Acta Tropica 116 (2010) 152–156

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Molecular differentiation of *Angiostrongylus* taxa (Nematoda: Angiostrongylidae) by cytochrome *c* oxidase subunit I (COI) gene sequences

Praphathip Eamsobhana[a,*], Phaik Eem Lim[b,c], Gabriela Solano[d], Hongman Zhang[e], Xiaoxian Gan[f], Hoi Sen Yong[c]

[a] *Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand*
[b] *Institute of Ocean and Earth Science, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[c] *Institute of Biological Sciences, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[d] *Facultad de Microbiologia, Universidad de Costa Rica, San Jose, Apartado 2060, Costa Rica*
[e] *Guangxi Zhuang Autonomous Region Center for Disease Control and Prevention, Nanning 530028, PR China*
[f] *Institute of Parasitic Diseases, Zhejiang Academy of Medical Sciences, Hangzhou 310013, PR China*

ARTICLE INFO

Article history:
Received 25 May 2010
Received in revised form 29 June 2010
Accepted 14 July 2010
Available online 21 July 2010

Keywords:
Angiostrongylus cantonensis
Angiostrongylus costaricensis
Angiostrongylus malaysiensis
Angiostrongylus vasorum
Parastrongylus
Molecular phylogeny
Phylogeography

ABSTRACT

Nematodes of the genus *Angiostrongylus* are parasites of rodents and carnivores. They reside in the pulmonary or mesenteric arteries of their hosts. Two species are pathogenic in humans – *Angiostrongylus cantonensis* causes eosinophilic meningitis or meningoencephalitis, and *Angiostrongylus costaricensis* produces abdominal angiostrongyliasis. In addition *Angiostrongylus malaysiensis* may have the potential of being pathogenic in humans. The mitochondrial gene cytochrome *c* oxidase subunit I (COI) of these *Angiostrongylus* species and three geographical isolates (China, Hawaii and Thailand) of *A. cantonensis* were studied by polymerase chain reaction amplification and DNA sequencing. COI sequences of *A. cantonensis*, *A. costaricensis* and *Angiostrongylus vasorum* in the GenBank were included for comparison. Phylogenetic analysis by maximum-likelihood (ML), maximum-parsimony (MP), neighbour-joining (NJ) and Bayesian inference (BI) produced similar tree topology except variation in the bootstrap support values. There were two major clades – (1) *A. cantonensis* and *A. malaysiensis*, and (2) *A. costaricensis* and *A. vasorum*. The three geographical isolates of *A. cantonensis* formed a clade with low to high bootstrap values, and consisted of two subclades: (a) China and Hawaii isolates, and (b) monophyletic Thailand isolate. The individuals of each isolate formed a distinct cluster. In the second major clade, the Europe isolates of *A. vasorum* were distinctly different from the Brazil isolates. For *A. costaricensis*, the Costa Rica isolate was distinct from the Brazil isolate with an uncorrected (*p*) distance of 11.39%, indicating the possible occurrence of cryptic species. The present results indicate that COI sequences might be a useful marker for differentiating geographical isolates of *A. cantonensis* and in uncovering cryptic species. Efforts are being made to carry out an extensive collaborative study to cover a wide range of *Angiostrongylus* species and geographical isolates.

1. Introduction

Nematodes of the genus *Angiostrongylus* Kamensky, 1905 are parasites of rodents and carnivores (Anderson, 2000). Under this classification, the component species are grouped into two subgenera, i.e. *Angiostrongylus* and *Parastrongylus* (Drozdz, 1970; Anderson, 1978). These two subgenera however have been elevated by some authors (Chabaud, 1972; Ubelaker, 1986) as full genera, but this taxonomic treatment has not been generally accepted. Currently, 13 species are from rodent hosts and two from carnivore hosts (Ubelaker, 1986; Costa et al., 2003; del Rosario Robles et al., 2008). These nematodes reside in the pulmonary arteries of their hosts, except *Angiostrongylus costaricensis* Morera and Céspedes, 1971 and *A. siamensis* Ohbayahi, Kamiya and Bhaibulaya, 1979 which are found in the mesenteric arteries.

