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Invasive *Pomacea* snails as important intermediate hosts of *Angiostrongylus cantonensis* in Laos, Cambodia and Vietnam: Implications for outbreaks of eosinophilic meningitis

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ABSTRACT

The rat lungworm *Angiostrongylus cantonensis* causes human eosinophilic meningitis and it is endemic in Southeast Asia, but little is known about its distribution in Laos, Cambodia and Vietnam. We conducted a multi-country survey for *A. cantonensis* in these countries to estimate its prevalence in snails along the Mekong River and the east coast of Vietnam. We identified *Angiostrongylus* species by morphological and molecular analysis. We found *A. cantonensis* in the invasive snail, *Pomacea* spp. The wide accessibility of *Pomacea* snails, along with their infection by *A. cantonensis*, indicates that this snail species could be used in surveillance for preventing outbreaks of eosinophilic meningitis.

1. Introduction

Angiostrongylus cantonensis is the causative agent of human cerebral angiostrongyliasis, most commonly manifested as eosinophilic meningitis. This helminth appears to have originated in Southeast Asia and has spread to many parts of the world, including Australia, Africa and the Americas. Humans acquire infection through ingestion of snails and other food sources that are contaminated with third stage larvae of *A. cantonensis*. In Asia, snail species commonly involved include both freshwater snails (e.g., species of *Pila* and *Pomacea*) and terrestrial snails (e.g., *Achatina fulica*). Notably, *Pomacea* spp., initially introduced from South America as a human food resource, became invasive in East and Southeast Asia, coincident with the emergence of angiostrongyliasis in some areas (Lv et al., 2009a, 2008). Although eosinophilic meningitis is commonly reported in Thailand and southern China (Lv et al., 2010), there are fewer reports Laos, Cambodia and Vietnam. Nevertheless, *A. cantonensis* may be an important cause of eosinophilic meningitis in the region, especially when considering its global range expansion (McBride et al., 2017; Xuan et al., 2007).

Angiostrongyliasis is a rare disease, and the most efficient means to assess for active transmission is through direct sampling of the intermediate hosts (snails) that are required for completion of the helminth life cycle. To investigate the species of infected snails and their helminth burden in this region, we conducted a survey in three countries, investigating the prevalence of *A. cantonensis* in freshwater snail populations.

2. Methods

2.1. Field survey

Three countries (Laos, Cambodia and Vietnam) were covered in the study area. The criteria for choosing sampling sites were: 1) the sampling sites were located along the Mekong River and the eastern coast of Vietnam in densely populated areas at low elevation, where the potential for transmission of *A. cantonensis* is likely to be greatest; 2) the sampling sites were distributed evenly in the study region; 3) the sampling sites were villages with poor sanitation, which could facilitate the life cycle of *A. cantonensis*. Each site consisted of a few villages where snails were collected, with a target of 50 snails per site (based on an expected prevalence of 1.2% (Lv et al., 2009a), to achieve an uncertainty interval of ± 3%). All target snail populations were close to

