimeric phosphoserine-binding proteins and members of a family of acidic regulatory molecules that participate in signal transduction, transport, and regulation of several eukaryotic biochemical processes.[10,11] In some parasites, such as *Echinococcus multilocularis* and *Schistosoma mansoni*, 14-3-3 proteins have been shown to be immunogenic and therefore have been promoted as potential vaccine targets.[12-14] For this reason, the *A. cantonensis* 14-3-3- protein was chosen for expression in the baculovirus system. SDS-PAGE and Western blot analyses revealed expression of a protein with the expected molecular mass (Figure 5). 14-3-3 was secreted during expression, which facilitated its purification. In the western Blot analysis, 14-3-3 was recognized by the *Angiostrongylus*-specific serum pool, though the reaction was very weak (Figure 5, B), which suggested that the carbohydrates incorporated during insect cell expression may not be the correct sugar moieties necessary for antigenicity.

Ultimately another expression system may be necessary to produce these recombinant glycoproteins. Further analysis of the glycosylation patterns of *Angiostrongylus* may be warranted.

Conflict of Interest

None of the authors identifies any conflict of interest.

Acknowledgements

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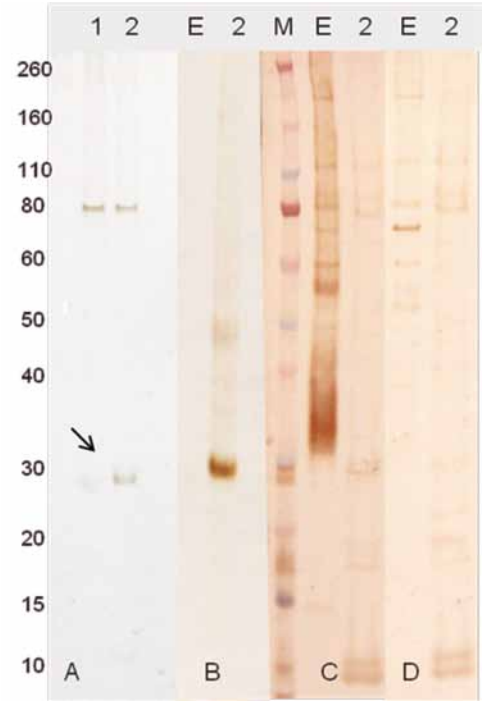

Figure 3. Expression and purification of ES7 protein. A - ES7 expressed in *E. coli* cells and purified by affinity chromatography to nickel: 1 - elution 1; 2 - elution 2. Western blot analyses were performed using monoclonal anti-His tag (B), *Angiostrongylus*-specific serum (C), and normal human serum (D). E - crude extract of *A. cantonensis* 0.8 µg. M - molecular weight in kDa. The arrow indicates the ES7 recombinant protein.

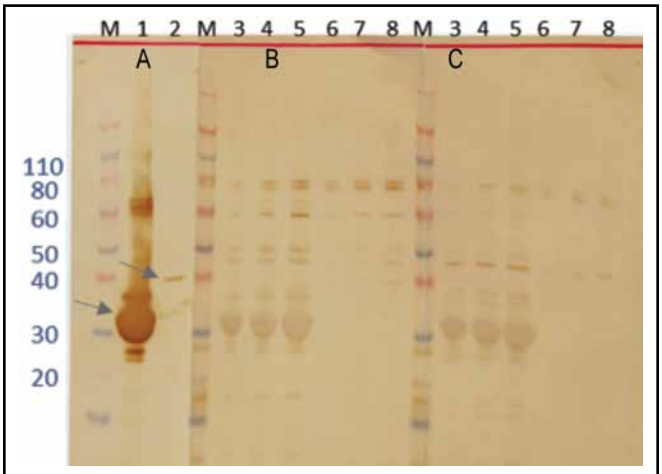

Figure 4. Expression and immunoblotting analyses of 14-3-3 and Lec5 recombinant proteins in *E. coli* cells. Western blot analyses were performed using monoclonal anti-His tag (A), *Angiostrongylus*-specific serum (B), and normal human serum (C). Lane 1 - 14-3-3 5 µg/lane; lane 2 - Lec5 0.8 µg/lane; lane 3 - 14-3-3 1 µg/lane; lane 4 - 14-3-3 3 µg/lane; lane 5 - 14-3-3 5 µg/lane; lane 6 - Lec5 0.1 µg/lane; lane 7 - Lec5 0.5µg/lane; lane 8 - Lec5 0.8 µg/lane M - molecular weight in kDa. The arrows indicate 14-3-3 (lane 1) and Lec5 (lane 2) recombinant proteins.

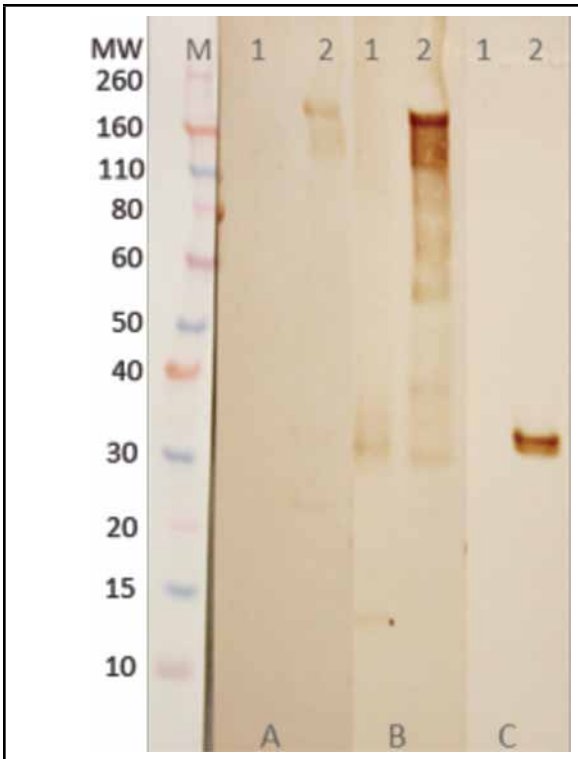

Figure 5. Expression of 14-3-3 recombinant protein in insect cells. Western blot analyses were performed using normal human serum (A), *Angiostrongylus*-specific serum (B), and monoclonal anti-His tag (C). Lane 1 - crude extract of *A. cantonensis* 0.5 µg/lane; lane 2 - 14-3-3 0.5 µg/lane. M - molecular weight in kDa.

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Detection of Rat Lungworm in Intermediate, Definitive, and Paratenic Hosts Obtained from Environmental Sources

Yvonne Qvarnstrom PhD; Henry S. Bishop BS; and Alexandre J da Silva PhD

Abstract

Angiostrongylus cantonensis *is the most common parasite causing human eosinophilic meningitis worldwide. The geographical distribution of this disease has changed dramatically in the last few decades. Various methods have been used to detect* A. cantonensis *in host animals around the world. A survey of mollusks collected on the island of Hawai'i in 2005 using PCR showed an infection rate of 24-78% depending on the mollusk species. In this study, samples from intermediate, definitive, and paratenic hosts were analyzed to further determine the presence of* A. cantonensis *in the United States. All samples were from Hawai'i, except for the apple snails (*Pomacea maculata*) that were collected in New Orleans, Louisiana.* Angiostrongylus cantonensis *was detected in the majority of species examined, including the apple snails from New Orleans and flatworms (planarians) from Hawai'i. Among the mollusks examined, the semi-slug* Parmarion martensi *had the highest parasite load, with an average larval burden of 445 larvae in 25 mg of tissue, as estimated by real-time PCR. In contrast, slime excreted from these highly infected mollusks contained no or very little* A. cantonensis *DNA. Analysis of definitive hosts (*Rattus *spp.) showed discrepancies between morphological and PCR-based identification; 54% of the rats were positive based on morphology, while 100% of tissue samples from these animals were positive by real-time PCR. This indicates that necropsies of rodents could underestimate the infection rates in definitive hosts of* A. cantonensis.

Keywords

Angiostrongyliasis, Angiostrongylus cantonensis*, Emerging infectious disease, Eosinophilic meningitis, Hawaii, Louisiana, Parasitology, Polymerase chain reaction, Rat lungworm disease, Snails, Slugs*

Introduction

From its original range in southeastern China, *A. cantonensis* spread throughout many tropical and sub-tropical regions of the world during the 20th century.[1] It now occurs in Southeast Asia, many Pacific Islands, Australia, India, Sri Lanka, the state of Louisiana in the United States, the Canary Islands, and some countries in the Caribbean, South and Central America, and Africa.[2-9] This rapid geographical spread has coincided with globalization and has probably been facilitated by the unintentional transport of infected host animals in cargo ships and planes.[1] Once introduced into a new area, the parasite may easily establish itself in the local fauna since a large number of mollusk species can act as intermediate hosts and rats are ubiquitous. Thus, it is plausible that *A. cantonensis* will continue to spread to new regions, especially coastal cities, increasing the risk for cerebral angiostrongyliasis in humans. Isolated cases of *A. cantonensis* eosinophilic meningitis have been reported in captive animals in Mississippi and Florida but since there have been no surveys of rodents and mollusks in those areas it is unclear to what extent the nematode has spread into these states.[10,11] Ascertaining the geographic presence of the parasite is an important public health need to prevent additional cases of the disease and to inform diagnosis of patients with eosinophilic meningitis. This report gives an overview of common detection methods and their use to detect *A. cantonensis* in biological samples obtained from environmental sources. It also presents data from surveys of intermediate (mollusks), definitive (rats) and paratenic (flatworm) hosts, mainly collected in Hawai'i.

Morphology-based Detection Methods

The ultimate evidence for occurrence is to find adult worms infecting the local rodent population. Traditionally, this is done by removal of adult worms from the pulmonary arteries of rodents during a careful necropsy and identification of species-specific morphological features.[12] Alternatively, formalin-fixed tissues from the heart, lungs, and brain can be used to prepare hematoxylin and eosin stained microscopy slides.[3] For situations in which trapping and killing rodents is not desirable, *A. cantonensis* infection of rodents can be inferred by detecting first-stage larvae (L1) in animal feces.[13] Isolation of L1 from fecal material can be accomplished by soaking the feces in water and collecting the migrating larvae using a Baermann apparatus.[12] Detection of *A. cantonensis* in intermediate and paratenic hosts can be performed by digesting their tissues with a HCl-pepsin solution and identifying the released third-stage larvae (L3).[14] However, this procedure requires access to live animals so it must be performed shortly after sample collection.

Morphological identification of *A. cantonensis* in host animals has two general disadvantages. First, it is time- and labor-consuming and requires highly trained parasitologists with skills to recognize the parasite's diagnostic features. Second, identification to the species level can only be done on adult male worms with intact posterior ends. Larval stages may display features allowing identification to the family or genus level, depending on stage and isolation technique. If detailed identification is required from larval stages, they can be passed through appropriate laboratory animals to develop into adult worms. Thus, L1 isolated from rat droppings can be used to infect mollusks so that they reach the L3 stage,[15-17] which can be used to infect either mice or rats, to produce immature adults developing in the mouse brain[15,18] or fully mature adult worms in the rat pulmonary arteries.[15,17,19-21]

A rapid method to detect *A. cantonensis* in large snails such as *Pomacea canaliculata* and *Achatina fulica* is to perform a visual inspection of the snail lung tissue.[22,23] The lungs are removed from the snail body, spread open and examined under a dissecting microscope for the characteristic nodules containing the L3 larvae.

