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REVIEW

Genetic Characterization and Detection of *Angiostrongylus cantonensis* by Molecular Approaches

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Abstract

Angiostrongylus cantonensis constitutes a major etiologic agent of eosinophilic meningoencephalitis. The detection methods for angiostrongyliasis mainly depend on morphology or immunology. A firmer diagnosis could be reached by directly detecting the parasite in the cerebrospinal fluid or through laboratory assays that are specific for *Angiostrongylus*-induced antibodies or the parasite's DNA. *A. cantonensis* detection could be carried out by larva release from the tissue upon pepsin digestion. However, the procedure requires live mollusks, which might complicate the analysis of large amounts of samples. Since morphological assays are limited, multiple molecular techniques have been put forward for detecting *A. cantonensis*, including PCR amplification of targets followed by fragment length or DNA sequence analysis. This allows rapid and accurate identification of *A. cantonensis* for efficient infection management and epidemiological purposes. In this study, we reviewed the current methods, concepts, and applications of molecular approaches to better understand the genetic characterization, molecular detection methods, and practical application of molecular detection in *A. cantonensis*.

Keywords: *Angiostrongylus cantonensis*, genetic characterization, molecular detection methods, detection

Introduction

Angiostrongylus cantonensis, also called rat lungworm, represents an important pathogen responsible for a zoonotic disease affecting humans (accidental host), rats (definitive host), and freshwater snails (intermediate hosts) (Himsworth et al. 2013, Barratt et al. 2016, Huang et al. 2019, Lima et al. 2020). This nematode causes angiostrongyliasis with the prominent symptom of eosinophilic meningoencephalitis in humans, which involves elevated cerebrospinal fluid (CSF) eosinophil amounts (Sawanyawisuth et al. 2012, Tsai et al. 2012, Aghazadeh et al. 2015a, Wang et al. 2020, Zhou et al. 2020).

Angiostrongyliasis-related symptoms result from the presence of larvae or developing worms and host response in the brain. Dead or dying worms in the central nervous system (CNS) can induce inflammation and cause disease (Cross and Chen 2007, Barratt et al. 2016). Major signs of eosinophilic meningitis include serious headache, neck stiffness, paresthesia, and gastrointestinal tract symptoms such as vomiting and nausea (Wilkins et al. 2013, Ansdell and Wattanagoon 2018). Moreover, encephalitic angiostrongyliasis can be fatal (Sawanyawisuth et al. 2009, Wang et al. 2012, Cowie et al. 2017).

Clinical detection of angiostrongyliasis remains challenging, including both patient examination and laboratory

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assessment of Angiostrongylus-specific antibodies or parasite DNA (Eamsobhana et al. 2018, Graeff-Teixeira et al. 2018, Somboonpatarakun et al. 2019, 2020). It is uncommon to detect *A. cantonensis* larvae in the CSF (Kuberski and Wallace 1979, Punyagupta et al. 1990, Prociv et al. 2000, Chye et al. 2004, Graeff-Teixeira et al. 2009). High circulating content of eosinophils is an indication of this disease, but is not definitive (Lv et al. 2017b). Computed tomography and magnetic resonance imaging could help detect brain lesions, but do not provide differential diagnosis (Kanpittaya et al. 2000, Tsai et al. 2003, Jin et al. 2005).

Researchers have examined the impact of *A. cantonensis* on human health by studying the epidemiology, morphology, pathology, diagnosis, and treatment of the associated disease (Kwon et al. 2013, Martin-Alonso et al. 2015, Aghazadeh et al. 2016, Chen et al. 2016, Waugh et al. 2016). In this review, we presented the current approaches and issues surrounding the genetic characterization of and molecular detection methods for *A. cantonensis*, as well as the practical application of molecular detection.