Of the 15 *Angiostrongylus* species, only two are of public health importance, causing human angiostrongyliasis. *Angiostrongylus cantonensis* (Chen, 1935) is a primary cause of human eosinophilic meningitis or eosinophilic meningoencephalitis in Asia and the Pacific Islands (Eamsobhana and Tungtrongchitr, 2005; Eamsobhana, 2006). Its occurrence has now been reported in many countries worldwide (Eamsobhana, 2006; Cross and Chen, 2007; Foronda et al., 2010). Furthermore, the parasites of different geographical locality show different infectivity, severity and pathogenicity in experimental hosts (Cross, 1979). The other species, *A. costaricensis* produces abdominal angiostrongylia-

* Corresponding author. Tel.: +66 (0) 24196468.
E-mail address: sipes@mahidol.ac.th (P. Eamsobhana).

0001-706X/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.actatropica.2010.07.005

sis and occurs throughout the Americas, from southern United States to northern Argentina in South America (Morera, 1985; Graeff-Teixeira et al., 2009; Graeff-Teixeira, 2010). In addition to *A. cantonensis* and *A. costaricensis*, two other species (*A. mackerrasae* Bhaibulaya, 1968 in Australia and *Angiostrongylus malaysiensis* Bhaibulaya and Cross, 1971 in Malaysia) may have the potential of being pathogenic in humans (Prociv et al., 2000). When first documented both species were referred to as *A. cantonensis* (Bhaibulaya, 1968; Bhaibulaya and Cross, 1971).

Of the two *Angiostrongylus* species that are pathogenic in humans, *A. cantonensis* has received greater attention in both laboratory and clinical studies (Eamsobhana, 2006; Cross and Chen, 2007; Graeff-Teixeira et al., 2009). Immunological diagnosis in particular has been extensively explored (Eamsobhana and Yong, 2009). Molecular differentiation of *A. cantonensis*, *A. costaricensis* and *Angiostrongylus vasorum* (Baillet, 1866) has been achieved with polymerase chain reaction-restriction fragment length polymorphism (Caldeira et al., 2003). The partial DNA sequences of a 66-kDa protein gene could unequivocally differentiate *A. cantonensis*, *A. costaricensis* and *A. malaysiensis*, and indicated that *A. cantonensis* and *A. malaysiensis* were closer related than to *A. costaricensis* (Eamsobhana et al., 2010a). The small subunit (SSU) ribosomal DNA sequences have been used for constructing phylogenetic tree of five species of *Angiostrongylus*—*A. cantonensis*, *A. costaricensis*, *A. dujardini* Drozdz and Doby, 1970, *A. malaysiensis* and *A. vasorum* (Fontanilla and Wade, 2008; Van Megan et al., 2009). Phylogenetic tree has also been constructed with internal transcribed spacer 2 (ITS-2) for *A. cantonensis*, *A. costaricensis* and *A. vasorum* (Jefferies et al., 2009) as well as *A. dujardini* (Foronda et al., 2010).

The nucleotide sequences of cytochrome *c* oxidase subunit I (COI) have been reported for *A. cantonensis* from China (GenBank accession number GQ398121 – complete mitochondrial genome), and *A. costaricensis* from Brazil (GenBank accession numbers GQ398122, NC013067 – complete mitochondrial genome). COI sequences have been briefly reported to clearly differentiate *A. cantonensis*, *A. costaricensis*, *A. malaysiensis* and *A. vasorum* (Eamsobhana et al., 2010a) and geographical isolates of *A. cantonensis* (Eamsobhana et al., 2010a; He et al., 2010). In contrast COI has been extensively studied in *A. vasorum* from Europe and South America (Jefferies et al., 2009, 2010).

The present study was undertaken to determine the usefulness and suitability of the COI gene for differentiating closely related species (e.g. *A. cantonensis* and *A. malaysiensis*) and geographical isolates of *A. cantonensis*, as well as to determine the phylogenetic relationship of *A. cantonensis*, *A. costaricensis*, *A. malaysiensis* and *A. vasorum*.