Molecular DNA-based Detection Methods

Molecular detection using the polymerase chain reaction (PCR) has been used to circumvent the problems associated with morphological identification of *Angiostrongylus* worms. Genomic

DNA suitable for PCR detection can be extracted from various types of material, including intact worms in all developmental stages, tissues from intermediate, definitive and paratenic hosts, and rat droppings. The first published PCR methods for *A. cantonensis* detection used broadly reactive PCR primers to amplify a genetic region from nematode worms, followed by either restriction length fragment polymorphism analysis or DNA sequencing to provide a species-specific identification. These methods targeted the small subunit ribosomal gene (SSU rRNA),[24,25] the internal transcribed spacer 2 (ITS2),[26] or the mitochondrial cytochrome oxidase I gene (CO1).[26] As DNA sequencing data became available from more *Angiostrongylus* species it has been possible to design species-specific PCR assays. Thus, an *A. cantonensis*-specific real-time PCR assay amplifying the ITS1 region detects only this species in infected mollusks and slime trails.[27] Another real-time PCR assay based on species-specific regions of ITS2 was developed to detect *A. cantonensis* L1 in rat feces.[28] More recently, the loop-mediated isothermal DNA amplification (LAMP) technique has been used to detect *A. cantonensis* in invasive snail species in China.[29,30] The main advantage compared to PCR is that the LAMP technique does not require special equipment; amplification of the target DNA can be performed in a water bath or heat block and the end-point analysis can be achieved by visual inspection of turbidity directly in the reaction tube. This makes LAMP very suitable for field studies.

Quantitative Assessment of Larval Burden in Intermediate and Paratenic Hosts

The ITS1 real-time PCR assay[27] was used to assess the larval burden in intermediate and paratenic hosts. In order to create a standard curve to use in the quantification, L3 larvae were obtained from naturally infected *Parmarion martensi* semi-slugs from the island of Hawai'i by pepsin digestion. The larvae were counted under a dissecting microscope and transferred to new tubes, so that the final numbers of larvae were 1, 5, 10, 100, 500, and 1000 per tube. Figure 1 shows a standard curve obtained by plotting the Ct values from the samples containing known number of larvae against the larvae counts. To test the ability of the standard curve to correctly estimate larval burden, foot tissue from two infected slugs was analyzed both morphologically (counting the larvae released after pepsin treatment) and by quantitative real-time PCR (using the standard curve developed). One slug contained 237 larvae based on the microscopic evaluation and 476 larvae estimated from the real-time PCR results, while the corresponding values for the other slug were 14 and 11 larvae. The difference in counts between the two methods could be due to uneven distribution of larvae in the tissue and the fact that real-time PCR detected DNA from different stages, including dead worms, while only live L3 larvae were included in the morphological method.

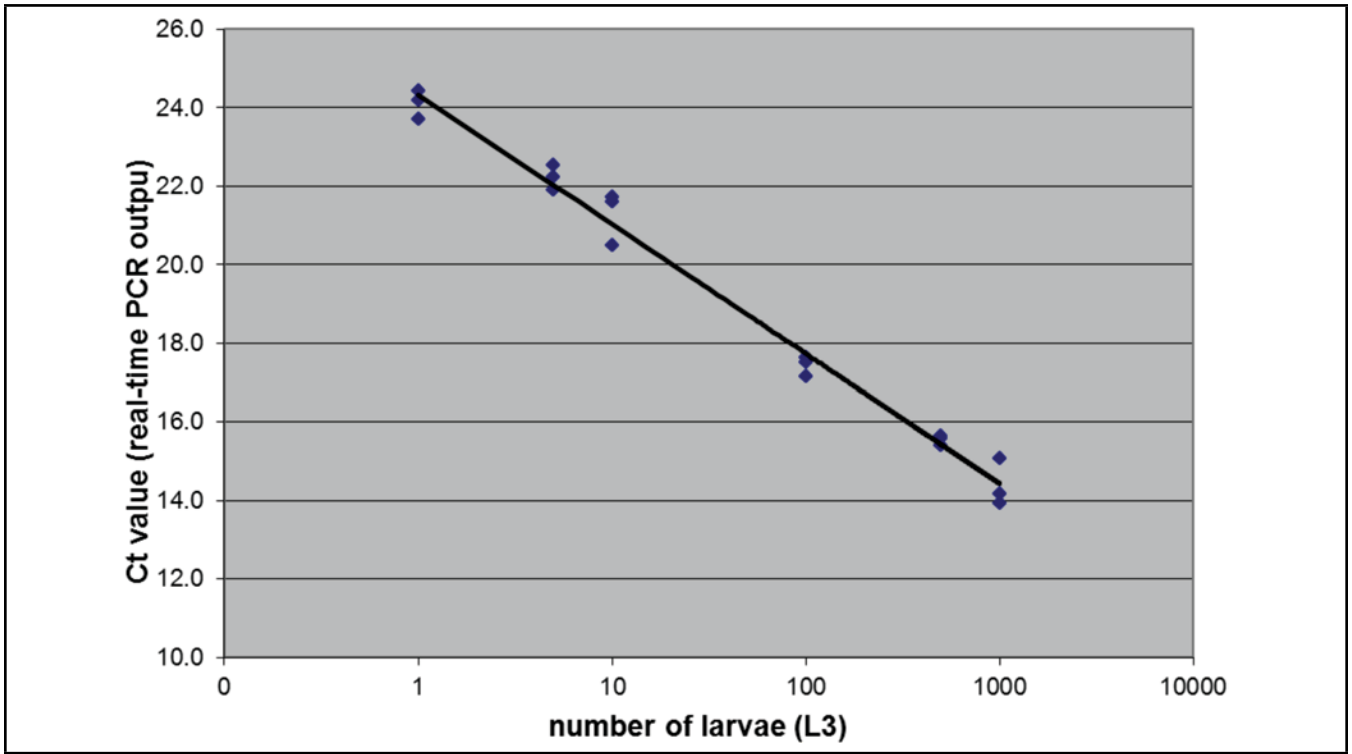

Figure 1. Standard curve for the quantitative estimation of larval burdens. The threshold cycle (Ct) values obtained from samples containing known numbers of larvae were plotted against the larval counts. The regression line has a slope of -3.302, corresponding to a PCR amplification efficiency of 101%, and a coefficient of determination R2 of 0.987.

The standard curve was then used to estimate the larval burden in mollusks and flatworms from Hawai'i and New Orleans. Most of these samples were screened for *A. cantonensis* in previous studies and have thus been described in detail elsewhere.[25,27,31] Locations of collection sites in Hawai'i are shown in Figure 2; details of those in New Orleans are given by Teem, et al.[34] In this study, 79% of *P. martensi* semi-slugs were infected with *A. cantonensis*. It seems that *P. martensi* is a common host of *A. cantonensis*; recent surveys found a 78% infection rate in Hawai'i and 20% in Japan.[23,31] Furthermore, quantification by real-time PCR indicated that *P. martensi* had much higher parasite loads than the other species examined, with an average burden of 445 larvae in 25 mg of tissue, compared to 1 to 205 larvae for other species (Table 1). In 17% (13 of 77) of the positive *P. martensi* samples, the real-time PCR produced a Ct value that was outside the range covered in the standard curve, indicating that this species frequently contained more than 1,000 larvae in 25 mg of tissue. This high parasite load means that even a relatively small piece might contain enough infective larvae to cause severe infection if accidentally ingested. Although high percentages of some other molluscan species were positive for *A. cantonensis*, none had estimated parasite loads as high as *P. martensi* (Table 1).

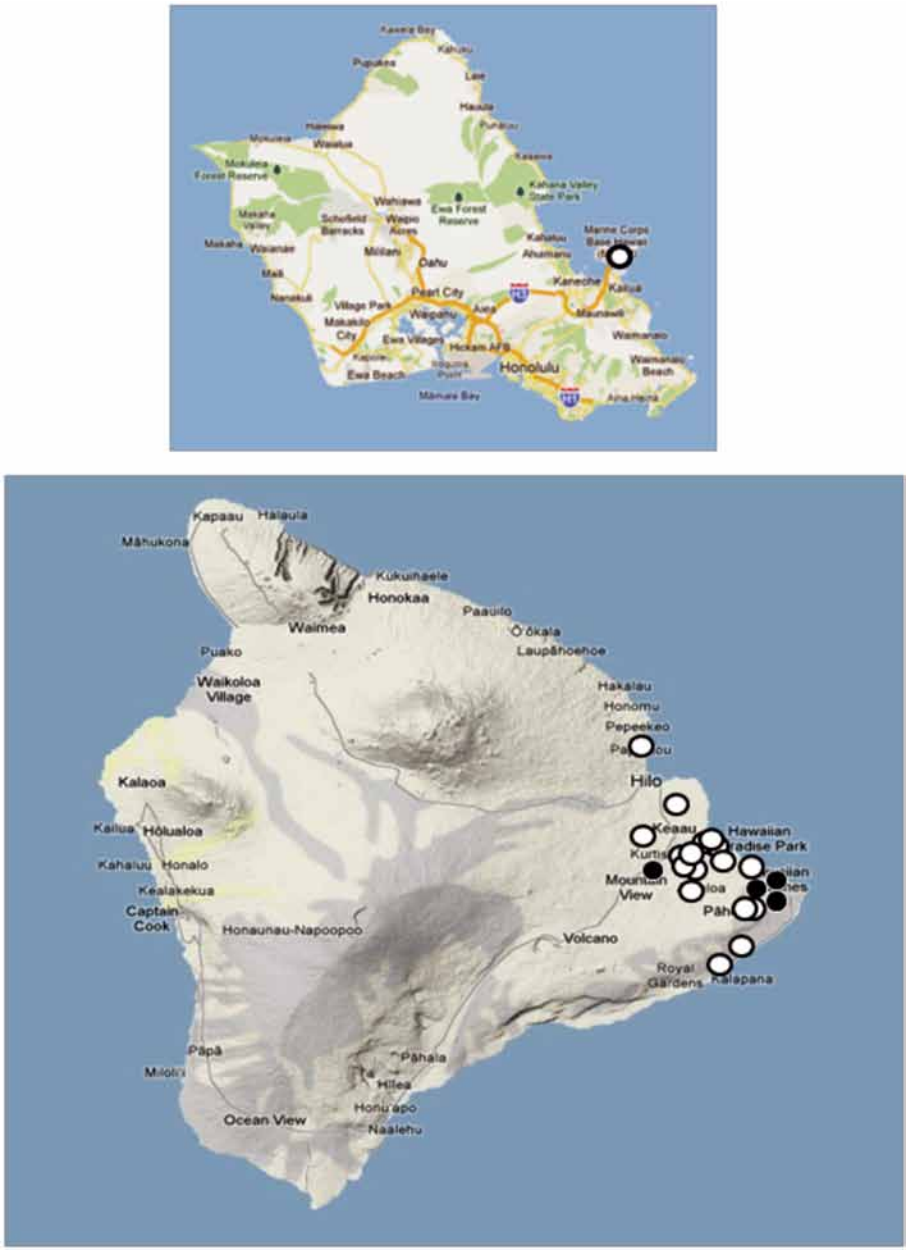

Figure 2. Location of collection sites for intermediate and paratenic hosts on the islands of O'ahu (top) and Hawai'i (bottom). Open circles: collection sites for mollusks only in 2005-2011. Closed circles: collection sites for flatworms and mollusks in 2009 and 2011.