Genotyping of *A. cantonensis*

Ribosomal DNA sequences

A ribosomal DNA (rDNA) represents a DNA sequence encoding ribosomal RNA (rRNA). The rDNA in eukaryotic cells comprises a tandem repeat of a single operon encompassing NTS, ETS, 18S, ITS1, 5.8S, ITS2, and 28S tracts (Alvarez and Wendel 2003). The various coding regions of these rDNA repeats frequently indicate distinct evolutionary levels. This difference subsequently provides phylogenetic information of species that belong to multiple systematic levels (Hillis and Dixon 1991, Salim and Gerton 2019).

Internal transcribed spacer sequences

Internal transcribed spacer (ITS) sequences refer to DNA spaces found between small-subunit (SSU) and large-subunit rRNA genes on DNA or the corresponding transcript (Lafontaine and Tollervey 2001, Coleman 2015). Previous reports have demonstrated that the first and second ITSs (ITS-1 and ITS-2) of rDNA could help identify *A. cantonensis* at the species level (Caldeira et al. 2003, Foronda 2010, Qvarnstrom et al. 2010, Liu et al. 2011, Carvalho et al. 2012, Fang et al. 2012, Eamsobhana et al. 2013, Lee et al. 2014, Guerino et al. 2017, Lv et al. 2017a).

Generally, to identify parasites, third instar larvae are obtained from naturally infected snails, and utilized for infecting vertebrate hosts by the oral route. Upon maturation of worms and migration to their habitat, parasite recovery is performed for morphological analysis. Such process is lengthy, and the vertebrate host must be sacrificed for worm recovery. ITS sequences, when used as biomarkers, allow the differentiation of larvae and adult worms in *Angiostrongylus costaricensis*, *A. cantonensis*, and *Angiostrongylus vasorum*. This in turn contributes to reducing morphological identification that generally requires $\sim$40 days (Caldeira et al. 2003).

According to ITS sequences, Lv et al. (2017a) analyzed the genetics and evolution of internal species in *A. cantonensis* in China. Singleton variable sites make up 67.3% and 70.0% of all variable sites in ITS1 and ITS2, respectively; these rates are markedly elevated than found for mitochondrial (mt) genes. The study also ruled out the possibility of group II constituting a distinct species, because there are no major differences in ITS sequences between this and other clades. Also striking intragenomic or within-individual heterogeneity was detected in ITS sequences and microsatellites, conflicting with the notion of concerted evolution (Ganley et al. 2007), but corroborating previously reported findings (Maslunka et al. 2014, Zhao et al. 2015).

Different local strains have been distinguished based on this gene. The DNA sequences of ITS-1 in *A. cantonensis* in China, United States, and Brazil have an intraspecific sequence variation of about 0.1–1.0%, whereas ITS-2 varies by 0.0–1.3% in isolates collected in China and Philippines (Eamsobhana et al. 2013). Moreover, the ITS gene of Hualien (H) *A. cantonensis* was shown to differ from those of counterparts in Pingtung (P) and three additional locations by 19%. H *A. cantonensis* was less infective and fecund in rats, with impaired development, causing a milder disease and reduced death rate in mice, compared with the P strain (Lee et al. 2014).

Furthermore, ITS has been utilized for the identification of the species of L3 stage *Angiostrongylus* larvae in snails (Foronda et al. 2010, Qvarnstrom et al. 2010, Liu et al. 2011, Carvalho et al. 2012, Guerino et al. 2017), as well as L1 stage larvae in mouse stool (Fang et al. 2012).

The SSU rRNA gene

18S rRNA, as a rRNA, is considered a basic cell component in eukaryotes. 18S rRNA sequences are broadly utilized for reconstructing the evolutionary history of organisms (Liu et al. 2015, Cooper et al. 2016, Harl et al. 2019). Because it slowly evolves, it can be utilized for reconstructing ancient divergences (Meyer et al. 2010, Sulaiman et al. 2014, Zhang et al. 2017b, Vitari et al. 2019, Kenmotsu et al. 2020).

