Journal of Helminthology (2016) **90,** 737–741
doi:10.1017/S0022149X15001042

Levels of infection with the lungworm *Angiostrongylus cantonensis* in terrestrial snails from Thailand, with *Cryptozona siamensis* as a new intermediate host

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(Received 27 August 2015; Accepted 5 November 2015; First Published Online 15 January 2016)

Abstract

Angiostrongylus cantonensis is primarily considered an emerging infectious agent of eosinophilic meningitis or meningoencephalitis with a worldwide distribution. Rodents and snails are important invasive hosts for transmission and expansion of *A. cantonensis*. The objective of this study was to investigate infection levels of *A. cantonensis* in snails, the most important natural intermediate host. Our study location was Mueang Kamphaeng Phet district, Kamphaeng Phet Province, and was undertaken between October and December 2012. A total of 2228 freshwater and terrestrial snails were collected, comprising 1119 *Filopaludina* spp., 409 *Pomacea caniculata*, 275 *Achatina fulica* and 425 *Cryptozona siamensis*. *Angiostrongylus* larvae were isolated by artificial digestion methods following Baermann's techniques. A low prevalence and intensity of *A. cantonensis* were observed in *A. fulica*, while higher numbers were found in *C. siamensis*. None of the *Filopaludina* spp. and *Pomacea caniculata* were infected with *A. cantonensis*. Molecular characterization was performed by analysing the 264 bp of cytochrome *c* oxidase subunit I (COI). Three COI sequences of *Angiostrongylus* were identical to *A. cantonensis* with 91–99% identity. *Cryptozona siamensis* has not previously been recorded as an intermediate host for *A. cantonensis* in Thailand. The infection of *A. cantonensis* identified in the natural intermediate hosts is new and important information to assist in the prevention and control of human angiostrongyliasis.

Introduction

Angiostrongylus cantonensis or the rat lungworm is the causative agent of human angiostrongyliasis. It is known to be a cause of eosinophilic meningitis and eosinophilic meningoencephalitis. Human angiostrongyliasis has been reported in more than 30 countries on all continents, but especially in Taiwan, China and Thailand (Wang *et al.*, 2008). Recently, infections in both natural hosts and human cases have been reported from North and South America (Maldonado *et al.*, 2010; Thiengo *et al.*, 2010; Espírito-Santo *et al.*, 2013; Teem *et al.*, 2013). In general, humans are an accidental host where the worms develop into the young adult stage without being able to develop into adult stages. Infection with *A. cantonensis* occurs following the ingestion of improperly cooked food from intermediate hosts such as *Pila* snails and *Filopaludina*

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snails, or from eating improperly cooked food from paratenic hosts such as shrimp, crab and yellow tree monitor, or from eating vegetables contaminated with infective larvae. In Thailand, the infection in patients usually occurs in communities that eat raw or undercooked *Pila* snails, often as an accompaniment to drinking alcohol (Eamsobhana, 2013). The most obvious clinical symptoms are severe headache and neck stiffness. Diagnosis can be made by several methods, such as knowing the history of eating intermediate or paratenic hosts, laboratory findings and clinical symptoms (Wang *et al.*, 2008; Vitta, 2012). There are no specific drugs available for the treatment of this infection.

