Experimental Parasitology 126 (2010) 564–569

Contents lists available at ScienceDirect

Experimental Parasitology

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

Molecular differentiation and phylogenetic relationships of three *Angiostrongylus* species and *Angiostrongylus cantonensis* geographical isolates based on a 66-kDa protein gene of *A. cantonensis* (Nematoda: Angiostrongylidae)

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ARTICLE INFO

Article history:
Received 15 January 2010
Received in revised form 28 May 2010
Accepted 1 June 2010
Available online 8 June 2010

Keywords:
Angiostrongylus cantonensis
Angiostrongylus costaricensis
Angiostrongylus malaysiensis
Molecular differentiation
Phylogenetic relationships
DNA sequences

ABSTRACT

The phylogenetic relationships and molecular differentiation of three species of angiostrongylid nematodes (*Angiostrongylus cantonensis*, *Angiostrongylus costaricensis* and *Angiostrongylus malaysiensis*) were studied using the AC primers for a 66-kDa protein gene of *A. cantonensis*. The AC primers successfully amplified the genomic DNA of these angiostrongylid nematodes. No amplification was detected for the DNA of *Ascaris lumbricoides*, *Ascaris suum*, *Anisakis simplex*, *Gnathostoma spinigerum, Toxocara canis*, and *Trichinella spiralis*. The maximum-parsimony (MP) consensus tree and the maximum-likelihood (ML) tree both showed that the *Angiostrongylus* taxa could be divided into two major clades – Clade 1 (*A. costaricensis*) and Clade 2 (*A. cantonensis* and *A. malaysiensis*) with a full support bootstrap value. *A. costaricensis* is the most distant taxon. *A. cantonensis* is a sister group to *A. malaysiensis*; these two taxa (species) are clearly separated. There is no clear distinction between the *A. cantonensis* samples from four different geographical localities (Thailand, China, Japan and Hawaii); only some of the samples are grouped ranging from no support or low support to moderate support of bootstrap values. The published nucleotide sequences of *A. cantonensis* adult-specific native 66 kDa protein mRNA, clone L5–400 from Taiwan (U17585) appear to be very distant from the *A. cantonensis* samples from Thailand, China, Japan and Hawaii, with the uncorrected *p*-distance values ranging from 26.87% to 29.92%.

1. Introduction

Angiostrongylus cantonensis, a nematode parasite inhabiting the pulmonary arteries of rats, is a primary cause of human eosinophilic meningitis or eosinophilic meningoencephalitis (cerebral angiostrongyliasis) in Asia and the Pacific Islands (Eamsobhana and Tungtrongchitr, 2005; Eamsobhana, 2006). *Angiostrongylus costaricensis* which inhabits the mesenteric arteries of rats produces abdominal angiostrongyliasis in humans in Central and South America (Morera, 1985). *Angiostrongylus malaysiensis* is very similar to *A. cantonensis* and also inhabits the lung of rats. It has been shown to produce neurologic abnormality in infected rodent host (Cross, 1979), but the potential of being pathogenic to humans need further elucidation.

Biochemical and immunological approaches had been used to discriminate antigenic variability in *Angiostrongylus* species (*A. cantonensis*, *A. costaricensis* and *A. malaysiensis*) (Eamsobhana et al., 1998; Sawabe and Mikiya, 1994). More recently, molecular analysis has been used to differentiate various *Angiostrongylus* species. Restriction fragment length polymorphisms (RFLPs) have proved valuable in differentiating *A. cantonensis*, *A. costaricensis,* and *Angiostrongylus vasorum* (Caldeira et al., 2003). The small-subunit ribosomal DNA sequences have been used for constructing phylogenetic tree of five species of *Angiostrongylus*, viz. *A. cantonensis*, *A. costaricensis*, *Angiostrongylus dujardini*, *A. malaysiensis* and *A. vasorum* (van Megan et al., 2009; Fontanilla and Wade, 2008). Phylogenetic tree has also been constructed with internal transcribed spacer 2 (ITS-2) for *A. cantonensis*, *A. costaricensis* (from Costa Rica and Brazil) and *A. vasorum* (from Brazil and Europe) (Jefferies et al., 2009). In addition other nucleotide sequences, including the mitochondrial cytochrome-*c* oxidase subunit I (COI) have been deposited in the GenBank.

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doi:10.1016/j.exppara.2010.06.001

Of the 15–16 species of *Angiostrongylus* in the world, nucleotide sequences are available in the GenBank for *A. cantonensis* (209 entries), *A. costaricensis* (12), *A. dujardini* (2), *A. malaysiensis* (1) and *A. vasorum* (22). There is only a single entry for the adult-specific muscle protein gene, namely for *A. cantonensis*. It is evident that there had been few studies of the pathogenic *A. cantonensis* in different endemic localities. In recent years, there have been evidences for wide geographic distribution of *A. cantonensis* and increasing incidence of human angiostrongyliasis worldwide (Wang et al., 2008). Furthermore, the parasites of different geographical locality show different infectivity, severity and pathogenicity in experimental hosts (Cross, 1979).