The identification of nematodes in the environment remains important for assessing their potential risks to humans. Most nematodes that are present in intermediate hosts are juvenile, making it difficult to differentiate species based on morphology. Nuclear 18S (SSU) rDNAs are used to differentiate multiple *Angiostrongylus* taxa and to conduct systematic and phylogenetic analyses of relationships among *Angiostrongylus* species and other metastrongyloid groups (Floyd et al. 2002, Fontanilla and Wade 2008, Tokiwa et al. 2012, Eamsobhana et al. 2015). It was suggested that two sequences are from a single species with 99.5–100% similarity for the first 450 bp of the SSU rDNA (Floyd et al. 2002).

Importantly, 18S (SSU) rDNAs precisely segregated the five *Angiostrongylus* spp., confirming that *A. cantonensis* and *Angiostrongylus malaysiensis* on the one hand, and *A. costaricensis* and *Angiostrongylus dujardini* on the other hand, are closely related (Eamsobhana et al. 2015). Thus, the identity of a species can be determined by matching the sequence to an accessible sequence database such as GenBank. It is important to evaluate the role of *Achatina fulica* in the spread of *A. cantonensis* and potentially other nematodes in Philippines, especially in the densely populated area of Metro Manila. This allows proper assessment of the epidemiology of the associated diseases.

Phylogenetic relationships of *A. cantonensis* from distinct geographical areas revealed multiple lineages. Two different SSU genotypes (G1 and G2) were detected in 17 individual *A. cantonensis* strains from 17 geographical areas in Japan, Mainland China, Taiwan, and Thailand (Tokiwa et al. 2012).

The mt genome

Mitochondrial DNA (mtDNA or mDNA) is found in mitochondria, which produce adenosine triphosphate from food-associated energy for cell utilization (Iborra et al. 2004, Yan et al. 2019). Unlike nuclear DNA, mtDNA does not generally vary from parent to offspring. Since mtDNA copies can recombine (Zhang et al. 2017b), mtDNA mutates more than nuclear DNA in animals (Tatarenkov and Avise 2007, Zhang et al. 2017c). Therefore, mtDNA acts as a potent biomarker in tracking ancestry through females (matrilineage) for multiple millennia.

Complete mt genome

The full mt genome of the rodent intra-arterial nematode *A. cantonensis* was reported in 2012 (Lv et al. 2012). It is 13,497 bp long, including 2 proteins, 22 transfer ribonucleic acid (tRNAs), and 2 rRNAs, all on the same strand. Meanwhile, *A. costaricensis*'s full mt genomes are 13,585 bp long, with 81.6% nucleotide similarity between both mt genomes, ranging between 77.7% and 87.1% in individual gene pairs. In 2015, Yong et al. (2015b) revealed the genetic diversity of *A. cantonensis* by analyzing the complete mt genome. The mt genomes of Thai (13,519 bp) and Chinese (13,497–13,502 bp) isolates differed in length, as well as five protein-coding genes, including atp6, cox1, cox2, cob, and nad2. Stop codons for four genes (atp6, cob, nad2, and nad6) differed between the Thai and Chinese isolates as well.

In addition, the Thai and Chinese isolates had 4 and 3 incomplete T stop codons, respectively. The Thai isolate had longer control (258 bp) in comparison with the Chinese (230–236 bp) and Taiwanese (237 bp) counterparts. The intergenic sequence between nad4 and cox1 in the Thai isolate was short 2 bp (indels) at 5′ in comparison with the Chinese counterpart, also differing at 7 additional sites. In the Thai, Chinese, and Taiwanese isolates, 18, 17, and 16 tRNAs lacked the whole TΨC-arm, respectively.

A phylogenetic analysis of 36 mt genes, 12 polycomb group (PCGs), 2 rRNA genes, 22 tRNA genes, and control region revealed the Chinese isolate had closer genetic affinity with the Taiwanese counterpart in comparison with the Thai isolate. On the basis of 36 mt genes, the interisolate genetic distance varied from $p = 3.2\%$ between the Chinese and Taiwanese isolates to $p = 11.6\%$ between the Thai and Chinese isolates. Hence, the mt genome could help in population, phylogenetic, and phylogeographical analyses.