Table 1. Quantitative real-time PCR estimations of larval burden in environmental samples.					
Sample	**Origin**	**Number of positives (%)**	**Estimated larval burden in 25 mg tissue[a]**		
			Average	**Median**	**Maximum**
Parmarion martensi (n = 97)	Hawai'i Island, Hawai'i	77 (79)	445	110	>1,000
Veronicella cubensis (n = 71)	Hawai'i Island, Hawai'i	27 (38)	35	<1	468
Pallifera sp. (n = 5)	Hawai'i Island, Hawai'i	2 (40)	<1	<1	<1
Laevicaulis alte (n = 5)	Hawai'i Island, Hawai'i	4 (80)	205	2	819
Achatina fulica (n = 8)	Hawai'i Island, Hawai'i	7 (88)	5	1	18
Achatina fulica (n = 9)	O'ahu, Hawai'i	5 (56)	7	3	25
Other mollusks[b] (n = 14)	Hawai'i Island, Hawai'i	6 (43)	<1	<1	<1
slime from infected *P. martensi* (n = 13)	Hawai'i Island, Hawai'i	1 (8)	-	-	<1
Flatworms (planarians) (n=12)	Hawai'i Island, Hawai'i	8 (67)	4	<1	30
Pomacea maculata (n=31)	New Orleans, Louisiana	5 (16)	19	2	71

[a]Values lower or higher than the range included in the standard curve are presented as <1 or >1,000, respectively. [b]Included three *Euglandina rosea*, three *Ovachlamys fulgens*, one *Bradybaena similaris*, and seven unidentified mollusks; two of the *O. fulgens* and four of the unidentified mollusks were positive for *A. cantonensis* by PCR.

It has been debated whether the slime trail from infected mollusks could transfer infective L3 larvae to humans if ingested. The real-time PCR assay gave a positive result on the slime from one of 13 naturally infected *P. martensi*; however, quantification using the standard curve indicated that this slime contained DNA corresponding to much less than one intact worm. This is in agreement with previous findings that slime from infected mollusks seemed to contain no or few larvae under normal circumstances, even when the mollusks themselves had a high parasite burden.[16,32] Nevertheless, more studies are needed to clarify the role of mollusk slime in human disease transmission, especially in regards to different environmental conditions.

The finding of *Pomacea maculata* (formerly known as *Pomacea insularum*)[33] from New Orleans infected with *A. cantonensis* concurs with previous reports about the potential for angiostrongyliasis transmission in this area.[5,16] These preliminary data prompted a more detailed assessment of the distribution of *A. cantonensis* in *P. maculata* populations in other states in the Southeastern United States, results of which are reported by Teem, et al.[34]

Flatworms (planarians) from Hawai'i were also included in this study. Eight out of twelve flatworms tested positive for *A. cantonensis*, with larval burdens estimated by real-time PCR of up to 30 larvae in 25 mg of tissue. Although the flatworms were not identified to species, at least one of them was almost certainly *Platydemus manokwari*, a species that feeds on slugs and snails and that has been widely disseminated across the Pacific in ecologically ill-considered attempts to control populations of the giant African snail.[35] Predatory flatworms that ingest infected mollusks are known to be paratenic (carrier) hosts of *A. cantonensis*.[23] In Okinawa, *P. manokwari* was suspected to be an important source of infection for humans because they hide in leafy vegetables and can contaminate fresh salads with larvae of *A. cantonensis* if these flatworms are sliced open during food preparation.

No other paratenic hosts were included in this study. Many species of amphibians, fish, and crustaceans can act as paratenic hosts for infective L3 and pose a threat to human health if ingested raw or undercooked. In Thailand several cases of angiostrongyliasis were attributed to the consumption of yellow tree monitor lizards, with *A. cantonensis* larvae in 21 out of 22 lizards collected from the wild in five provinces.[36] Through careful dissection of various organs it was determined that the liver was the main organ where L3s were found in these lizards. Species of amphibians have been surveyed in New Caledonia and Japan and found to be paratenic hosts for *A. cantonensis*.[23,37] A recent national survey in China looked for L3 in various paratenic hosts from markets and restaurants, including frogs, shrimps, crabs, toads, and fish but all samples were negative for *A. cantonensis*.[38]

Detection in Rats in Hawai'i

Rats were trapped at six sites on the island of Hawai'i (Figure 3). Three species of rats were trapped: 26 *Rattus rattus*, 10 *R. exulans*, and one *R. norvegicus*. The arteries of their lungs and hearts were examined for the presence of adult *A. cantonensis* worms, and if present they were removed and counted. The remaining lung tissues were saved for real-time PCR detection. The majority, 20 out of 37 (54%), of the rats were positive for adult *A. cantonensis* worms: 14 of the *R. rattus*, 5 of the *R. exulans*, and the single *R. norvegicus*. The infection rate in rats in other countries varies between 3 and 100%.[8] Between 1 and 30 adult worms were detected in each positive rat in Hawai'i. It is not uncommon for rats to have many worms in their bodies; previous studies in other countries have found up to 100 worms in individual rats.[28]

The lungs from all examined rats were positive for *A. cantonensis* based on real-time PCR, including the rats that were negative for adult worms according to the morphological examination. In general, tissue from the rats in which adult worms had been detected resulted in a stronger real-time PCR signal

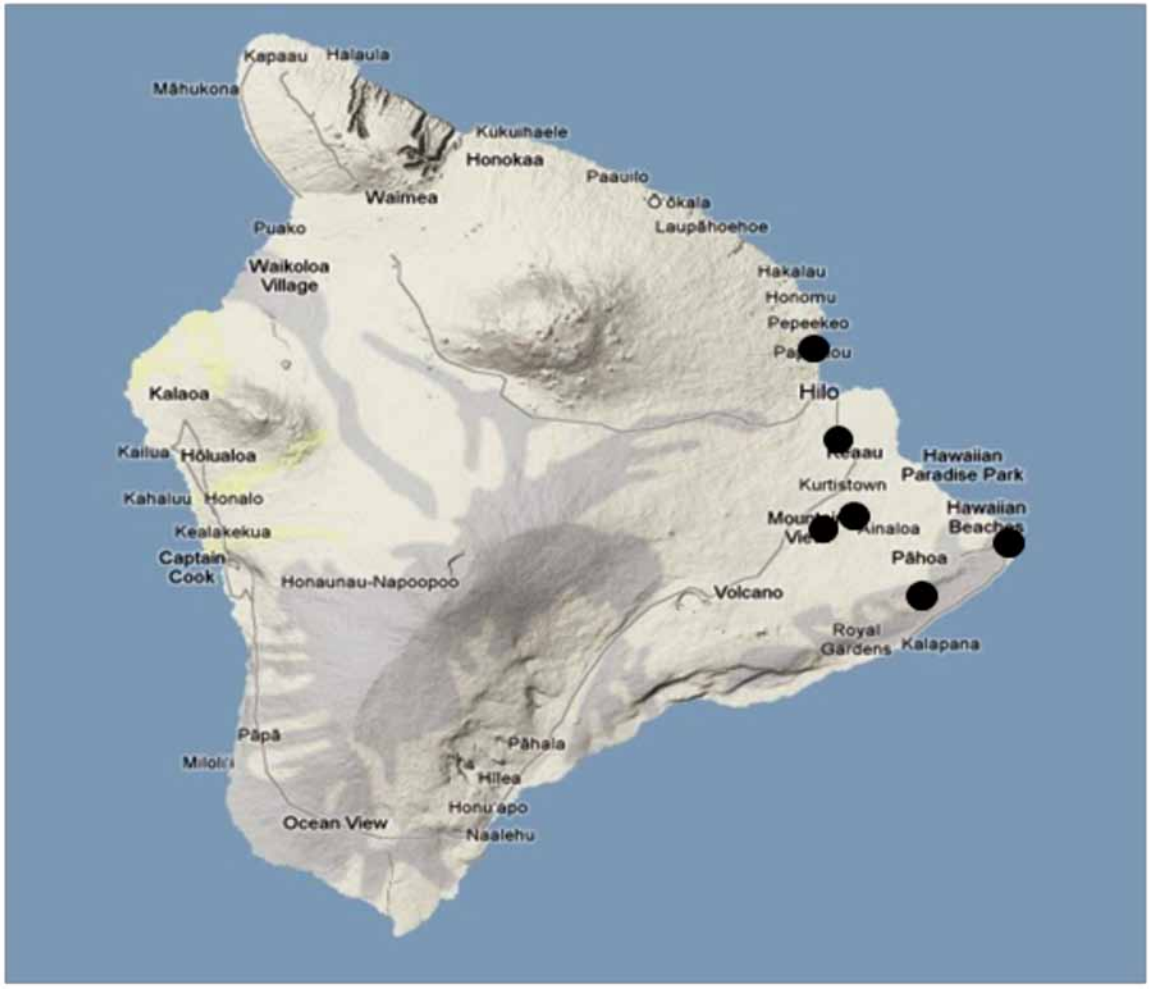

Figure 3. Locations of rat traps on Hawai'i Island (closed circles). Rats were trapped in 2009 and 2011.

compared to tissue from rats without adult worms. PCR does not distinguish between the different life stages of the parasite and will produce a positive result for any material containing *A. cantonensis* DNA, including eggs, fragments of worms, and residual cells shed from adult worms. The lung tissues contained enough genetic material to produce a real-time PCR signal, even after all visible worms had been removed from the arteries. The lungs from rats in which no adult worms were detected could have contained eggs and/or L1 larvae left by adult worms that had since died or escaped detection during the necropsy. Alternatively, these rats could have been recently infected so that L3 larvae were present in their blood stream and were passing through the pulmonary arteries on their way to the brain. In any case, all the rats examined in this study displayed evidence of active or recent infection with *A. cantonensis*.

Future Developments

Molecular detection by PCR or LAMP offers the possibility of screening samples from environmental sources more efficiently for the presence of *A. cantonensis*. However, environmental samples are often challenging for DNA amplification, since they may contain substances that co-purify with the DNA during the extraction process and inhibit downstream applications.[39,40] Also, environmental samples must be collected in a very systematic way to avoid cross-contamination and therefore false-positive results. Since high-quality DNA is essential for successful molecular detection, it is crucial to carefully validate the DNA extraction method to avoid false negative results. In this study, PCR inhibition was common in DNA extracted from mollusks and required dilution or further purification of some samples before accurate PCR amplification could occur. PCR inhibitors were detected by including a PCR reaction in a separate vessel spiked with DNA extracted from adult *A. cantonensis* worms. Another way of testing for PCR inhibition is to co-amplify an internal control in every sample. This internal control can be a genetic target in the sample matrix or an artificial template that is added to the sample prior to extraction.[41]

Another challenge for environmental surveys is that they often involve large sample sizes. One way to facilitate testing of large numbers of samples could be to pool samples and analyze them

in batches.[42,43] This has the potential to substantially reduce the number of procedures required. However, pooling samples can also compromise the performance of the detection method, especially reducing the sensitivity, so the pooling strategy has to be carefully evaluated.[44]

In Hawai'i and other regions where it is not customary to eat raw snail meat, the route of infection in humans often remains unknown. The most commonly proposed route of infection is accidental ingestion of infected neonate mollusks or mollusk pieces hidden in food items such as leafy vegetables.[8,23,45,46] The role of slime trails and mollusk feces in the transmission of infective larvae to humans is still uncertain. It has also been speculated that humans can become infected by consuming water contaminated with larvae released from decaying mollusks.[47,48] These hypotheses could be addressed by exposing laboratory rats or an animal model to contaminated foods. Molecular detection methods are of limited use for direct examination of potential contamination in foods and water, since they do not distinguish between infective and non-infective larvae.