2. Materials and methods

2.1. Angiostrongylus worms

The Thailand isolate of *A. cantonensis* was maintained in the Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, with passages through albino rats as definitive rodent host and *Biomphalaria glabrata* as intermediate snail host. The Hawaii isolate of *A. cantonensis* was kindly provided by Dr. Akira Ishih of Hamamatsu University, Japan and maintained in the Department of Parasitology, Mahidol University. Both the Thailand and Hawaii adult worm samples have been kept in absolute ethanol since August 2008. The China specimens of *A. cantonensis* were obtained from rodents caught in Guangxi Province, PR China in September 2009. Individual worm homogenate was applied and dried onto the FTA card before shipment to Bangkok for DNA preparations. *A. malaysiensis* male and female worms were obtained from the pulmonary arteries of wild caught *Rattus tiomanicus* on 26 June 2008 in Pahang, Peninsular Malaysia and stored in absolute ethanol until DNA was extracted. *A. costaricensis* adult specimens preserved in RNA*later* (RNA stabilization solution) were a gift from Dr. Elizabeth Abrahams, Department of Parasitology, University of Costa Rica.

2.2. DNA extraction

The FTA card method (Whatman BioScience) which is more rapid and convenient to perform than the commercial DNA extraction kits was employed for DNA preparation in the present study.

Genomic DNA extraction from individual adult female and male worms of *A. cantonensis* (Thailand, Hawaii and China isolates), *A. costaricensis* and *A. malaysiensis*, was carried out using FTA technology (Whatman BioScience) following the manufacturer's instruction. In brief, individual worm was homogenized in 100–150 μL of sterilized PBS, pH 7.4. The homogenate was applied and dried onto the FTA card according to the Whatman FTA tissue protocol. A sample disc was taken using a 2.0 mm diameter Harris micro punch, washed with FTA purification reagent, and used in PCR for DNA analysis.

2.3. PCR amplification and DNA sequencing

The DNA amplification by polymerase chain reaction was conducted using the previously described primers COI_F 5′ TAAAGAAAGAACATAATGAAAATG 3′ and COI_R 5′ TTTTTTGGGCATCCTGAGGTTTAT 3′ for a partial region of the COI gene (Bowles et al., 1993; Hu et al., 2002; Jefferies et al., 2009). The reaction mixture was prepared in a total volume of 25 μL containing 2.5 μL of 10× PCR buffer (TrisCl, KCl, $(NH_4)_2$ SO_4, 15 mM $MgCl_2$, pH 8.7 (QIAGEN), 0.5 μL of dNTP mix (10 mM each), 0.5 μL of each primer (12.5 ng/μL), 0.1 μL of Taq DNA polymerase (5 Units/μL), 21.4 μL of dH_2O and a 2.0 mm DNA punched disc (FTA card) that contained extracted DNA from individual worm samples in a DNA thermal cycler (PerkinElmer Cetus). The thermocycler was programmed to incubate the samples for 5 min at 94 °C, followed by 40 cycles, each at 94 °C for 30 s, at 55 °C for 30 s, at 72 °C for 1 min, and final extension at 72 °C for 5 min. The reaction products were separated by electrophoresis on 1.5% (w/v) agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. Amplified products were purified using a QIAquick PCR Purification Kit (QIAGEN). Sequencing reactions were performed using an ABI PrismDyeTerminator Cycle Sequencing Core kit (Applied Biosystems, USA).

2.4. COI sequences from GenBank

COI sequences were obtained from the GenBank as follows: (1) *A. cantonensis* of China – GQ398121; (2) *A. costaricensis* of Brazil – GQ398122, and NC013067; and (3) *A. vasorum* of Europe – EU493161 to EU493167. The sequences for *A. vasorum* isolates Brazil 5421, Brazil 5641 and Brazil 5642 were constructed from published data on their variable nucleotide positions (Jefferies et al., 2009). Sequences of *Ancylostoma duodenale* (GenBank accession number AJ417718) and *Ancylostoma tubaeforme* (GenBank accession number AJ407940) were used as outgroup.

2.5. Sequence alignment and phylogenetic analysis

Sequences from this study were preliminarily aligned using the CLUSTAL X program (Thompson et al., 1997) and subsequently manually aligned. The aligned sequences were subjected to maximum-likelihood (ML), maximum-parsimony (MP) and neighbour-joining (NJ) analyses using PAUP* 4.0b10 (Swofford, 2002). ML analyses of the COI data were performed using PAUP* and a best-fitting evolution model. The model of sequence evolution