Furthermore, next-generation sequencing analysis of *A. cantonensis* may help better understand rat lung worm invasion and parasite detection. Novel *A. cantonensis* mt genomes in Sydney (Australia), Hawaii (United States), Canary Islands (Spain), and Fatu Hiva (French Polynesia) were comparatively assessed with those outside of Southeast Asia, showing low genetic diversity (0.02–1.03%). Both cox1 and cox2 are preferred biomarkers for detecting *A. cantonensis* haplotypes. Low-coverage whole-genome sequencing enables standardized identification of *A. cantonensis* (laboratory and field strains) (Červená et al. 2019).

Of the 13 *Angiostrongylus* species (Spratt 2015), *A. cantonensis* (Lv et al. 2012), *A. costaricensis* Brazil taxon (Lv et al. 2012), *A. costaricensis* Costa Rica taxon (Yong et al. 2015b), *A. mackerrasae* (Aghazadeh et al. 2015b, Valentyne et al. 2020), *A. malaysiensis* (Yong et al. 2016), and *A. vasorum* (Gasser et al. 2012) have complete mt genomes available. The mitogenome is therefore useful for phylogenetic and systematic analyses of *Angiostrongylus* lung worms and other Metastrongyloid nematodes.

Cytochrome c oxidase subunit I gene

Cytochrome c oxidase subunit I (COI) represents the major cytochrome c oxidase subunit. COI belongs to respiratory complex IV, which also includes cytochrome coxidase 2 and 3. Complex IV represents the third and final enzyme of the electron transport chain during the process of mt oxidative phosphorylation. Cytochrome c oxidase constitutes a major enzyme involved in aerobic metabolism. Heme–copper oxidases (proton pumps) are terminal, energy-transfer enzymes of prokaryotic and eukaryotic respiratory chains (Kosakyan et al. 2012).

The *A. cantonensis* nematode was first described in China. Lee et al. (2014) analyzed the cox1 gene of Taiwanese strains, where sequences of the H strain differed from those of the P and other three strains by 19% and 11%, respectively. A study conducted by Lv et al. (2017) reported the cox1 genes of totally 130 *A. cantonensis* samples from 32 distinct sites covering the entire endemic area of angiostrongyliasis in China, which were successfully sequenced. There were 39 cox1 haplotypes (Hd, 0.8114; Pi, 0.0284). Among the 843 sites of the complete nicotinamide adenine dinucleotide dehydrogenase subunit 1 (nad1) gene and 1577 sites of cox1, there were 171 variable sites per gene. Parsimony-informative sites were 94.7% in cox1 (162/171).

No substitution saturation was detected in mt cox1 gene in *A. cantonensis* (Wang and Lv 2014). Reports have examined the phylogenetic features of this nematode in other Asian countries, such as Japan, Lao PDR, Cambodia, Myanmar, and Thailand, besides China. Haplotypes ac1 (Tokyo, Chiba, Kanagawa, Amamioshima Island, and Taichung), ac2 (Ishikawa, Shenzhen, and Lianjiang), ac5 (Okinawa and Ogasawara), and ac7 (Miyagi, Aichi, and Kanagawa) were the commonest. The above regions are separated from one another by high mountains or seas (Tokiwa et al. 2012). Differences in the cox1 genes of *A. cantonensis* and *A. malaysiensis* have also been identified. Larvae were obtained from land snails in Lao PDR, Cambodia, Myanmar, and Thailand.

The regional distributions of both species showed a broad overlap. Publicly available databases were queried to infer phylogenetic relationships in *Angiostrongylus* species. COI showed the highest intraspecific variation in study regions (five and four *A. cantonensis* and *A. malaysiensis* haplotypes, respectively) (Rodpai et al. 2016). This parasite was also reported in the Americas. *A. cantonensis* isolates from distinct geographical Brazilian regions were shown to be genetically variable, based on COI gene sequences. Phylogenetic analysis revealed that *A. cantonensis* obtained in Pará (Brazil) and the Japanese stain are similar. Also, the above specimens once had a single haplotype with high bootstrap support with the Rio de Janeiro's isolate (Moreira et al. 2013).