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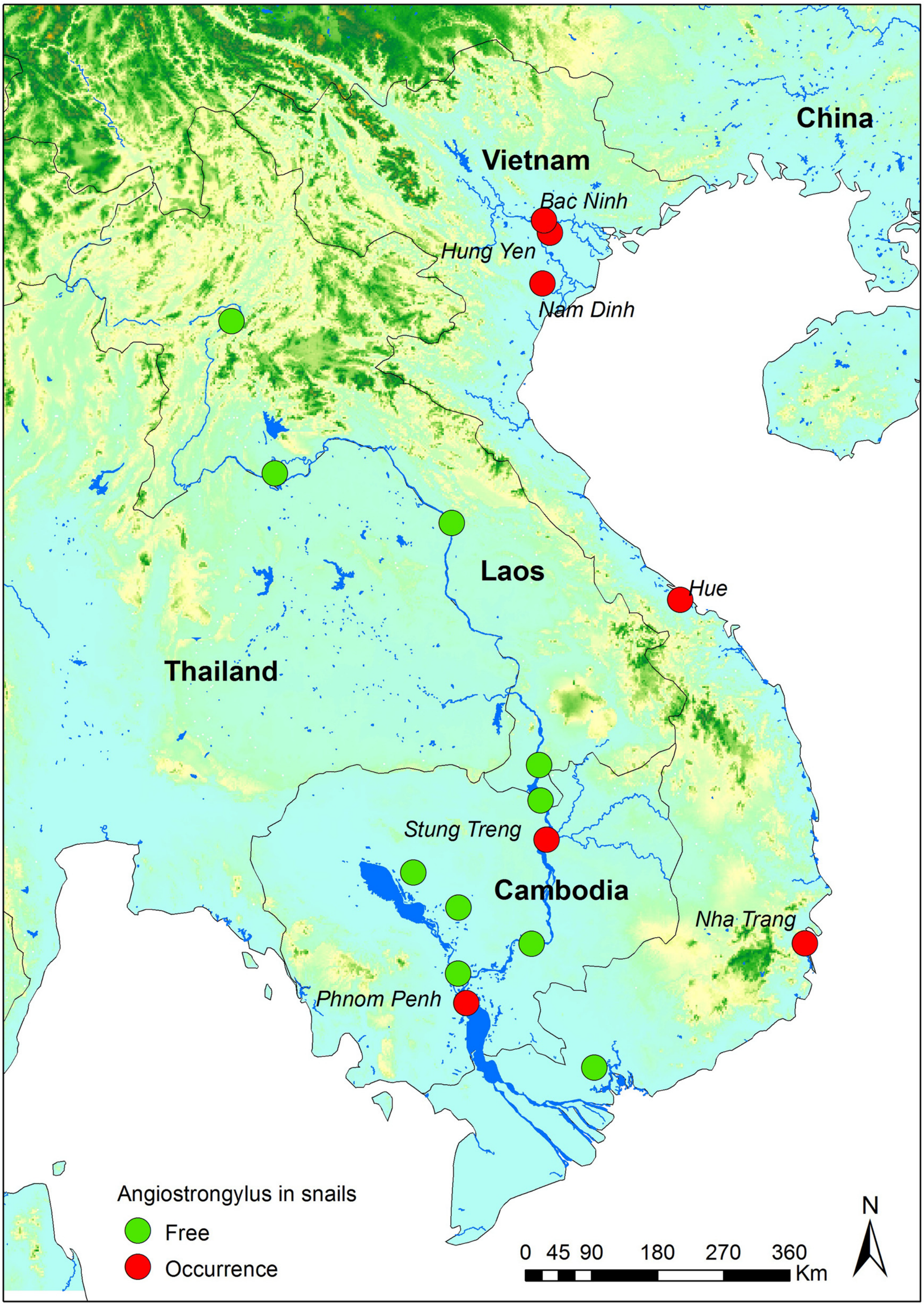

Fig. 1. The distribution of *Angiostrongylus cantonensis* in Laos, Cambodia and Vietnam. Sites in which infected snails were found are in italics.

residential settings since the definitive hosts of *A. cantonensis* are commensal *Rattus* species. The environments for collecting freshwater snails were ponds, rice fields, channels and streams. In two sites (in Laos and Cambodia), the snails were bought from local sellers who collected snails nearby, which could be part of the chain of transmission.

2.2. Snail examination

Snail species were identified to the genus level by shell morphology. The lung or mantle cavity of *Pomacea* and *Pila* snails was separated from the soft body. The cavity was then opened and examined under the microscope for nodules on the lung wall containing *A. cantonensis* larvae. Infection was confirmed if a nematode larva emerged from any nodule pricked by needle. The larvae can be identified preliminarily according to the morphology and behavior described in detail by Lv et al. (2009b).

2.3. Parasite identification

To confirm the *Angiostrongylus* species, we infected lab rats with the third-stage larvae harvested from the *Pomacea* snails. Four *A. cantonensis* isolates from Hung Yen (Vietnam), Bac Ninh (Vietnam), Hue (Vietnam), and Phnom Penh (Cambodia) were established in the lab. Four or five female adult worms from each isolate were randomly selected for DNA extraction. PCR targeting of the complete cytochrome c oxidase subunit I (*cox*1) marker was performed by two pairs of primers. COI_F1: 5′-GGTGATTATAATGTTTAATG-3′, COI_R1: 5′-CGTAGGAACCGCAATAAC-3′; COI_F2: 5′-TATGGTTTATGCTATTTTAAG-3′, COI_R2: 5′-GGCACTACACAACGATTATC-3′. The PCR products were sequenced using an ABI 3730xl DNA Analyzer (Applied Biosystems, USA). The sequences were visualized and edited in Vector NTI v.9.1.0 (Invitrogen Corp., USA).

A phylogeny based on Bayesian inference was generated using MrBayes v. 3.2. The sequences used in this study included JX268542 (Avas), GQ398122 (Acos), KT947979 (Amal), AB684358 (ac1), AB684364 (ac2), AB684367 (ac3), AB684368 (ac4), AB684369 (ac5), AB684374 (ac6), AB684375 (ac7), HQ440217 (ac8), JX471055 (ac9), KU532147(ac10), KU532143 (ac11), KU532148 (ac12), KU532146 (ac13), KY779735 (HY; present study), KY779736 (PP; present study), KY779737 (HUE; present study), KY779738 (BN; present study). The species of *Angiostrongylus* was identified by comparing to known sequences.