Angiostrongylus cantonensis requires both an intermediate and a definitive host to complete its life cycle. Previously, gastropods from 44 families, 99 genera and 140 species, identified as intermediate hosts, naturally or experimentally infected with *A. cantonensis*, were found to have worldwide distribution (Kim *et al.*, 2014). In Thailand specifically, several species of freshwater snails have been reported as natural intermediate hosts, namely, *Pila ampullacea*, *P. polita, P. gracilis, P. scutata, P. pesmei, P. turbinis*, *Pomacea canaliculata, Clea helena, Bithynia siamensis goniomphalos, Melanoides tuberculata, Filopaludina martensi martensi* and *F. sumartrensis polygramma* (Punyagupta, 1965; Crook *et al.*, 1968; Setasubun *et al.*, 1968; Pipitgool *et al.*, 1997; Tesana *et al.*, 2009). The terrestrial snails and slugs included *Achatina fulica, Hemiplecta distincta, H. siamensis, Macrochlamys* (*Sarika*) *resplendens* and *Veronicella siamensis,* all of which have been documented as natural intermediate hosts for the rat lungworm in Thailand, including *Cryptozona siamensis* as a new intermediate host (Crook *et al.*, 1968; Pipitgool *et al.*, 1997; Tesana *et al.*, 2009; Vitta *et al.*, 2011). Rodents are definitive hosts for *A. cantonensis*. Five species of rat, namely *Rattus rattus, R. exulans, R. norvegicus, Bandicota indica* and *B. bengalensis*, have been found to be naturally infected with *A. cantonensis* (Setasubun *et al.*, 1968; Namue & Wongsawad, 1997; Pipitgool *et al.*, 1997; Vitta *et al.*, 2011). Paratenic hosts observed naturally were the reptile yellow tree monitor (*Varanus bengalensis*) (Radomyos *et al.*, 1994), prawns and the clawed frog (*Xenopus laevis*) and its tadpoles (Cross & Chen, 2007).

The high susceptibility of its hosts is considered to be an important factor in the distribution of *A. cantonensis* throughout these areas. Thus the aim of the present study was to investigate the infection levels of *A. cantonensis* in natural intermediate hosts from Kamphaeng Phet Province, Thailand. As part of this survey, *Cryptozona siamensis*, a terrestrial snail, was collected and helminth larvae were isolated. This snail is found in several regions of Thailand because the environment is suitable for its development. Molecular characterization of the *A. cantonensis* worm was also performed to confirm the morphological identification.

Materials and methods

Collection of larval nematodes from snails

Freshwater snails *Filopaludina* spp. and *Pomacea caniculata* and terrestrial snails *Achatina fulica* and *Cryptozona siamensis* were collected randomly in the Trai Trueng sub-district, Mueang Kamphaeng Phet district, Kamphaeng Phet Province, between October and December 2012. Snails were maintained in aerated boxes and transported to the Department of Microbiology and Parasitology at the Faculty of Medical Science, Naresuan University, where they were fed on lettuce leaves for a short time in the laboratory. The shell of each snail was cracked open with a hard stone and the entire soft parts removed, dissected and minced in a blender with 0.7% pepsin solution containing 7 g of pepsin powder (Acros Organics, Geel, Belgium), 10 ml of HCl and 990 ml of distilled water. The snail contents and pepsin solution were then incubated in a water bath at 37°C for 90 min. The Baermann technique was used for isolating the *Angiostrongylus* larvae, which were identified morphologically by the following characteristics: possessing a long, thin body with two chitinous rods at the anterior end, transverse striations on the cuticle and a cone-shaped, slightly curved and pointed tail (Eamsobhana, 2006).

DNA extraction

Genomic DNA was extracted from the larvae. Approximately 50 larvae were put in a 1.5-ml microcentrifuge tube containing 5 µl ATL buffer (Qiagen, Germany). The worms were ground with a sterile tip. Another 45 µl of ATL buffer was added. The tube was vortexed vigorously for about 1 min. The lysate was frozen at −20°C for 1 h, thawed at room temperature and digested with 15 µl of 10 mg/ml proteinase K. The mixture was incubated at 65°C overnight and then at 95°C for 1 h. Subsequently, the tube was centrifuged at 12,000 rpm for 2 min. The supernatant containing DNA was precipitated with 1/10 volume of 5 M NaCl followed by 2 volumes of cool absolute ethanol and centrifuged at 12,000 rpm for 30 min. The pellet was washed by centrifugation with 100 µl 70% ethanol, dried in air and dissolved with 15 µl of distilled water. One microlitre of purified genomic DNA was examined on 0.8% agarose gel electrophoresis running in 0.5 × TBE (Tris–borate–EDTA) buffer at 100 V. The gel was stained with 10 µg/ml ethidium bromide for 1 min and destained with distilled water for 30 min. The DNA band was visualized and compared to a 100 bp molecular size marker (GeneDireX, Taoyuan, Taiwan). The DNA was kept at −20°C until used.