The Brazilian haplotype ac5 and Japanese isolates cluster, whereas the Brazilian haplotype ac8 (Rio de Janeiro, São Paulo, Pará, and Pernambuco) constitute a different clade. The Brazilian haplotype ac9 has close relationships to the

Chinese and Japanese ac6 and ac7, respectively (Monte et al. 2012). There were also 7 Thai haplotypes, including AC4, AC10, AC11, AC13, AC14, and AC15 (Apichat et al. 2016, Eamsobhana et al. 2017). *A. cantonensis*, *A. costaricensis*, and *A. vasorum* COI sequences in GenBank were utilized for comparison. Two major clades were included: *A. cantonensis* and *A. malaysiensis* on one hand, and *A. costaricensis* and *A. vasorum* on the other hand.

The three *A. cantonensis* stains obtained in different geographical regions constituted a clade showing low-to-high bootstrap values, encompassing two subclades, namely the Chinese and Hawaiian isolates on one hand, and the Thai strain on the other hand. In the other main clade, European *A. vasorum* strains differ from Brazilian isolates. For *A. costaricensis*, the Costa Rican and Brazilian isolates differed, with an uncorrected (p) distance of 11.39%, suggesting the potential occurrence of cryptic species (Eamsobhana et al. 2010).

Nicotinamide adenine dinucleotide dehydrogenase subunit 1 (nad1) gene

Nad1 belongs to the NAD(P)H dehydrogenase (quinone) family that is translated into a cytoplasmic 2-electron reductase, which homodimerizes and catalyzes quinone reduction into hydroquinones and other redox substances. It preferentially transforms short-chain acceptor quinones, including ubiquinone, benzoquinone, juglone, and duroquinone (Sparla et al. 1996). Nad1 has a critical paralog, that is, NAD(P)H dehydrogenase quinone 2. This protein is cytosolic (Ross et al. 2000).

In China, the nad1 genes of 130 *A. cantonensis* strains obtained in 32 distinct sites covering the entire reported endemic region for angiostrongyliasis were accumulated. There was significant variation in nad1 sequences, with 75 haplotypes (Hd: 0.9260; Pi: 0.0314). Of the 843 nad1 sites, 171 variable ones were identified. Parsimony-informative sites were most frequent, representing 60.2% (103/171). However, there were multiple singlets in nad1. In addition, three compound parsimony-informative sites (three or four nucleotide types concurrently found more than twice) were identified. The nicotinamide adenine dinucleotide 1 protein consisted of 281 amino acids, with 17 variable sites. The corresponding synonymous variations were 73. The rates of singleton variable sites markedly differed between nuclear and mt genes, as well as among mt genes (Lv et al. 2017).

Cytochrome b gene

Cytochrome b (Cytb), a mt protein in eukaryotes, is involved in the electron transport chain as the major subunit of transmembrane cytochrome bc1 and b6f complexes (Esposti et al. 1993). Cytb is an important constituent of respiratory chain complex III (bc1 complex or ubiquinol-cytochrome c reductase) found in mitochondria of eukaryotes as well as aerobic prokaryotes. Due to the sequence variability of Cytb, it is frequently utilized to determine phylogenetic relationships among organisms, notably at the family and genus levels. Comparative analyses of cytochrome b produced novel classification patterns and helped assign new species to a genus and provided insights into evolutionary relationships (Castresana 2001).

In phylogeographical researches, the Cytb gene has been used in studying *A. cantonensis* in southern China (including Taiwan) and some Southeast Asian regions (Thailand). Peng et al. (2017) analyzed 520 distinct *A. cantonensis* strains from 13 localities, which were sequenced and grouped into 42 haplotypes. Genetic variations among populations were prominent, but demographic expansion was not observed through neutrality assays or mismatch distribution analysis, indicating low local gene flow in various sample collection sites. Two distinct *A. cantonensis* lineages were found in Taiwan, indicating that the parasite came to the island from many locations. Hekou (China) and Laos populations had the highest genetic variations, as reflected by genetic diversity parameters and analysis of molecular variation.