3. Results

We visited 17 sites along the Mekong River and eastern coast in Laos, Cambodia and Vietnam (Fig. 1) and collected freshwater snails, both *Pomacea* and *Pila*. *Pomacea* was widely distributed in the study region. Fifteen out of the 17 sites were infested with *Pomacea* (Table 1). In contrast, the native species of *Pila* was mainly distributed in the lower reaches of the Mekong River, particularly in Cambodia.

We examined 1365 snails for the presence of *A. cantonensis*. We found that 41% (7 of 17) of sites were positive for *A. cantonensis;* no infected snails were found in Laos. Among the *Pomacea* snails, 2.5% (32/1291) were infected, with prevalence ranging from 1.3–16.2% in the seven collecting sites in Cambodia and Vietnam in which any infected snail was found. We did not find *A. cantonensis* in any *Pila* snails.

We observed significant sequence difference among the four isolates, although all sequences within a single collecting site were identical. All haplotypes from this study fall in the known clades of *A. cantonensis* identified in previous studies (Rodpai et al., 2016; Tokiwa et al., 2012), indicating that the specimens were *A. cantonensis* (Fig. 2).

4. Discussion

Our study found that the invasive snails, *Pomacea* spp., which have spread throughout Southeast Asia, are important intermediate hosts for *A. cantonensis* in Cambodia, Laos and Vietnam. We found multiple sites in Cambodia and Vietnam with infected *Pomacea* snails, which highlights the health concern regarding eosinophilic meningitis caused by consumption of these snails. *Pomacea,* introduced from South America around 1980, has been a notorious invasive species in freshwater ecosystems in East and Southeast Asia (Joshi and Sebastian, 2006). It was first confirmed as an intermediate host of *A. cantonensis* in Taiwan in 1986 (Nishimura et al., 1986) and became the leading intermediate host in China (Lv et al., 2009a, 2008). Eleven out of 15 major outbreaks recorded in East and Southeast Asia have been attributed to *Pomacea* (Odermatt et al., 2010). In view of this wide accessibility of *Pomacea* snails in the study area, we recommend that the extent of consumption of *Pomacea* snails be assessed and that hospital-based surveillance be implemented to detect potential outbreaks of eosinophilic meningitis.

Our findings extend knowledge of the geographical distribution of *A. cantonensis* in Southeast Asia. Although *A. cantonensis* was reported in rats and *Achatina fulica* in Cambodia and Vietnam as early as the 1960s, few surveys of the presence of the parasite in animals have been published. Our finding of *A. cantonensis* in multiple areas suggests a broader geographical distribution in these countries than has been previously recognized. We did not find any infections in snail populations in landlocked Laos. By contrast, *A. cantonensis* was present in 5 of 6 sites in Vietnam, most of which were coastal or near rivers near to the coast. This finding implies that *A. cantonensis* is likely to become established in low elevation aquatic environments.

Table 1
Sampling sites for snails and prevalence of *A. cantonensis* within *Pomacea* snails in Laos, Cambodia and Vietnam.

Sites	Number of villages	Number of snails screened		Number (%) of *Pomacea* infected	Larval burden[a]
		Pomacea	*Pila*		
Vientiane, Laos	2	54	0	0 (0)	
Luang Prabang, Laos	2	99	0	0 (0)	
Thaknek, Laos	3	68	8	0 (0)	
Mid-Paske-Island, Laos	1	19	1	0 (0)	
Som Island, Laos	1	34	1	0 (0)	
Stung Treng, Cambodia	2	71	18	2 (2.8)	L(2)
Krakie, Cambodia	1	10	0	0 (0)	
Siem Reap, Cambodia	1	0	30	0 (0)	
Kampong Thom, Cambodia	1	0	16	0 (0)	
Kampang Cham, Cambodia	1	36	0	0 (0)	
Phnom Penh, Cambodia	4	159	0	3 (1.9)	H(1[b]), L(2)
Saigon, Vietnam	3	118	0	0 (0)	
Nha Trang, Vietnam	3	153	0	2 (1.3)	
Hue, Vietnam	3	217	0	6 (2.8)	H(5[b]), M(1)
Nam Dinh, Vietnam	4	105	0	4 (3.8)	L(4)
Hung Yen, Vietnam	1	74	0	12 (16.2)	H(4[b]), M(3), L(5)
Bac Ninh, Vietnam	1	74	0	3 (4.1)	M(2[b]), L(1)
Total	34	1291	74	32(2.5)	

[a] Worm burden based on larval nodules visualized on the lung wall. H(high) denotes more than 10 nodules, M(middle) 6–10, L(low) 1–5. The number of snails are included in the bracket.

[b] Individual snails were found to harbor more than 100 larvae after digestion in artificial gastric juice (0.2% pepsin in 0.7% hydrochloric acid).