Finally, to facilitate collaborations and comparisons of studies it would be beneficial to standardize the methodology to detect *A. cantonensis* in samples from environmental sources. Such standardized methods should incorporate quality controls and be validated in several laboratories around the world. A recent multi-center validation study for the standardization of PCR for Chagas disease could serve as a model for such a project.[49]

Conflict of Interest

None of the authors identifies any conflict of interest.

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Pathways for Transmission of Angiostrongyliasis and the Risk of Disease Associated with Them

Robert H. Cowie PhD

Abstract

Angiostrongylus cantonensis*, the rat lungworm, is a major cause of eosinophilic meningitis in humans. This short paper reviews what is known about the pathways of infection and assesses the probable importance of each in causing disease. Rats are the definitive hosts. People can become infected by eating, both deliberately and inadvertently, raw or under-cooked intermediate hosts (snails or slugs) or paratenic hosts such as freshwater shrimp, crabs and frogs. Food preparation prior to cooking can leave debris from which infection can also occur. It may be possible to become infected by consuming snail/slug slime (mucus) on produce or by transferring mucus from hands to mouth after handling snails/slugs. Infection from consuming drinking water contaminated by snails/slugs and infection via open wounds may be theoretically possible but no cases have been reported. The severity of the disease is probably related to the number of infective larvae ingested as well as to the precise location of the worms in the host and the host's inflammatory response. Strategies for reducing human infection should include snail and slug control to reduce chances of accidental ingestion, cooking of intermediate and paratenic hosts, and public education on food preparation.*

Keywords

Angiostrongyliasis, Angiostrongylus cantonensis*, Emerging infectious disease, Eosinophilic meningitis, Parasitology, Rat lungworm disease, Snails, Slugs*

Introduction

Eosinophilic meningitis caused by the parasitic nematode *Angiostrongylus cantonensis*[1,2] is an emerging infectious disease.[3-6] The natural life cycle involves rats as the definitive host and snails and slugs (Figure 1) as the intermediate hosts.[7,8] Adult worms reproduce in the pulmonary arteries of rats, where the females lay eggs. The eggs travel passively via the circulatory system to the lung, where they hatch into first stage larvae (L_1). The larvae break through the walls of the bronchioles and alveoli, move up the trachea, and are swallowed and passed out in the feces. Feces containing L_1 larvae are then ingested by the intermediate hosts and develop to the third, infective larval stage (L_3) in the snail or slug. The natural cycle then involves rats ingesting the infected intermediate hosts. The L_3 larvae migrate from the rat's gut via the circulatory system to the central nervous system, eventually reaching the brain. In the brain they develop to the fifth stage (young adult) before returning via the circulatory system to the pulmonary arteries, where they mature fully.

Human infection (angiostrongyliasis) results when L_3 larvae are ingested by people, as accidental hosts.[4,5] The cycle then follows the same trajectory as in the definitive rat host, with worms migrating to the brain and developing to the young adult stage. However, at this stage instead of returning to the circulatory system and continuing the cycle, they die. Tissue damage is caused by the movements of the worms in the brain, and an inflammatory immune response is triggered, predominantly by dead worms.[8-10] The damage and inflammation result in eosinophilic meningitis, the symptoms of which range from severe headaches, through various neurological malfunctions, to coma, and occasionally death.[11-13]

Eosinophilic meningitis caused by rat lungworm infection is a potentially serious disease. It is spreading geographically and the number of cases reported is increasing.[4] To reduce the risk of people becoming infected, it is important to understand the pathways via which infection may occur. This short paper reviews what is known about these pathways and assesses the probable relative importance of each in causing disease.

Figure 1. Two important intermediate hosts of *Angiostrongylus cantonensis*: left - the giant African snail, *Achatina fulica* (photo R.H. Cowie); right - an apple snail, *Pomacea canaliculata* (photo K.A. Hayes); both photographed in Hawai'i where they are invasive alien species. Scale bars approximately 2 cm.

Raw or Under-cooked Snails or Slugs

Deliberate Ingestion

People become infected with *A. cantonensis* through intentionally consuming raw or under-cooked snails and slugs. The majority of reported cases of disease are from southern China and other parts of South-east Asia, notably north-east Thailand, where raw or slightly cooked snails are a delicacy.[14] An early report from Thailand implicated the apple snail *Pila ampullacea* as the cause of eosinophilic meningitis, after the snails had been consumed raw.[15] *Pila* species continue to be important in causing angiostrongyliasis in Thailand.[15-17] However, with the introduction to Asia around 1980 of non-native apple snails from South America (*Pomacea canaliculata* and *P. maculata*) followed by their spread and dramatic increase in abundance,[3,18,19] these snails, especially *P. canaliculata*, became the popular dietary choice, especially in southern China where there is a strong association between the presence of *P. canaliculata* and *A. cantonensis*.[3] Disease outbreaks have occurred in Taiwan among immigrant Thai workers as a result of eating raw *P. canaliculata*,[10] although this is not a common practice among Taiwanese people. An outbreak occurred in 1980 among a group of Korean fishermen aboard a boat docked in American Samoa, following ingestion of raw giant African snails (*Achatina fulica*); eating raw snails is not normal among Samoans but is traditional in Korea.[11]

In other countries, snails are not widely eaten raw, but are cooked, rendering them non-infective. Occasionally, however, there have been instances of infection and resultant disease following deliberate ingestion of raw snails or slugs. The first clinically diagnosed case in Hawai'i was in 1961 when a man became ill after eating two raw veronicellid slugs for health reasons.[20] Also in Hawai'i, a case of eosinophilic meningitis was traced to the consumption of apple snails[21] (identified as *Pomacea paludosa*, which may be incorrect as this species is not known for certain to have ever been present in the wild in Hawai'i[22]). In Brazil, the first recorded cases of eosinophilic meningitis caused by *A. cantonensis* occurred following ingestion of a live veronicellid slug,[23,24] on a dare when drunk. Shortly thereafter two cases were associated with eating raw snails, probably giant African snails.[24] Similarly, a man in Australia became ill with eosinophilic meningitis following ingestion of a slug on a dare,[25] and the first case in North America was of a boy who consumed a snail on a dare.[26] More recently, a man in Hawai'i contracted the disease after eating a live giant African snail for a bet.[27] Children may deliberately swallow snails[28] and this may have been the cause of a fatal case of a 14-month old boy in Jamaica, although it may also have resulted from inadvertent ingestion of contaminated fruit or vegetables.[29]

Inadvertent Ingestion

Snails are safe to eat once thoroughly cooked. However, it may be possible to become infected via contact with the debris associated with preparing the snails for cooking. This was thought to have been the case in Taiwan, where *A. cantonensis* is one of the most important zoonotic parasites. Natural infections have been detected in introduced populations of the apple snail *Pomacea canaliculata* in Taiwan, but, although these snails are collected for food, they are rarely eaten raw.[30,31] Crushing giant African snails (*Achatina fulica*) by hand, and presumably ingesting debris or mucus, has been implicated in Okinawa.[28] However, where snails are not deliberately eaten raw, the most likely pathway of infection is by accidentally consuming a small slug or snail, or portion of one, in uncooked vegetables, especially salads.[2,12,28,32-35] This was the possible cause of an outbreak of eosinophilic meningitis in Jamaica, where a small slug was found in a head of lettuce purchased in a market.[3,35] This pathway also appears to be the most likely mode of transmission in most cases in Hawai'i.[36,37] In Taiwan, an outbreak occurred associated with drinking raw vegetable juice.[33] Small species and especially juveniles may be most easily overlooked in produce.[36,37] If the host present in the produce is damaged or decomposed, with infective larvae still viable, there may be a greater chance that it will not be noticed during food preparation and consumption, particularly in the case of green leafy vegetables.[13]

Mucus

It has been suggested that shedding of *A. cantonensis* larvae in the mucus of infected slugs and snails may result in pathways for human infection.[33,38] The main pathway has been thought to be through consumption of vegetables or other produce contaminated by infected mucus.[12,33,35,39] It has also been suggested that infection may result from handling snails and transferring larvae in the mucus from hand to mouth, especially among children playing with snails.[5] Most cases of eosinophilic meningitis in Taiwan were among children, mostly associated with contact with the giant African snail, *Achatina fulica*, including raising them as pets.[40-42] Handling *A. fulica* has also been implicated in transmission of the parasite in Okinawa.[28]

Although larvae have been found in the mucus of two veronicellid slug species[33] and the semi-slug *Parmarion martensi*,[39] the numbers of larvae were low and this may not represent a significant mode of transmission. In other species (the slug *Limax maximus*[43] and the giant African snail[44]), no larvae were detected in mucus slime trails. Identification of *A. cantonensis* larvae in the slime of the Malaysian slug *Microparmarion malayanus*[38] was probably a misidentification of *Angiostrongylus malaysiensis*,[12] and the numbers of larvae found were low (less than 1 per infected slug). *Angiostrongylus costaricensis*, the cause of human abdominal angiostrongyliasis, has been found in mucus secreted by slugs but also only in very low numbers.[45] While slime trails may represent a source of sporadic infections the evidence suggests that this is not one of the main routes of transmission of the parasite nor a major cause of disease.

Contaminated Water

Contaminated drinking water has been suggested as another potential source of infection for humans.[9,21] When damaged and even undamaged *Achatina fulica* and another snail species, *Subulina octona*, were immersed in water, infective stage *A.*

cantonensis were released and survived in the water for up to 72 h.[46] Similar results were found for *A. costaricensis* released from the freshwater snail *Biomphalaria glabrata*.[47] However, only few larvae were released from the veronicellid slug *Laevicaulis alte* when drowned.[46] In a market in Jamaica vendors rinse vegetables in buckets of water prior to displaying them, and it has been suggested that if larvae are present in slime or feces of snails they could be washed off into the water.[35] Similarly, if dead infected snails were rinsed into the buckets, larvae could be released into the water. In both cases, produce could be cross-contaminated.[35]

Open Wounds

Theoretically, *A. cantonensis* could also infect a person through an open wound.[13] However, there is no evidence for human infection via this route.

