POPULATION STRUCTURE OF ANGIOSTRONGYLUS CANTONENSI S (NEMATODA: META STRONGYLIDAE) IN THAILAND BASED ON PCR-RAPD MARKERS

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Abstract. Angiostrongylus cantonensis is the causative agent of angiostrongyliasis, which is widely distributed throughout the world. It can specifically infect many species of intermediate and definitive hosts. This study examined the genetic differentiation and population structure using the RAPD-PCR method of parasites obtained from 8 different geographical areas of Thailand. Based on 8 primers, high levels of genetic diversity and low levels of gene flow among populations were found. Using genetic distance and neighbor-joining dendrogram methods, A. cantonensis in Thailand could be divided into two groups with statistically significant genetic differentiation of the two populations. However, genotypic variations and haplotype relationships need to be further elucidated using other markers.

Keywords: Angiostrongylus cantonensis, population structure, PCR-RAPD, Thailand

INTRODUCTION

Angiostrongylus cantonensis (rat lung-worm) is a nematode of rodents, and adult worms live inside the pulmonary arteries (Alto, 2001). Humans occasionally acquire infection by ingesting raw or undercooked snail, slug, prawn, tadpole, or contaminated vegetables, containing infective A. cantonensis larvae (Bronstein et al, 1978; Oku et al, 1980; Wang et al, 2008). The parasitic worm is neurotropic in humans and can cause eosinophilic meningitis (Alto, 2001). A. cantonensis is distributed widely in 30 countries, and outbreaks are frequently reported in many countries in Asia and in the Pacific and Caribbean Islands (Wang et al, 2008).

Many kinds of freshwater snails (Pila spp, Pomacea canaliculata, Biomphalaria alexandrina, Lymnaea natalensis, and Melanoides tuberculata) and land snails (Achatina fulica and Helix pomatia) are reportedly paratenic hosts (Tasana et al, 2009). In China, P. canaliculata and A. fulica are believed to be closely associated with angiostrongyliasis. In Thailand, the high rate of angiostrongyliasis is linked to the custom of eating raw Pila snails (Wang et al, 2008). However, the highest infection rate (44.3%) of A. cantonensis is from A. fulica, while the rate among Pila spp is around 0.9% (Pipitgool et al, 1997). A. fulica is also a major source of
infection worldwide (Kliks and Palumbo, 1992). *Rattus rattus* and *R. norvegicus* are considered the most common definitive hosts of this parasite, while other rodents can also serve as parasitic hosts (Cross and Chen, 2007).

The broad spectrum of specific intermediate and definitive hosts is related to the population genetic structure and diversity of disease-causing organisms. These are used to draw inferences about their evolutionary history and potential to generate trait variations that determine interactions with their hosts (Barrett et al., 2008). Accurate measurements of genetic variations and their distribution within host-parasite systems are an important parameters needed to assess the re-emergence of disease and the effects of infection on host mortality and parasite reproduction (Nadler, 1995).

Recently, several molecular markers have been developed to study the population genetics and diversity of various parasites such as a randomly amplified polymorphic DNA (RAPD) markers based on PCR technique (Nadler et al., 1995; Chrisanfova et al., 2000; Guizani et al., 2002). RAPD method is useful for studying intraspecific species where no study has previously been undertaken, with low cost and can handle extensive numbers of samples (Li et al., 2006). Therefore, it is possible to conduct a preliminary study of a large sample size from each population.

In this study, RAPD technique was used to examine the genetic diversity of *A. cantonensis* in various regions of Thailand that have not yet been reported. This is the first PCR-RAPD investigation of the genetic variation and intraspecific variants among 8 populations of *A. cantonensis* collected from *Ac. fulica* in Thailand.

**MATERIALS AND METHODS**

**Sample collection**

More than 200 *Ac. fulica* were collected from each of 8 different geographical areas of Thailand (Fig 1). The snails were killed and their shells were cracked. The soft tissues of individual snails were homogenized and digested with 0.25% pepsin A (BDH, Leicestershire, England) for one hour at 37°C. The digested fluid containing nematode larvae from each snail was collected using Baermann’s apparatus, and L3 *A. cantonensis* larvae were identified under a dissecting microscope. The L3 larvae from each positive snail were pooled and infected into Wistar rats (*Rattus norvegicus*). After 4 months, the infected rats were sacrificed and the adult *A. cantonensis* worms were isolated from the rat pulmonary arteries. All parasite infection procedures with the rats were approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (no. FTM-ACUC001/2008). Twenty-five adult *A. cantonensis* worms from each locality were preserved in 70% EtOH at -20°C for further use.

**RAPD-PCR**

DNA was extracted from individual adult worms using a Genomic DNA Mini kit (Tissue) (Geneaid, Taiwan), according to the manufacturer’s instructions. DNA of each worm was amplified using preselected oligonucleotide from the Operon A series (Operon Technologies, Alameda, CA). PCR was performed in 20 μl reaction volume containing 10 μl of Top Taq™ Master Mix Kit, 3 μM arbitrary primer, and 20 ng of genomic DNA. Into the reaction, MgCl₂ was adjusted to 2.5 mM for greater specificity. RAPD-PCR was conducted by heating at 95°C for 3 minutes, followed by 39 cycles of 94°C for 1 minute, 34°C for 1 minute, and 72°C for 1 minute, with
of 30 primers, 8 (10-mers) were selected (Operon Technologies) after screening for intense and reproducible bands for further PCR amplifications. The 8 selected primers (Table 1) were used for RAPD-PCR of 112 *A. cantonensis* samples from 8 populations and 135 loci (between 165 and 1,015 bp) were obtained overall, and 116 (86%) of these loci were polymorphic. The RAPD band patterns from 8 populations are shown in Fig 2. Within a given population, however, most *A. cantonensis* had monomorphic bands (completely identical) except the population from PK (69%). There were high levels of genetic diversity among these populations \[H\] (Nei’s gene diversity) = 0.3176 ± 0.1554; \[I\] (Shannon’s information index) = 0.1157 ± 0.218). Gene flow among the subdivided populations was calculated according to Slatkin and Barton’s formula (Slatkin and Barton, 1989), revealing a very low gene flow \([Nm = 0.059]\).