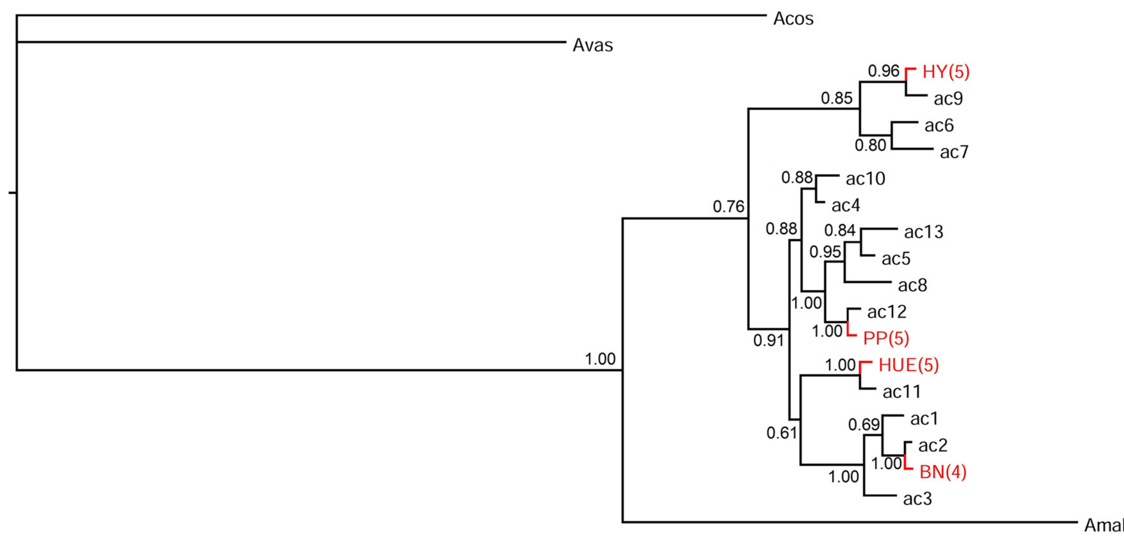

Fig. 2. Phylogenetic tree based on Bayesian inference.
Numbers at node branches indicate the Bayesian support for the node. HY: Hung Yen, PP: Phnom Penh, HUE: Hue, BN: Bac Ninh. Acos: *Angiostrongylus costaricensis*, Avas: *Angiostrongylus vasorum*, Amal: *Angiostrongylus malaysiensis*. Codes ac1-13 indicate the unique clades of *Angiostrongylus cantonensis* identified in previous studies. The isolates are highlighted, followed by the number of specimens in brackets.

Pila species are native apple snails in Southeast Asia and also play an important role in transmission of *A. cantonensis* to humans (Eamsobhana, 2014). Although *Pila* has been found to be more susceptible to *A. cantonensis* than *Pomacea* (Tesana et al., 2008), the native species is being replaced by *Pomacea* because of the latter's striking ecological adaptability (Chaichana and Sumpan, 2015). *Pomacea* may therefore be of increasing importance in this region. Additionally, *Pomacea* may be a useful index of occurrence of *A. cantonensis* for several reasons. First, it is much easier to find and collect *Pomacea* than other snails because of its highly visible bright pink eggs that are laid above the water surface on emergent hard surfaces. Second, a special detection technique for *Angiostrongylus* in *Pomacea* has been described (Lv et al., 2009a,b). The microscopic discovery of larval nodules on the wall of snail lung or mantle cavity has proven to be a sensitive indicator of infection, particularly in mild infections. Together, these properties and the findings of this study indicate that surveillance for *Pomacea* snails may be valuable for monitoring the risk posed by this pathogen in the region.

These findings should be understood within the limitations of the study design. First, the convenience-based sampling method precluded investigation of associations between occurrence of *A. cantonensis* and environmental factors. Second, we did not have data concerning human cases of eosinophilic meningitis in the region to determine whether these findings correlate with local risk of human disease. Third, we used morphologic diagnosis of *A. cantonensis* rather than PCR, due to the latter being unavailable in the field, and our results therefore may underestimate prevalence. Finally, our original snail sample size target was not met in several villages due to flooding. Additional sampling and phylogenetic analysis may further elucidate the distribution patterns *A. cantonensis* in this region and determine whether other species, such as *A. malaysiensis,* are present in this region.

In conclusion, our findings demonstrated that invasive *Pomacea* snails are playing a key role in the lifecycle of *A. cantonensis* in Cambodia and Vietnam, indicating that this snail species could be used as a routine risk assessment indicator for eosinophilic meningitis. Surveillance to detect potential outbreaks of eosinophilic meningitis caused by consumption of *Pomacea* snails should be strengthened.

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