Paratenic Hosts

Paratenic (or transport or carrier) hosts are animals that can be infected with larval parasites but in which the parasite does not develop through the stages it would in its normal intermediate host. People can become infected by ingestion of a paratenic host infected with third stage *A. cantonensis* larvae. This was considered the most likely pathway of infection in Tahiti in the early days (1960s) of developing an understanding of the causes of eosinophilic meningitis, with freshwater prawns being the major source of infection.[2,9] Freshwater shrimp have also been postulated as a source of infection in Jamaica.[35] In addition to prawns and shrimp, various other animals have been identified as paratenic hosts, including land crabs, frogs and toads (including tadpoles), monitor lizards, and planarians.[9,17,28,44,48] Some of these paratenic hosts are eaten intentionally by people in certain parts of the world, and have been identified as pathways of infection by *A. cantonensis*, for instance frogs in Taiwan, China, and the USA,[2,49] and monitor lizards in Thailand, Sri Lanka, and India.[2] Some species of planarians are snail predators and become infected by feeding on infected snails.[33] Such planarians have been thought to be important in transmission to people,[2] as they are readily consumed inadvertently in vegetables, fruit, and other produce, especially green salad vegetables, for example, in New Caledonia[32] and Okinawa.[28]

Other animals that are important human food resources have been experimentally infected with *A. cantonensis*, including fish, pigs, and calves.[48,50] If these were to become infected naturally, then they might pose a threat of infection to humans, although only if eaten uncooked or inadequately cooked.

Risk Associated with Pathways

The number of infective third-stage *Angiostrongylus cantonensis* larvae necessary to cause disease in humans is not known.[12,51] When pigs and calves were infected with 20,000 to 70,000 larvae, respectively, cerebral pathology resulted in one of five pigs and in all of the calves tested, although only calves manifested clinical signs of disease. A dog developed temporary, partial paralysis of the hind legs 12-16 days after infection from a dose of 2,000 larvae, but a dose of several hundred larvae produced eosinophilic meningitis in monkeys.[51] It is not unusual for individual *Achatina fulica* and veronicellid slugs to harbor several thousand infective larvae.[12,51]

It has been implied that the number of infective larvae ingested is important in the severity of disease,[2,40] which seems likely. The greater the number of worms ingested, the more that are likely to reach the brain, and if large numbers reach the brain, greater damage will ensue and the more serious the symptoms that will be manifested. But it is not known what parasitic burden is necessary to cause a particular degree of severity of symptoms; nor is it known how many must be ingested for a certain number to reach the brain. Nonetheless, the risk associated with the various possible pathways of infection is probably correlated with the number of infective larvae likely to be ingested. Mild cases of eosinophilic meningitis, the mildest involving just a short-lived headache, may be so mild as to not be diagnosed as eosinophilic meningitis and probably result from very few larvae reaching the brain. Severe cases probably result from a large number reaching the brain.

Therefore, the most important pathways of infection likely to result in disease are those involving ingestion of the hosts (intermediate or paratenic), and especially those host species that carry large parasite loads. So, deliberate ingestion of a large host such as a giant African snail, apple snail (*Pomacea* spp., *Pila* spp.), or veronicellid slug, which can carry thousands of infective larvae,[21] probably has the highest likelihood of causing serious disease. Infection via inadvertently eating a much smaller snail or slug, containing a smaller total number of infective larvae, may pose less of a disease risk, but in many parts of the world where raw snails are not eaten, is probably the most likely path for infection. Nonetheless, even small snails or slugs may carry thousands of infective larvae. The potential parasite load of the various possible paratenic hosts is not known, but if it is as much as that of the snail and slug intermediate hosts, then eating these paratenic hosts raw poses a great risk.

The only pathway that has received much attention other than eating intermediate and paratenic hosts, is ingestion of snail and slug mucus, either by direct transfer from hand to mouth after handling these hosts or through ingestion of produce contaminated with mucus. Although there are implications that the latter pathway has been important, particularly among children,[5,28,40-42] no case of disease caused by infection via mucus on produce has been definitively demonstrated. And given the low numbers of larvae in the mucus of those species so far investigated, these mucus mediated pathways are probably less likely to cause serious disease. However, in cases in which disease is ascribed to ingestion of contaminated produce it is rarely possible to determine definitively whether the contamination was snails/slugs or only mucus.[3] Given the limited knowledge of these mucus pathways, it would be irresponsible to consider them negligible.

Contaminated drinking water probably poses little risk, depending on the number of hosts and volume of water involved. Because of dilution, the number of larvae likely to be ingested

may be low and insufficient to cause serious illness. No cases have been linked to this pathway of infection.

No cases of disease caused by infection through open wounds have been reported. Though it may be theoretically possible to become infected via this route, the chance of a large number of larvae entering the blood stream seems minimal. The risk of contracting serious disease therefore seems very low.

Recommendations

Strategies for reducing human infection should include public education so that people do not deliberately eat raw intermediate and paratenic hosts and that they take care to clean vegetables/produce prior to consuming them. People, especially children, should be cautioned not to handle snails and slugs, and if they do to wash their hands thoroughly afterwards. Control of definitive and intermediate hosts, and management of intermediate and paratenic hosts to reduce chances of accidental ingestion, may also be undertaken.

Conflict of Interest

The author identifies no conflict of interest.

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Control Measures for Slug and Snail Hosts of *Angiostrongylus cantonensis*, with Special Reference to the Semi-slug *Parmarion martensi*

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Abstract

Slugs and snails (class Gastropoda) are the obligate intermediate hosts of the rat lungworm, Angiostrongylus cantonensis*. This nematode is the causative agent of human angiostrongyliasis and the most common cause of human eosinophilic meningoencephalitis. Humans can become infected by accidental consumption of slugs or snails and possibly flatworms (or a portion of one of these animals) in fresh produce, but the slime from these animals can contain nematodes and may also constitute a disease risk. Gastropod carriers in Hawai'i include, among other species, giant African snails, veronicellid slugs, and the semi-slug* Parmarion martensi*. This latter species was first discovered on the island of Hawai'i in 2004 and is now common in the area where the majority of the state's documented cases of human angiostrongyliasis occurred between 2005 and 2011. This species is considered a high risk carrier of* A. cantonensis *because of its climbing behavior, abundance around human dwellings, and high worm burdens. One individual collected from east Hawai'i Island contained >6,800 infective third stage* A. cantonensis *larvae. Common and efficient control methods for slugs and snails include sanitation (eg, removal of objects that serve as hiding places) and the use of poison food baits, such as those containing metaldehyde and iron. An iron-containing bait that is relatively safe to non-target organisms was effective in controlling semi-slugs in cage experiments, although it killed more slowly than a metaldehyde-containing bait and the majority of slugs affected did not die until 1-2 weeks following ingestion.*

Keywords

Angiostrongylus cantonensis*, Hawaii, Iron phosphate,* Parmarion martensi*, Rat lungworm, Semi-slug, Slug baits*

Introduction

Angiostrongylus cantonensis was first discovered in rats in Canton, China, in 1935. It was recognized as causing human disease (meningitis) in 1944. However, the scientific report published the following year was in Japanese and was largely overlooked until reported by Beaver and Rosen in 1964.[1] In 1955 the role of slugs and snails as intermediate hosts of *Angiostrongylus cantonensis* was reported.[2] The information proved correct although it was later discovered that the authors were actually studying *A. mackerrasae*, a species almost identical to *A. cantonensis*.[3] In the early 1960s, outbreaks of eosinophilic meningitis occurred in human populations in a number of Pacific islands. University of Hawai'i parasitologist Joseph Alicata correctly inferred that *A. cantonensis* infection was the cause of those outbreaks, and it was subsequently demonstrated that *A. cantonensis* was the usual cause of eosinophilic meningitis in South-East Asia and the Pacific islands.[4,5]

Although slugs or snails are the obligatory intermediate hosts of *A. cantonensis*, first to third stage larvae can also be found in the tissues of other, paratenic, hosts that are passive carriers. Regarding risk to humans, the most important paratenic hosts are crustaceans (such as prawns and land crabs) and predacious land planarians (such as flatworms in the genus *Platydemus*).[5] *Platydemus* spp. are predators of slugs and snails, excreting digestive juices that externally digest their prey. These flatworms are considered high risk carriers of nematodes because they are small, commonly found on lettuce, cabbage, and fruits, and easily overlooked while preparing food that is consumed without cooking.[6-8] Hosts of *A. cantonensis* such as snails, slugs, and prawns are considered safe for human consumption if thoroughly cooked (heated to an internal temperature of 74°C or 165°F).[9]

A key unresolved question,[5] is whether the mucus (slime) deposited by slugs and snails infected with *A. cantonensis* on fresh produce constitutes a human disease risk. Observations of veronicellid slugs showed that they rarely shed larvae in their mucus, and when they did, the larvae survived for only a few hours.[7] *Angiostrongylus cantonensis* larvae were not detected in mucus rinsed off infected *Lissachatina fulica* (giant African snail; also referred to as *Achatina fulica*).[10] *Angiostrongylus* larvae shed in the mucus of *Microparmarion malayanus* were viable and could be used to successfully infect rats, although the numbers of larvae were low.[3,11] *Angiostrongylus costaricensis*, the cause of human abdominal angiostrongyliasis, has been found in slug mucus but only in very small numbers.[12] A study using PCR (polymerase chain reaction) and microscopic analysis found that 3 of 25 naturally infected *Parmarion martensi* shed *A. cantonensis* larvae in their mucus when prodded, although only one to four larvae per individual were observed.[13] In a different study, levels of parasite DNA detected by quantitative PCR in the mucus of a naturally-infected *P. martensi* individual were low compared with levels in various parts of the semi-slug body. An equivalent of 0.83 larvae were detected per mg of mucus compared with 41.3 larvae per mg of tail tissue and 49.3 larvae per mg of midsection tissue.[14] As all these studies detected only very low numbers of larvae, mucus probably poses minimal risk of human infection.

The prevalence of *A. cantonensis* in intermediate hosts can be assessed by digesting slug or snail tissue in artificial gastric juice (1% pepsin with 1% HCl) and the third-stage larvae in the sediment identified morphologically under the microscope using the key of Ash.[15] PCR tests have now been developed to identify *A. cantonensis* in tissues of slugs and snails and quantify the parasite load.[14,16]

Parmarion martensi as a Host in Hawai'i

In 2004, the semi-slug *Parmarion martensi* (family Helicarionidae; Figure 1), native to southeast Asia and introduced to Hawai'i, was discovered on a residential property in the Puna district of Hawai'i Island.[17] The three residents at this property

Figure 1. Parmarion martensi. (A) eggs and neonates. Eggs are about 2.5 mm in diameter. (B) Adult with yellowish-brown flattened fingernail-shaped shell visible on the dorsum. The distinct keel along the posterior dorsal midline helps distinguish this species from similar-looking species in Hawai'i. (C) Adult with shell covered by mantle folds. Adults are about 5 cm in length.

had developed symptoms of angiostrongyliasis (infection by *A. cantonensis*) after consuming home-grown lettuce reportedly contaminated by immature semi-slugs.[17] Semi-slugs from the property were heavily infected with *A. cantonensis* larvae. An island-wide survey in 2005 found that *P. martensi* was common in residential properties throughout the lower Puna area.