The above findings jointly indicate that the region surrounding Thailand or Hekou in Yunnan province, China, might be the most plausible origin of *A. cantonensis*. In 2015, Dusitsittipon et al. (2015) and Yong et al. (2015a) used this gene to determine *A. cantonensis*' genetic structure in Thailand. Phylogenetic assessment revealed two major clades in Thailand, which comprised four subclades or clusters. Genetic variation of gene flow and geographical location were not overtly related. Such low genetic variation and geographical distribution of *A. cantonensis* in various locations indicated a founder effect, likely due to many independent origins, suggesting haplotype migration from endemic areas through anthropogenic factors (Dusitsittipon et al. 2015).

Moreover, Cytb haplotypes from different Thai regions have been established, with some apparently restricted to particular geographical areas. Incomplete Cytb DNA sequences could allow an unequivocal separation of *A. cantonensis* strains collected in Thailand, China, and Hawaii, as well as the congeners *A. malaysiensis*, *A. costaricensis*, and *A. vasorum*, with *A. malaysiensis* and *A. costaricensis* grouped with *A. cantonensis* and *A. vasorum*, respectively (Yong et al. 2015a). However, CYTB mtDNA contains pseudogenes (Dusitsittipon et al. 2018).

Molecular Identification and Diagnosis of *A. cantonensis*

To address the limitations of phenotypic assays, genotypic strategies have been utilized for identifying and differentiating *A. cantonensis* (Ash 1970, Lindo et al. 2002, Cowie 2013, Qvarnstrom et al. 2013, Heather et al. 2017). Beside the development of conventional PCR detection in *A. cantonensis* (Eamsobhana et al. 2010, Lv et al. 2012, Lee et al. 2014, Yong et al. 2015a), several PCR-based approaches, including PCR-linked restriction fragment length polymorphism, PCR-random amplified polymorphic DNA (PCR-RAPD), multiplex PCR, quantitative PCR (qPCR), and loop-mediated isothermal amplification (LAMP) assays, are currently available for the accurate identification of *A. cantonensis* (Caldeira et al. 2003, Wei et al. 2010, Chen et al. 2011, Liu et al. 2011, Cui et al. 2012, Jarvi et al. 2012, 2015, Thaenkham et al. 2012, Qvarnstrom et al. 2016) (Table 1).

For example, according to the differentiation of COI and ITS2 regions of mtDNA and rDNA, respectively, in *A. cantonensis*, *A. costaricensis*, and *A. vasorum*, and subsequent fragment digestion by *Rsa*I, *Hap*II, *Alu*I, *Hae*III, *Dde*I, and *Cla*I, species can be differentiated and identified; *Rsa*I and

Table 1. Molecular Assays for Detecting and/or Genetically Differentiating *Angiostrongylus cantonensis*