Molecular analysis

The polymerase chain reaction (PCR) was performed to amplify a partial region of cytochrome *c* oxidase subunit I (COI) according to Jefferies *et al.* (2009). Specific primers were: CO1_F 5′-TAAAGAAAGAACATAATGAAAATG-3′ and CO1_R 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′. The PCR reaction was carried out in 30 µl volumes which consisted of 3 µl of 10 × buffer, 4.2 µl of 25 mM $MgCl_2$, 0.6 µl of 200 mM deoxynucleoside triphosphates (dNTPs), 1.2 µl of of each primer (5 µM), 0.6 µl of 100 units *Taq* DNA polymerase (Sigma, St. Louis, Missouri, USA), 11.7 µl of distilled water and 7.5 µl of DNA template. PCR was performed using a thermal cycler (Applied Biosystems Life Technologies, Carlsbad, California, USA) with an initial denaturation at 94°C for 5 min; followed by

https://doi.org/10.1017/S0022149X15001042 Published online by Cambridge University Press

30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s and extension at 72°C for 1 min; and a final extension at 72°C for 5 min. One microlitre of PCR products was analysed by 1.2% agarose gel electrophoresis in 0.5 × TBE buffer at 100 V and compared to a 100 bp standard ladder. The gels were stained with ethidium bromide for 1 min and destained with distilled water for 30 min. The bands were visualized and photographed under UV light.

PCR products were purified using a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd, Taiwan). The PCR product (30 µl) was transferred into a 1.5-ml microcentrifuge tube. Five volumes of Gel/PCR buffer were added to the tube and vortexed. The mixture was then transferred into a DFH column in a 2-ml collection tube and centrifuged at 12,000 rpm for 30 s and the flow-through was discarded. The tube containing the DFH column was centrifuged at 12,000 rpm for 3 min to dry the column matrix. The DFH column was transferred to a new 1.5-ml microcentrifuge tube. Twenty microlitres of elution buffer were added into the centre of the column matrix. The tube was allowed to stand for 2 min in order to completely absorb the elution buffer. To elute the purified DNA, the tube was then centrifuged at 12,000 rpm for 2 min. The purified PCR product was checked by running 1.2% agarose gel electrophoresis in 0.5 × TBE buffer at 100 V and comparing to a 100 bp standard ladder. The gels were stained with ethidium bromide for 1 min, destained with distilled water for 30 min and visualized and photographed under UV light. Purified PCR product was sent to Korea for sequencing by Macrogen Inc. (Korea).

To characterize species of *Angiostrongylus*, a BLASTN search against a nucleotide database was performed (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All sequences were edited using SegManII software (DNASTAR, Madison, Wisconsin, USA). A 264 bp region of the COI gene was used for analysing the species, using ClustalW (Thompson *et al.*, 1994) and MEGA software version 5.0 (Tamura *et al.*, 2011).

Results

Infection levels of Angiostrongylus cantonensis *in snails*

Of the freshwater snails, a total of 1119 *Filopaludina* spp. and 409 *P. caniculata* were collected but neither snail species was infected, in contrast to both the terrestrial species, *A. fulica* and *C. siamensis*. Nevertheless the majority of these two terrestrial species were free of infection with *A. cantonensis*, and up to 3 of 275 (1.1%) of *A. fulica* were infected with a range of 5–15 larvae, representing an average intensity of 0.1. Higher levels of infection were found in *C. siamensis*, where 45 of 425 (10.6%) snails were infected with a range of 1–4858 larvae. An average intensity of 23.0 was recorded, where larval numbers of 1–10 and 11–100 were found in 3.5% and 4.2% of infected snails, respectively.

Molecular characterization of Angiostrongylus cantonensis

A partial region of COI sequence from *Angiostrongylus* larvae from *C. siamensis* was determined by PCR and sequenced. PCR products were approximately 450 bp in size when compared with the standard DNA ladder. Two COI sequences of *Angiostrongylus* larvae collected from two *C. siamensis* and one COI sequence from an adult worm were obtained. After a BLASTN search of the trimmed sequence of 264 bp, the two sequences from larvae were found to be closely related to *A. cantonensis* from Miyagi, Sendai, Japan (GenBank accession number AB684375) with 91–92% identity, and the sequence of the adult worm was 99% identical to *A. cantonensis* from Bangkok, Thailand (GenBank accession number AB684368).