An average of 78% of semi-slugs collected from residential properties were infected with *A. cantonensis*, compared with only 24% of Cuban slugs (*Veronicella cubensis*, family Veronicellidae) collected from the same properties. Residents reported that semi-slugs were more often found climbing on structures such as exterior house walls, drain pipes, and water tanks than other slug species. Some residents reported finding semi-slugs in outdoor sinks, on dishes, and in food preparation areas. They were frequently abundant under plastic and in piles of compost, fallen palm leaves, and in other types of rotting organic matter.[17]

There is both direct evidence[17] and circumstantial evidence that *P. martensi* has been responsible for an increase in the number of human cases of rat lungworm disease on Hawai'i Island. The State of Hawai'i Department of Health investigated 38 cases between 2005 and 2011, many of which were considered as probable cases only, as they were not confirmed by visualization of the larvae in the cerebrospinal fluid. The majority of these cases occurred on the east side of Hawai'i Island (M. Dixon, State of Hawai'i, Department of Health, letter to SIJ, August 24, 2012). This area includes the center of the distribution of *P. martensi* as determined in the 2005 survey.[17] Since that time, the distribution of *P. martensi* has expanded and the Hilo area is now also infested (RGH, personal observations). Isolated populations are also present along the Hamakua coast north of Hilo (RGH, personal observations) and in Waimea and Kailua-Kona.[17]

Based on the biology and behavior of this species, it appears to represent an unusually high risk for infecting people and animals with *A. cantonensis* relative to other slug and snail species. Very high parasite loads have been found in some individuals. For example, over 6,800 larvae were extracted from an individual semi-slug from Puna (SIJ, unpublished). This, combined with their common climbing behavior and ability to move quickly and cover large distances across dry substrates (including wood, concrete, tree bark) to locate food sources such as bird food, dog food, cat food, fish entrails, and papaya fruits[17] increases the likelihood that people and pets will come into contact with them and the parasitic nematodes they carry.

The environmental fate of infective (third-stage) larvae of *A. cantonenis* in individuals of *P. martensi* after death is poorly known. *Angiostrongylus cantonensis* larvae can exit the bodies of drowned snails and live up to 72 h in water.[18,19] Both field observations and cage studies (RGH, unpublished) suggest that *P. martensi* may have an annual life cycle. Observations on semi-slugs held individually revealed that an egg clutch is sometimes produced by a semi-slug a day or two before it dies. In the field, clutches have been found mainly during spring or summer, sometimes in the immediate vicinity of dead adults. Larvae of *A. cantonensis* in the bodies of the dead semi-slug adults could be acquired by immature semi-slugs directly feeding on the former or via contact of immature semi-slugs with materials in the environment contaminated by *A. cantonensis* larvae passively liberated or exiting the tissues of the dead semi-slugs.

In Okinawa, *P. martensi* and a flatworm predator of slugs and snails, *Platydemus manokwari*, were carriers of *A. cantonensis* associated with an outbreak of human cases of rat lungworm disease in 2000, the flatworms presumably having become infected by eating infected slugs or snails.[8] Both species are found in Hawai'i, sometimes in association with one another (RGH, personal observation).

Management and Control of Slugs and Snails around Homes and Gardens

In parts of the world, including Hawai'i, where slugs and snails are carriers of *Angiostrongylus cantonensis*, controlling these animals around homes, gardens, and in the landscape is recommended and should reduce disease risk. Control is most easily accomplished using a combination of sanitation and chemical control.[9,20]

Sanitation and Non-chemical Control

Slugs and snails are mainly active at night, which helps preserve water balance, a critical survival factor for these animals. Slugs in particular can quickly become dehydrated and die if they forage on a hot, sunny day and are unable to find cover. Therefore, a good method for reducing their populations is to limit the number of moist hiding places. This may include removing unnecessary ground cover, cutting back vegetation, removing rocks and fallen wood, and raising items in the landscape off the ground. For example, plant pots and storage sheds can be placed on blocks instead of directly on the ground.

Conversely, a home owner, gardener, or farmer might want to *provide* hiding places along the perimeter of the area to be protected in order to lure and trap the slugs or snails so that they can be more easily disposed of. Some slugs and snails show homing behavior, returning to the same resting sites each day after a night's feeding.[21] Lures may be as simple as boards or pieces of plywood placed on the surface of the soil. Other lures include overturned flowerpots, overturned melon rinds, or orange peels. These lures can be checked frequently to collect slugs and snails for disposal. It is important to use gloves or a tool (such as disposable chopsticks or tweezers) to handle the slugs and snails, as they carry other parasites besides rat lungworms. Hand-picking of snails and slugs when they are out of their hiding places can be effective and supplement other methods if the species is relatively large. It is best done at night or early in the morning following rains or watering, which increase moisture levels in the ground, foliage, and air. Burch[22] considered hand-picking impractical for most species, yet the only satisfactory method for reducing populations of the giant African snail (*Lissachatina fulica*), partly because these snails sometimes feed high in foliage where they would not encounter poison bait pellets applied on the ground.

Drowning slugs or snails for several days in a covered bucket filled with soapy water or a 15% solution of salt water is a convenient and safe way to kill slugs and snails. In the case of salt water, this treatment would also be expected to kill any *A. cantonensis* larvae that might exit the bodies or remain within them (SIJ, unpublished). Simply smashing slugs and snails and leaving them on the ground is not recommended, as the disease-causing nematodes might be eaten by other animals (such as pets or surviving slugs and snails).

Chemical Control

In Hawai'i, the most common formulations of molluscicides (chemicals used to control slugs and snails) around homes and gardens are food bait pellets or granules that can be broadcast over the area to be protected, or selectively placed in crop borders or underneath objects where slugs and snails take refuge (Figure 2). These typically contain metaldehyde or iron phosphate as the active ingredient. Placing the baits underneath boards or other objects protects them from the direct effects of rain (which can cause baits to fall apart) and may slow the development of mold, which can make baits unpalatable. It also reduces the chance that domestic animals will consume them. Using molluscicides as the only control measure seldom produces adequate results. Frequently a bait application might kill only half of the slugs or snails in the treated area. Others will survive the treatment because either they were buried or hidden at the time of treatment, they were not attracted to the bait, or they did not eat enough to kill them.

Food bait pellets attract snails and slugs, but they may also attract domestic pets. If consumed, metaldehyde products are very toxic to dogs, cats, and other animals, and poisoning incidents are common. Partly for this reason, metaldehyde products are also available as granules without the food attractants. Granular

Figure 2. Common products used to control slugs and snails in Hawai'i in commercial and residential settings. From left to right: Sluggo® (food bait containing 1% iron phosphate) (Neudorff, Emmerthal, Germany), Durham® metaldehyde granules 7.5 (AMVAC Chemical Corp., Los Angeles, CA), Metarex® (metaldehyde food bait) (De Sangosse, Pont Du Casse, France) and Deadline® (metaldehyde food bait) (AMVAC Chemical Corp., Los Angeles, CA).

formulations are finer than the pellets. Slugs or snails accidentally contacting granules will respond by producing copious amounts of slime. Under the right environmental conditions (hot and dry), the associated loss of moisture leads to death of the snail or slug.[23] Liquid forms of metaldehyde are used as a foliar spray or pot drench but are not allowed on edible crops. Metaldehyde products must be kept out of waterways and cannot be used legally around water or in swampy areas. For all pesticide products, directions for use are indicated on product labels, and directions must be strictly followed as a matter of federal law.

Alternatives to metaldehyde products are food baits with 1% iron phosphate or a chelated form of iron (sodium ferric EDTA) as the active ingredient. These products are considered generally safer for use around domestic animals and wildlife than those containing metaldeyde. Slugs or snails that feed on iron phosphate baits stop feeding immediately but may not die for several days, so the control will not be immediately apparent. Iron-containing baits can be just as effective, or more so, as metaldehyde baits. However, the scientific consensus is that metaldehyde baits outperform iron phosphate baits under normal conditions.[20] Relative performance will vary by pest species (which can affect bait acceptance), bait formulation, and environmental conditions following application (eg, temperature and moisture), which affect survival associated with contact exposure to metaldehyde and mold growth on bait.

Experimental Assessment of Baits for Chemical Control of Semi-slugs

As a preliminary step in determining the acceptability and efficacy of three food bait products for control of *Parmarion martensi*, experiments were conducted in which semi-slugs were exposed to baits in mesh cages (~46 x 46 x 48 cm high). Each cage had a plastic pot as a hiding place, a stick to elevate the lip of the pot to allow access, and a piece of cardboard moistened by spraying with water every 1-3 days (Figure 3). Ten adult semi-slugs were placed in each cage. The cages were set up on tables in a covered outdoor area. Each experiment used four cages, one as the control (no bait or other food provided), while the other three were provisioned with 140 pellets each of one of three types of bait pellets. Additional pellets were added as necessary to ensure that bait was always available. The tests were replicated three times.

Tests were set up on a Monday, and mortality and bait consumption were monitored usually each weekday following test set up for a period of two weeks (two replicates) or three weeks (one replicate). Products tested were Deadline® MP's™ (4% metaldehyde, AMVAC Chemical Corp., Los Angeles, CA), Sluggo® (for details see Figure 2), and Ferroxx®, a newly available iron-containing product (5% sodium ferric EDTA, Neudorff, Emmerthal, Germany). For each of the products, almost all bait consumption occurred within the first two days. Although feeding by individual semi-slugs was not tracked, the results suggested that all three products were generally accepted as food. The average number (± SE) of pellets consumed by the ten semi-slugs was 17.7 (± 1.4), 41.7 (± 5.5) and 75.7 (± 15.9), corresponding to 0.48, 0.88 and 0.91 g dry weight for Deadline, Sluggo, and Ferroxx, respectively Cumulative mortality of semi-slugs after 7 and 14 days is shown in Table 1. Deadline performed best, killing an average of 90% of semi-slugs within 7 days and 100% by 14 days. Ferroxx performed better than Sluggo, killing an average of 63% (compared to 30% for Sluggo) by the end of the second week. Results for the test monitored over a three-week period are shown in Figure 4. Although all semi-slugs were eventually killed in the Ferroxx treatment in this test, 100% mortality was not observed until 16 days after the start of the trial. These results were in sharp contrast to results obtained in a single test using Cuban slugs (*Veronicella cubensis*), in which Deadline and Ferroxx produced 100% mortality by day 4 and Sluggo produced 100% mortality by day 7 (Figure 5).

While Deadline was the most effective product tested, it would also be the most toxic to domestic animals and must be used with discretion. The choice of bait for home users should take into consideration efficacy, cost, and the potential risks of each bait. Given favorable weather, a sufficient amount of bait, and repeated treatments, any of the three products could result in complete control of semi-slugs over time.

Figure 3. Inside view of cage in which semi-slugs were offered food baits.

Table 1. Cumulative percent mortality of *Parmarion martensi* semi-slugs exposed to poisoned food baits.

Days[a]	Replicate	Deadline	Sluggo	Ferroxx	Control
7	1	100	0	0	0
7	2	100	30	10	10
7	3	70	10	10	0
14	1	100	10	70	0
14	2	100	70	60	20
14	3	100	10	60	0

[a]The number of days post treatment.