	Source of samples							
		Definitive host (rat)			*Accidental host (human)*			
Molecular approaches	*Intermediate host (snails and/or slugs)*	*Lung*	*Feces*	*Blood and peripheral tissues*	*Cerebrospinal fluid*	*Blood*	*DNA target regions*	*References*
Conventional PCR	√						ITS1	Qvarnstrom et al. (2010)
	√						ITS2	Foronda et al. (2010), Rodpai et al. (2016), Guerino et al. (2017)
		√					ITS	Eamsobhana et al. (2013), Lv et al. (2017)
	√				√		ITS	Lee et al. (2014)
		√					SSU rRNA	Fontanilla and Wade (2008), Tokiwa et al. (2012), Eamsobhana et al. (2013), Eamsobhana et al. (2015)
	√						SSU rRNA	Foronda et al. (2010), Rodpai et al. (2016)
		√					complete mitochondrial genome	Lv et al. (2012), Yong et al. (2015b), Červená et al. (2019), Valentyne et al. (2020)
		√					COI	Eamsobhana et al. (2010), Monte et al. (2012), Tokiwa et al. (2012), Eamsobhana et al. (2013), Lee et al. (2014), Wang and Lv (2014), Lv et al. (2017), Eamsobhana et al. (2017), Dusitsittipon et al. (2018)
	√						COI	Qvarnstrom et al. (2013), Apichat et al. (2016), Rodpai et al. (2016)
	√	√					COI	Moreira et al. (2013)
		√					nad1	Lv et al. (2017)
		√					Cyt b	Dusitsittipon et al. (2015), Yong et al. (2015a), Peng et al. (2017), Dusitsittipon et al. (2018)
Multiplex PCR	√						SSU rRNA	Wei et al. (2010)
PCR-RFLP	√						COI and ITS2	Caldeira et al. (2003)
	√						ITS2	Carvalho et al. (2012)
PCR-RAPD	√						Genomic DNA	Thaenkham et al. (2012)
Quantitative PCR			√				ITS-2	Fang et al. (2012)
	√						ITS-1	Qvarnstrom et al. (2010), Jarvi et al. (2012)
						√	ITS-1	Wilkins et al. (2013), Jarvi et al. (2015)
					√		ITS-1	Qvarnstrom et al. (2016)
	√	√					ITS-1	Qvarnstrom et al. (2013), Stockdale et al. (2017)
	√				√			Sears et al. (2020)
LAMP	√						18S rRNA	Chen et al. (2011)
	√						ITS-1	Liu et al. (2011)
	√						18S rRNA and ITS-1	Martin-Alonso et al. (2015)
RAA	√						ITS1	Zhang et al. (2020)
RPA-EXO	√						ITS1	Jarvi et al. (2020)
RPA-LFA	√						ITS1	Jarvi et al. (2020)

COI, cytochrome c oxidase subunit I; ITS, internal transcribed spacer; LAMP, loop-mediated isothermal amplification; RAA, recombinase-aided isothermal amplification; PCR-RAPD, PCR-random amplified polymorphic DNA; PCR-RFLP, PCR-linked restriction fragment length polymorphism; RPA-EXO, recombinase polymerase amplification Exo; RPA-LFA, recombinase polymerase amplification lateral flow assay; rRNA, ribosomal RNA; SSU, small subunit.

*Cla*I were most potent in discriminating these two genes (Caldeira et al. 2003).

RAPD markers are 10-nt DNA fragments obtained by amplifying random segments of genomic DNA using a single primer of a random nucleotide sequence, which can distinguish genetically distinct individuals, although not reproducibly. They are utilized for assessing genetic diversity for an individual. In a study conducted by Thaenkham et al. (2012), *A. cantonensis* was collected in 8 Thai geographical areas. The results showed high genetic diversity and low gene flow among populations. Thai *A. cantonensis* was shown to comprise two groups with genetic differentiation. However, genotypic changes and haplotype relationships deserve further investigation utilizing additional markers.

qPCR could be applied in multiple research areas such as genotyping, and gene expression, microRNA, genetic variation and protein analyses. qPCR has higher efficiency and sensitivity compared with microscopy, providing a novel tool for quantifying *A. cantonensis* larvae in the intermediate host snails (Qvarnstrom et al. 2010) and potentially other sources (*e.g.*, semi-slugs) as well (Jarvi et al. 2012).

A. cantonensis DNA could also be detected by qPCR in peripheral blood at various times during rat lungworm infection (Jarvi et al. 2015), as well as in human CSF specimens (Qvarnstrom et al. 2016, Sears et al. 2020). The AcanR3990 qPCR instrument has a limit of detection (LOD) of 1 fg (DNA equivalent of 1/100,000 dilution of 1 third instar larva), with no cross-reaction with CSF specimens from individuals with definite neurocysticercosis, toxocariasis, gnathostomiasis, and baylisascariasis (Sears et al. 2020). LAMP represents an isothermal single-tube method utilized for DNA amplification. Unlike PCR that uses alternating temperature steps and/or cycles, LAMP is operated at constant temperature, with no need of a thermal cycler (Notomi et al. 2000).