The purpose of this study was to determine the phylogenetic relationships of three *Angiostrongylus* species (*A. cantonensis*, *A. costaricensis* and *A. malaysiensis*). Different geographical isolates of *A. cantonensis* from Thailand, Hawaii, Japan and China were also determined to see the level of their molecular variation, and to assess the potential of the mRNA nucleotide sequences of a 66-kDa muscle-associated protein gene of *A. cantonensis* as a candidate marker for species and isolates differentiation.

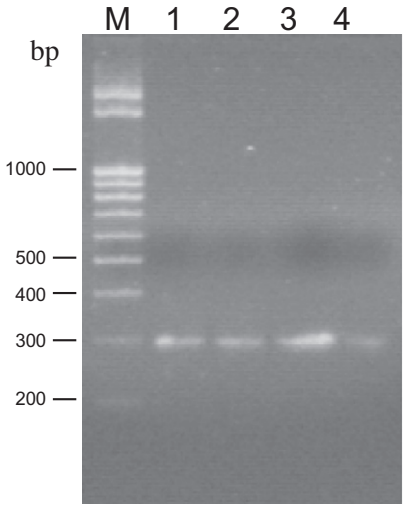

Fig. 1. Electrophoretic patterns of PCR products using the specific primers AC1 and AC2 for adult *A. cantonensis* on DNA extracted by FTA card method (Whatman BioScience) – Lanes 1 and 2, and QIAamp tissue kit (QIAGEN, Germany) – Lanes 3 and 4. Lane M, molecular weight markers (100 bp ladder).

2. Materials and methods

2.1. Angiostrongylus worms and other nematodes

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 alcohol 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 extractions. The *A. cantonensis* worms of Japan, collected in May 2006 by Dr. Asato and stored in absolute ethanol, were kindly provided by Professor Ichiro Miyagi and Professor T. Toma, University of the Ryukyus, Okinawa, Japan. *A. malaysiensis* male and female worms were obtained from the pulmonary arteries of wild caught *Rattus tiomanicus* on 26 June 2008 in Pahang, 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 and Dr. Maria Solano, Department of Parasitology, University of Costa Rica. We were not able to obtain specimens of *A. vasorum* and other *Angiostrongylus* species. Adult worms of *Ascaris suum* from pig, *Ascaris lumbricoides* from a human patient, *Toxocara canis* from cat, larvae of *Anisakis simplex* from marine fish, *Trichinella spiralis* from experimentally infected mouse and *Gnathostoma spinigerum* from naturally infected eels, were used as out-groups. All these nematode samples have been kept in absolute ethanol at the Department of Parasitology, Faculty of Medicine Siriraj Hospital, since 2008.

2.2. DNA extraction

Genomic DNA extraction from individual adult worms of *A. cantonensis* (Thailand isolate – 2 males, 2 females; Hawaii isolate – 2 males, 2 females) was tested using a QIAamp tissue kit (QIAGEN, Germany) and the FTA card method (Whatman BioScience). Both procedures yielded similar DNA banding patterns (Fig. 1) after PCR amplification using specific primers AC1 and AC2 for adult *A. cantonensis*. The FTA card method which is more rapid and convenient to perform was employed for DNA preparation in the present study.

Genomic DNA extraction from individual adult female and male worms of *A. cantonensis* (Thailand isolate – 6 males, 6 females; Hawaii isolate – 6 males, 6 females; Japan isolate – 2 males, 2 females; China isolate – 4 males, 4 females), *A. malaysiensis* (2 males, 2 females) and *A. costaricensis* (3 males, 3 females), 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 amplification by polymerase chain reaction was conducted using the primers AC1: 5′ CTCGGCTTAATCTTTGCGAC-3′ and AC2: 5′ AACGAGCGGCAGTAGAAAAA-3′ (Silva et al., 2003). The sequence of the primers was designed based on the adult-specific native 66 kDa protein mRNA, clone L5–400 of *A. cantonensis* (GenBank Accession No. U17585) (Bessarab and Joshua, 1997).

The PCR reaction was performed as described by Silva et al. (2003) with slight modification. The amplification was carried out in 50 μL containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 200 μM each of dATP, dGTP, dCTP and dTTP, 150 pmol of each primer, and 1 U of *Taq* DNA polymerase, with a 2.0 mm DNA punched disc (FTA card) that contained extracted DNA from individual worm samples in a DNA thermal cycler (Perkin–Elmer Cetus). Amplification of 35 cycles consisted of denaturation at 94 °C for 2 min, annealing at 58 °C for 3 min, and extension at 72 °C for 3 min. Negative and positive controls were run with each round of amplification.

The PCR products were electrophoresed in 1.5% agarose gel. After electrophoresis, the agarose gel containing DNA fragments was stained with 0.5 μg/mL of ethidium bromide and visualized by ultraviolet transilluminator. Amplified products were purified using a QIAquick PCR Purification kit (QIAGEN, Germany). Sequencing reactions were performed using an ABI PrismDyeTerminator Cycle Sequencing Core kit (Applied Biosystems, USA).