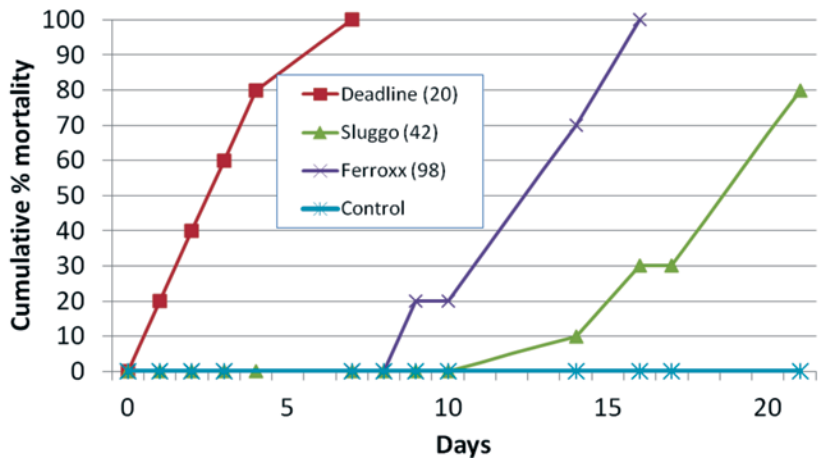

Figure 4. Mortality of semi-slugs (*Parmarion martensi*) offered different types of food baits. Numbers in parentheses indicate the number of bait pellets consumed by the 10 semi-slugs.

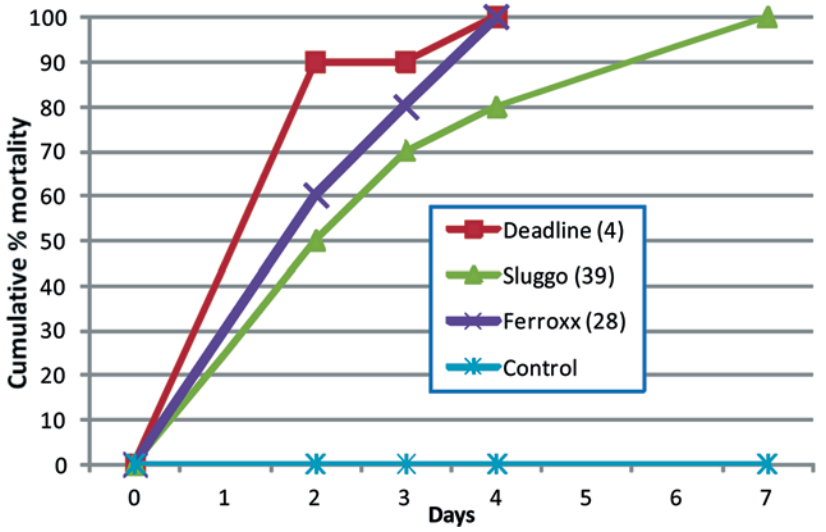

Figure 5. Mortality of Cuban slugs (*Veronicella cubensis*) exposed to different types of food baits. Numbers in parentheses indicate the number of bait pellets consumed by the 10 slugs.

Conflict of Interest

None of the authors identifies any conflict of interest.

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Rat Lungworm Disease in Hawai‘i: Community Outreach and Education on the Island of Hawai‘i (the ‘Big Island’)

Marlena Castro Dixon MS

Abstract

Due to an increase in severity of cases of rat lungworm disease and increased media attention, community outreach efforts on the island of Hawai‘i (the Big Island) were revisited in 2009, to include an updated flier, radio interviews, and community presentations. The Puna district of the island has been impacted the greatest by rat lungworm disease. The biggest challenge in disseminating information was that residents could not accept that limited information, testing, and treatment options were available. Some people wanted basic information while others requested great detail. Some responded better to information in “pidgin” but others preferred English. Another challenge was to provide information to communities where residents did not read newspapers or watch television news. As a result, a community education group formed and assisted in disseminating information to these communities. But some residents never received information and there has been no decrease in cases. Information must be sent repeatedly and through different media, including free journals, local community newspapers, local television stations, and even social networking.

Keywords

Angiostrongyliasis, Angiostrongylus cantonensis*, Public education*

Conflict of Interest

The author identifies no conflict of interest.

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Telling Consumers, Gardeners, and Farmers about the Possible Risk of Rat Lungworm in the Local Food Supply in Hawai‘i

James R. Hollyer MS

Abstract

The rat lungworm, Angiostrongylus cantonensis*, uses various species of rats as its definitive hosts and a wide range of slug and snail species as its intermediate hosts.[1,2] When ingested by humans, this nematode is a major cause of eosinophilic meningitis.[3] However, the risk of consuming the rat lungworm, is extremely low, especially when compared with other food-borne disease agents such as* E. coli *and* Salmonella*, or illegal pesticide residues. That said, ingesting a potentially harmful microscopic worm is an unpleasant thought, especially when consumers have seen the potential impact on television, which in serious cases can lead to coma and death, or to long-term neurological malfunction.*

On July 8, 2009, the Discovery Channel (a US television channel) ran a 15 minute story on rat lungworm, calling it "one of the most feared parasites on the planet."[4,5] The program covered the story of American tourists who had accidently eaten the parasite in a salad while on vacation in Jamaica in early 2000. While the victims eventually recovered, the story was seen by a large number of viewers. In late 2008 in Hawai‘i, two unrelated individuals had eaten insufficiently washed local produce and had developed the serious eosinophilic meningitis that results from ingesting harmful quantities of the parasite.[6] Finally, a November 2011 story in Australia covered the death of an infant who had apparently consumed the rat lungworm parasites while in the family garden.[7] The parents of the child were angry with public officials because they did not do more to notify the public of the potential risk in Sydney neighborhoods.

Alerting the public to the presence of a very rare non-communicable yet potentially serious disease is a double-edge sword, especially if it causes them to worry about potential risk associated with their fresh produce. Statistically speaking, consumers in Hawai‘i are much more likely, for example, to get hit by a car while walking across a street than to be infected by the rat lungworm. At the same time, there is a significant push to eat more fresh green vegetables, start school gardens, and bring the vegetables grown into the classroom and/or cafeteria, and for residents and visitors to buy more locally-grown produce. Reducing the use of pesticides that might kill rats and slugs and snails, seems also to be a priority for many. Large media campaigns to inform all consumers (residents and visitors) about a relatively small risk in the local food supply could have a significant economic impact on local produce growers. Health officials continue to struggle with this delicate situation, though the families of those who have been harmed by rat lungworm generally believe that all consumers should be informed of the potential risk as they have seen at first hand the devastating impact of the parasite on a family member.

While public policy should be left to policy makers and implementers, general advice for consumers and food growers can be shared via a range of media. Regarding consumers, first, all vegetables should be inspected for signs of snail or slug damage and discarded if there is any damage. If the plant is intact, for leafy vegetables, such as lettuce, Swiss chard, and celery, all leaves should be pulled apart and all surfaces inspected. This is important because slugs and snails, especially very small juvenile ones, can be found living deep among the leaves and stalks of the plant. Simply rinsing off the outer leaves of produce and then chopping the entire plant, for example for a fresh salad, might just lead to dicing up a live slug or snail, which may (or may not) be infected with rat lungworm. Once the plant has been disassembled and thoroughly inspected, all surfaces should be rinsed and rubbed with drinkable, running, cool water. This is the most effective way to reduce risk, but it is not an absolute guarantee. If a water sanitizing or produce "cleaning" product is used, it may or may not reduce the risk of a slug, snail, or the nematodes themselves being on the food that has been prepared and is about to be served.

As rat lungworms originate in the lungs of rats and undergo part of their development in snails and slugs before being re-ingested by rats in order to complete their development,[3] the best way to control or eliminate the risk of infection is to control it at the source by killing the rats and controlling the snails and slugs in the production and processing areas. Unlike pesticides used against other crop and garden pests, which can be spread over an entire field or plant, pesticides for killing rats and slugs and snails are more targeted and come in bait forms. They are therefore generally less effective, and the baits can be highly susceptible to disintegrating in heavy rain. Thus, a methodical program to keep a farm or garden free of rats and slugs and snails needs to be in place.

Keywords

Angiostrongyliasis, Education, Eosinophilic meningitis, Farmers, Gardeners, General public, Hawaii, Prevention, Rat lungworm

Conflict of Interest

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Effects of Washing Produce Contaminated with the Snail and Slug Hosts of *Angiostrongylus cantonensis* with Three Common Household Solutions

Norine W. Yeung PhD; Kenneth A. Hayes PhD; and Robert H. Cowie PhD

Abstract

The emerging infectious disease angiostrongyliasis (rat lungworm disease) is caused by ingesting snails and slugs infected by the nematode Angiostrongylus cantonensis*. The definitive hosts of* A. cantonensis *are rats and the obligatory intermediate hosts are slugs and snails. Many cases result from accidentally ingesting infected snails or slugs on produce (eg, lettuce). This study assessed three readily available household products as washing solutions for removing snails and slugs from produce (romaine lettuce) to lower the probability of accidentally ingesting them. The solutions were acetic acid (vinegar), sodium hypochlorite (bleach), and sodium chloride (domestic salt). Snail and slug species known to be intermediate hosts and that are common in the Hawaiian Islands were used in the experiments: the alien snail* Succinea tenella*, the alien semi-slug* Parmarion martensi*, and the alien slugs* Veronicella cubensis *and* Deroceras laeve*. None of the products was any more effective than washing and rinsing with tap water alone. Most snails and slugs were removed after treatment but some remained on the lettuce even after washing and rinsing the produce. Only washing, rinsing, and then rinsing each leaf individually resulted in complete removal of all snails and slugs. The study did not address removal of any remaining slime left by the snails and slugs, nor did it address killing of worms.*

Keywords

Angiostrongyliasis, Eosinophilic meningitis, Food safety, Hawaii, Infectious disease, Lettuce, Postharvest produce, Rat lungworm

Introduction

Emerging infectious diseases are having an increasing impact on the global economy and on public health, brought about by a diversity of social, economic, and environmental factors.[1] Proper public health management, including management of hosts of parasites that cause disease, is a key component of the prevention of future proliferation of such diseases.

Infection by the nematode *Angiostrongylus cantonensis* (angiostrongyliasis) is one of the main causes of eosinophilic meningitis, the disease commonly called rat lungworm disease.[2,3] As a disease that is not widely known or understood by medical practitioners, and because mild cases may not be diagnosed and the aetiological agent therefore remains undetected, it may spread rapidly without being noticed.[4] Severe cases may result in long term neurological damage, coma, or death. The parasite and cases of the disease have been reported from many Pacific Islands as well as widely in southern and eastern Asia and elsewhere,[5] with recent outbreaks leading to considerable attention in Hawai'i.[6,7]

Rats are the definitive hosts and the intermediate hosts of the parasite are snails and slugs (henceforth "snails", as slugs are simply snails that have lost or internalized their shell through evolution). Humans become infected as accidental hosts by ingesting third stage *A. cantonensis* larvae carried by snails. As in the natural definitive rat host, these third stage larvae penetrate the intestine wall and enter the circulatory system, from where they cross into the central nervous system, eventually reaching the brain where they mature to the fifth (subadult) stage.[8] However, instead of returning to the circulatory system to mature and reproduce in the pulmonary artery, as in the rat, with newly hatched first stage larvae passing up the rat's trachea to be swallowed and expelled in the feces, these subadult worms move around within the human brain, eventually dying there. The resulting neurological damage within the brain, combined with the immunological response to the worms, especially the dead worms, causes eosinophilic meningitis.[8,9]

People can become infected by intentionally eating raw or under-cooked snails, which in some countries are considered a delicacy, notably Thailand[10] and China.[11] Other cases have involved acquiring the infection by ingesting a snail on a dare or for a bet.[12,13] More widely, however, infection results from accidental ingestion of raw snails, or paratenic hosts such as flatworms, on some types of produce.[14-17] New thrusts to eat more fruit and vegetables, grow your own, and buy produce from local growers, for instance at farmers markets, mean that it is important to increase public awareness of the possibility of being infected by this route and of how to reduce that possibility.