LAMP represents a suitable technique for routine *A. cantonensis* detection within the intermediate hosts, that is, *Pomacea canaliculata* and *A. fulica* due to its simplicity, sensitivity, and specificity. It has great prospect for monitoring *A. cantonensis* in endemic regions. Reactions amplified *A. cantonensis*' 18S rRNA and ITS-1 sequences, with high sensitivity, that is, only 1 fg of DNA detected in *P. canaliculata* specimens (Chen et al. 2011) and 10 times higher sensitivity versus conventional PCR in *A. fulica*. Furthermore, no cross-reactivity with other parasitic organism was detected (Liu et al. 2011).

The recombinase-aided isothermal amplification (RAA) assay was also applied in the nucleic acid detection of *A. cantonensis*. *A. cantonensis*' ITS1 gene served as the target sequence for detection. RAA was performed for successful nucleic acid detection in *A. cantonensis*, achieving real-time and specific amplification within 20 min at 37°C. The lowest LODs of the fluorescent RAA assay were 10 copies/μL and 100 pg/μL of recombinant plasmid and genomic DNA, respectively. This technique could not detect the genomic DNAs of *A. cantonensis*, *Schistosoma mansoni*, *Ascaris lumbricoides*, *Clonorchis sinensis*, *Echinococcus granulosus*, *Ancylostoma duodenale*, *P. canaliculata*, and *Biomphalaria straminea* in snail tissues (Zhang et al. 2020).

Currently, the recombinase polymerase amplification Exo (RPA-EXO) assay and recombinase polymerase amplification lateral flow assay (RPA-LFA) with routine qPCR have been developed. These three methods have been applied to assess 35 slugs in Hawai for *A. cantonensis* DNA, with consistent, discordant, and equivocal results in 23/35 (65.7%), 7/35 (20%, <0.01 larvae/mg tissue), and 5/35 (14.3%), respectively. They consistently detected 50–100 copies/μL versus $\sim$13 copies/μL for qPCR. RPA-EXO could detect 25 copies/μL, whereas RPA-LFA only amplified consistently above 50 copies/μL. Although both RPA-EXO assay and RPA-LFA appear less sensitive compared with qPCR at low DNA levels, they are more convenient than qPCR (Jarvi et al. 2020).

Application of Molecular Detection of *A. cantonensis* Infection

In *A. cantonensis*, first instar larval worms are expelled in the feces of rats (definitive host) (Alicata et al. 1965). The infected feces is then ingested by many species of snails and slugs (intermediate hosts); however, larvae might also enter the snail through the body wall or through the respiratory pore (Alicata 1991). These larvae develop into third instar larvae in snails, remaining at this stage until the snail is eaten or dies. After snails with third instar larvae are eaten by rats, the larvae move to the small intestine through the rat gut. They next penetrate the intestinal wall and enter the circulation, with some eventually entering the CNS and reaching the brain. Humans (incidental host) contract the infection mostly like rats through deliberate or accidental ingestion of infected snails or slugs (Lindo et al. 2002).

For molecular detection of *A. cantonensis* infection, blood and tissue specimens from rats (Jarvi et al. 2015), larvae in the stool of rats (Fang et al. 2012), L3 stage larvae in snails or slugs (Zhang et al. 2006, Qvarnstrom et al. 2007, 2010, Wei et al. 2010, Carvalho et al. 2012, Chan et al. 2015, Guerino et al. 2017), CSF samples from humans (Eamsobhana et al. 2013), and abdominal angiostrongyliasis in humans (da Silva et al. 2003) have been tested.

Conclusions

In summary, most of the current knowledge of *A. cantonensis* epidemiology has stemmed from multiple observational and morphological analyses. Nevertheless, molecular epidemiologic studies reported in recent years have provided important insights into *Angiostrongylus* taxonomy and evolutionary biology. Importantly, these molecular detection methods have been widely used in diagnosing angiostrongyliasis. The application of such molecular methods, coupled with classical epidemiologic approaches, imparts greater resolution and accuracy in describing the epidemiology of *Angiostrongylus*.

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