Discussion

Based on both morphological and molecular characterization of the larvae, *A. cantonensis* was identified and then isolated from *A. fulica* and *C. siamensis*. We demonstrated the prevalence and intensity of *A. cantonensis* in *C. siamensis* collected from Kamphaeng Phet Province. Having not been previously reported, this land snail is considered to be a novel intermediate host for this worm in Thailand. At present, over 140 species of molluscs have been reported as natural or experimental intermediate hosts for *A. cantonensis* (Kim *et al.*, 2014). This indicates that *A. cantonensis* occurs in a wide range of intermediate hosts. The terrestrial snail is also distributed in Laos, Cambodia and Malaysia. Two species of this genus, *Cryptozona imperator* and *Cryptozona bistrialis*, have previously been reported as hosts of *A. cantonensis* (Ko, 1991; Cross & Chen, 2007). This terrestrial snail can transmit larvae to natural hosts when eaten and, although not a favourite food for Thai people, the snail is found in close proximity to humans in agricultural areas, thereby being a source of infection.

The low prevalence of *A. cantonensis* in *A. fulica* was shown in the present study, which is in contrast to previous studies in which a high prevalence of *A. cantonensis* in *A. fulica* collected from Thailand was recorded. The high level of prevalence previously reported ranged from 36.4% to 94.4% (Harinasuta *et al.*, 1965; Setasubun *et al.*, 1968; Pipitgool *et al.*, 1997). The prevalence of *A. cantonensis* in *A. fulica* in this study was lower than that found by Vitta *et al.* (2011) (12.38%) and Tesana *et al.* (2009) (7.55%).

Achatina fulica is considered to be an important intermediate host for transmission of *A. cantonensis*. Recently, this giant African land snail was introduced to Brazil and was considered as the source of the spread of human angiostrongyliasis (Graeff-Teixeira, 2007). On the other hand, freshwater snails, namely *P. polita*, *F. martensi martensi*, *F. sumatrensis polygramma*, *P. pesmei*, *C. helena* and *B. siamensis goniomphalos* have been reported to have a low prevalence of less than 5% (Setasubun *et al.*, 1968; Pipitgool *et al.*, 1997; Tesana *et al.*, 2009), while *P. ampullacea* was negative for larval infection (Pipitgool *et al.*, 1997). Our results agree with those above, as no *P. canliculta* and *Filopaludina* spp. specimens in our sample were infected with *A. cantonensis*. This may be due to these two snails being hosts with low susceptibility.

The average intensity of *A. cantonensis* in *C. siamensis* was 23 larvae/snail and the highest intensity was 4858

https://doi.org/10.1017/S0022149X15001042 Published online by Cambridge University Press

larvae in one snail. It is possible that larvae of *A. cantonensis* are restricted to the faecal pellets of the definitive host. In addition the dispersion of the larval stage within the snail is typically overdispersed or aggregated within the host population and likely to conform to a negative binomial distribution, where only a small number of snail hosts are infected with high numbers of larvae (Pal & Lewis, 2004). This was also observed in several species of parasites, such as *A. cantonensis* in intermediate hosts (Tesana *et al.*, 2009) and *Opisthorchis viverrini* in secondary intermediate hosts (Haswell-Elkins *et al.*, 1991).

In conclusion, the present study has recorded a new, previously unidentified, intermediate host for *A. cantonensis* in Thailand. The terrestrial snail *C. siamensis* should be included in the list of intermediate hosts for *A. cantonensis*. Prevalence and intensity of *A. cantonensis* infective larvae were reported to be low in the studied area. However, this investigation provides us with basic knowledge about the biology of *A. cantonensis* which is new and important information to assist in the prevention and control of human angiostrongyliasis.

Acknowledgements

We would like to thank the Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Thailand for provision of facilities. We are very grateful to Mr Roy Irvine Morien, Naresuan University Language Centre (NULC), Naresuan University, for assistance in editing the English of the manuscript.

Financial support

We would like to thank the Thailand Research Fund for financial support (grant number MRG5580044).

Conflict of interest

None.

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