To prevent infection it is therefore important to ensure that produce is free of infected hosts. Careful washing of produce is recommended, especially of green leafy vegetables that grow in close proximity to places where snails live.[18,19] The aim of the present study was to test various commonly available household products to assess their efficacy, compared to tap water, as washing solutions for removing snails from produce, in the present case from romaine lettuce.

Methods

Snails

When snails are active, they produce mucus that allows them to adhere better to produce. Therefore, it was necessary that the experimental snails were active during the washing experiments. However, some of the snails were to be placed within the lettuce head such that it would not be possible to observe their activity. Preliminary experiments were therefore performed to determine the mean time from being placed on a lettuce head for the snails to become active. A fresh romaine lettuce head was used for each trial. Having been stored in a refrigerator to keep them fresh, lettuce heads were first allowed to reach room temperature (around 25 °C). The snails were then placed on top of the lettuce head and the time it took for all snails to become active was recorded. Snails were considered active

when their tentacles were out and they were crawling. Adults and juveniles of four gastropod species were used (Table 1), all of which are introduced agricultural, horticultural, or garden pests in Hawai'i and commonly encountered.[20,21] Each trial consisted of three snails from one of the eight categories (4 species, 2 age classes) and each trial was replicated three times (72 snails in total). The mean time to activity of the nine snails in each category was used in the washing experiments as the time from placing the snails in/on the lettuce head to the start of the washing treatment.

Table 1. Gastropod species used in the experiments, with age class and size.

Species	Juvenile	Adult
Veronicella cubensis (slug)	10-20 mm	21-40 mm
Succinea tenella (snail)	4-5 mm	6-7 mm
Deroceras leave (slug)	10-15 mm	16-25 mm
Parmarion martensi (semi-slug)[a]	10-20 mm	21-35 mm

[a]Semi-slugs have a rudimentary shell into which they are not able to retract

Washing Experiments

The three washing solutions used were sodium hypochlorite (0.09%), acetic acid (1%), and sodium chloride (3%), with tap water as the control. These concentrations were chosen because experimentally 0.09% sodium hypochlorite (equivalent to 1.5% household bleach) and 1% acetic acid both reduce infectivity of the closely related parasite *A. costaricensis* by 100%, and a 3% cooking salt solution inactivates other parasites.[22] Dilutions of all solutions were prepared with tap water.

Four 15 l tubs were used for the washing experiments, one per wash solution, with 7.5 l of each solution in each designated tub, maintained at room temperature (ca. 25 °C). Each of the eight snail categories was tested separately. As above, a fresh lettuce head was used for each trial. Three individuals of the same age class were placed on/in the lettuce head: one in the center of the lettuce head, one outside the center but among the inner leaves, and one on the outside of the lettuce head. Each trial was replicated three times. Thus, for each treatment 72 snails were used, for a total of 288 snails across the four treatments. For each trial, the lettuce head with the snails was submerged completely under the wash solution, allowing all areas of the produce to be exposed to the solution, and left to soak for 10 min. Since the lettuce heads tended to float, they were turned over after 5 min so that the entire head was thoroughly soaked. The lettuce head was then removed and rinsed under cold flowing tap water for 10 s (from top to base of lettuce). The lettuce was then turned upside down and shaken. The numbers of snails that fell off during the washing and rinsing and their locations were recorded. Each leaf was then removed individually to record snails that had not been removed. Lastly, each individual leaf was rinsed under cold running water and the snails still remaining recorded. Each snail and lettuce head was used for one trial only. Logistical regression (MiniTab 16) was used to determine how removal rates varied according to species, age class, location on produce, and wash solution.

Results

Complete removal of all snails was not achieved for any species using any of the wash solutions (Table 2). However, several solutions were successful in completely removing certain age classes of particular species after washing and rinsing (Table 2). Washing and rinsing with tap water was effective in completely removing juveniles of *Veronicella cubensis* and *Deroceras laeve* and both juveniles and adults of *Parmarion martensi*. Bleach was effective in completely removing adult *Succinea tenella* and juvenile *Veronicella cubensis*. Vinegar was only effective in completely removing juvenile *Veronicella cubensis*. Salt was effective in removing adult *Succinea tenella* and *Veronicella cubensis*. All (100%) snails were removed only after washing, rinsing the whole lettuce head under cold running tap water, and then washing each individual leaf under cold running tap water. Almost all the snails placed on the outside of the lettuce heads were removed by the washing procedure (Table 3); only seven of the 96 snails were not removed by the washing procedure: two in the tap water control, four in the sodium hypochlorite, and one in the sodium chloride treatments (Table 3). However,

Table 2. Percentage of snails removed by species and age class after washing only and after washing and rinsing.

Snail species	Age class	Percentage of snails removed							
		Tap Water		Bleach		Vinegar		Salt	
		After wash	After wash + rinse	After wash	After wash + rinse	After wash	After wash + rinse	After wash	After wash + rinse
Succinea tenella	Juvenile	44	78	22	56	33	56	44	67
Succinea tenella	Adult	22	33	22	100	44	89	33	100
Veronicella cubensis	Juvenile	11	100	22	100	33	100	0	100
Veronicella cubensis	Adult	33	78	33	89	22	89	33	67
Deroceras laeve	Juvenile	44	100	33	89	33	78	33	78
Deroceras laeve	Adult	22	89	33	67	33	78	33	89
Parmarion martensi	Juvenile	89	100	44	67	33	67	78	89
Parmarion martensi	Adult	67	100	56	67	67	67	44	78

Table 3. Numbers of snails removed by washing alone and numbers removed by washing and rinsing.

Location of Lettuce	Numbers of snails removed							
	Tap Water		Bleach		Vinegar		Salt	
	After wash	After wash + rinse	After wash	After wash + rinse	After wash	After wash + rinse	After wash	After wash + rinse
Center	2	17	0	15	2	10	1	13
Inner	8	22	2	20	11	23	6	22
Outter	22	24	20	24	24	24	23	24

these seven remaining snails were subsequently removed during the tap water rinsing process. In contrast, of the 96 snails in the center of the lettuce head, only five were removed by the washing procedure: two in the control, two in the acetic acid, and one in the sodium chloride treatments. Another 50 were removed during the subsequent tap water rinsing (Table 3) but this meant that 41 out of the 96 snails remained in the center of the lettuce head following the entire washing and rinsing procedure.

The results of the logistic regression indicated very few significant differences among species, adults and juveniles, or position on/in the lettuce head with regard to the likelihood of being removed. However, there were a small number of significant differences among categories regarding the probablity of being removed after only the washing: juvenile *Veronicella cubensis* ($P = .026$) and *Parmarion martensi* ($P = .043$), *Parmarion martensi* in the inner ($P = .001$) and outer ($P = .015$), but not center locations, and *Succinea tenella* ($P = .011$) on the outer location all had a higher probability of being removed than other snail/age class/position combination.

Discussion

The three experimental washing solutions were chosen as being readily available in most domestic residences, sodium hypochlorite (bleach), acetic acid (vinegar), and sodium chloride (cooking salt). Simply washing and rinsing with water was just as effective overall as washing and rinsing with any of the experimental solutions. The procedure was not perfect, as some snails did remain in the lettuce head following washing and rinsing.

Although a few snail species and age class combinations were identified by the logistic regression analysis as being more readily removed than most others, overall, there were no general trends. However, snails in the center of the lettuce heads tended to be less readily removed, presumably because the solutions did not fully penetrate to the center and/or they were less readily dislodged by washing and rinsing, being wedged more tightly between the leaves.

Numerous snail species can act as hosts of *A. cantonensis*[23] and they probably differ considerably in the ease with which they can be removed from produce by washing and/or rinsing. The fact that some individuals of all species were not removed remains a cause for consideration. Only just over half the snails placed in the center of the lettuce were removed by the washing and rinsing. Thus if each leaf were not washed and rinsed separately, snails would remain in the lettuce head. If the lettuce, or similar produce, were subsequently chopped up and prepared for consumption, these snails would go unnoticed. Even if the snails themselves were chopped up the worms would probably remain viable and infective for some time. This may be particularly important when blending raw produce for drinks.[24] During the experimental washing treatments, in particular the acetic acid and to a lesser extent the sodium chloride treatments, the snails produced a lot of slime. Ingestion of slime containing infective *A. cantonensis* on produce has been thought of as a possible way people could become infected, but is generally considered of minor significance.[25-27] Nonetheless, production of large amounts of slime, possibly containing infective larvae, during washing with these solutions further indicates that washing with water alone, although some slime may still remain, is the most appropriate treatment. This study did not address killing the worms.

Conflict of Interest

None of the authors identifies any conflict of interest.

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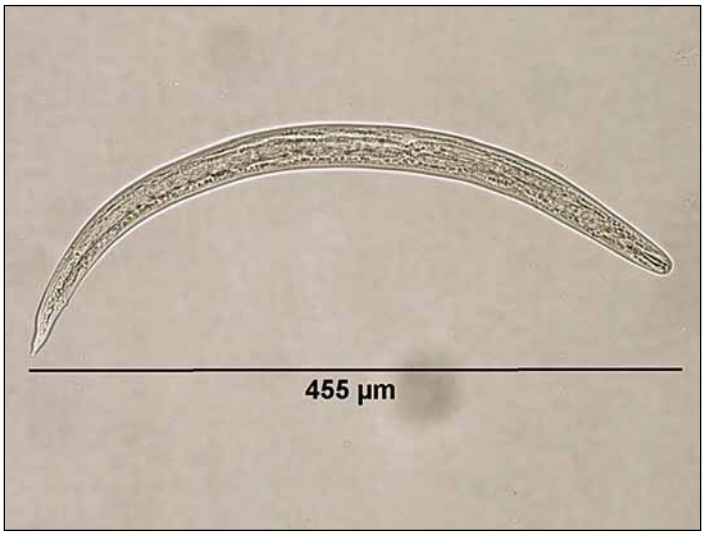
Newly hatched semi-slugs, Parmarion martensi. *These almost translucent, very small (approximately 4 mm long) semi-slugs could become hosts of infectious third stage larvae of* Angiostrongylus cantonensis *and could easily be overlooked on produce. [Photo: R.G. Hollingsworth]*

Two adult female Angiostrongylus cantonensis *isolated from a rat captured in Hawai'i. The worms are approximately 35 mm long. [Photo: CDC-DPDx]*



Third stage larva of Angiostrongylus cantonensis *isolated from* Parmarion martensi *collected in Hawai'i. [Photo:CDC-DPDx]*

Three color forms of the Caribbean slug Veronicella cubensis, *a known host of* Angiostrongylus cantonensis *in Hawai'i and elsewhere. The largest of the three slugs is about 6 cm long. [Photo: R. H. Cowie]*

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