**Genetic distance, identity and neighbor-joining dendrogram**

From the 8 *A. cantonensis* populations, Nei’s genetic distance ranged between 0.069 and 1.409 (Table 2) indicated that the *A. cantonensis* populations studied could be divided into 2 major groups: group 1...
Table 2
Nei’s identity and genetic distance for the eight *A. cantonensis* populations in Thailand. Genetic identity values are above diagonal and genetic distances are below.

<table>
<thead>
<tr>
<th>Population</th>
<th>KB</th>
<th>NK</th>
<th>NR</th>
<th>CM</th>
<th>LB</th>
<th>PC</th>
<th>KK</th>
<th>CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB</td>
<td>-</td>
<td>0.274</td>
<td>0.296</td>
<td>0.244</td>
<td>0.778</td>
<td>0.659</td>
<td>0.259</td>
<td>0.274</td>
</tr>
<tr>
<td>NK</td>
<td>1.294</td>
<td>-</td>
<td>0.918</td>
<td>0.896</td>
<td>0.452</td>
<td>0.735</td>
<td>0.881</td>
<td>0.896</td>
</tr>
<tr>
<td>NR</td>
<td>1.216</td>
<td>0.085</td>
<td>-</td>
<td>0.904</td>
<td>0.459</td>
<td>0.746</td>
<td>0.859</td>
<td>0.933</td>
</tr>
<tr>
<td>CM</td>
<td>1.409</td>
<td>0.109</td>
<td>0.101</td>
<td>-</td>
<td>0.422</td>
<td>0.745</td>
<td>0.852</td>
<td>0.882</td>
</tr>
<tr>
<td>LB</td>
<td>0.251</td>
<td>0.794</td>
<td>0.778</td>
<td>0.862</td>
<td>-</td>
<td>0.682</td>
<td>0.422</td>
<td>0.422</td>
</tr>
<tr>
<td>PC</td>
<td>0.417</td>
<td>0.308</td>
<td>0.294</td>
<td>0.294</td>
<td>0.383</td>
<td>-</td>
<td>0.755</td>
<td>0.768</td>
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<tr>
<td>KK</td>
<td>1.349</td>
<td>0.126</td>
<td>0.152</td>
<td>0.160</td>
<td>0.862</td>
<td>0.281</td>
<td>-</td>
<td>0.911</td>
</tr>
<tr>
<td>CP</td>
<td>1.294</td>
<td>0.109</td>
<td>0.069</td>
<td>0.126</td>
<td>0.862</td>
<td>0.264</td>
<td>0.093</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations are listed in legend of Fig 1.

Table 3
AMOVA.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f</th>
<th>Sum of squares</th>
<th>Variance component</th>
<th>Percentage variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among groups</td>
<td>1</td>
<td>1,525.845</td>
<td>34.323</td>
<td>79.77</td>
</tr>
<tr>
<td>Among populations within a group</td>
<td>6</td>
<td>504.905</td>
<td>5.803</td>
<td>13.49</td>
</tr>
<tr>
<td>Within a population</td>
<td>104</td>
<td>301.741</td>
<td>2.901</td>
<td>6.74</td>
</tr>
<tr>
<td>Total</td>
<td>113</td>
<td>2,332.64</td>
<td>43.03</td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis (*p* = 0.05)

composing of KB and LB populations and of NK, NR, CM, KK, and CP populations for group 2. However, the PK population seemed closely related to both groups. The genetic relationships are illustrated by a dendrogram, constructed by neighbor-joining method (Fig 3). The PK population was grouped with group 1. The cluster diagram clearly showed the separation of the 8 populations into 2 groups.

**Genetic differentiation**

The two divided groups were tested for genetic differentiation (Table 3) and as revealed by the variances among the populations relative to total variance (*Fst* = 0.936; *p* = 0.00), variance among populations within groups (*Fsc* = 0.667; *p* = 0.00), and variance among groups relative to total variance (*Fct* = 0.798; *p* = 0.035), all *F*-statistics show significant genetic differentiation (*p* < 0.05) among the *A. cantonensis* studied for all variables. Percent variation was high between the 2 groups (80%) with low values within parasite groups (7%).

**DISCUSSION**

In Thailand, *A. cantonensis* is distributed in many different geographical
localities (Komalamisra et al, unpublished), and infection has been reported in a broad spectrum of intermediate and definitive hosts (Tesana et al, 2009). In this study, RAPD-PCR method used to reveal the first picture of *A. cantonensis* genetic structure among 8 populations in Thailand. *A. cantonensis* can be divided into 2 major groups, based on genetic distance. Group I comprising parasite populations from Chiang Mai (CM), Nong Khai (NK), Khon Kaen (KK), Chanthaburi (CB), and Narathiwat (NR) and group II from Kanchanaburi (KB) and Lop Buri (LB) Provinces. Group I contains the majority of genotypes distributed throughout most of the country, while group II is restricted to parasite populations around central and western Thailand. Based on variance among groups relative to total variance, the two *A. cantonensis* population groups in Thailand are significantly genetically different. Moreover, genetic differentiations among parasite populations within the same group are also significantly different with high levels of genetic differentiation among the 8 populations. Genetic differentiation among *A. cantonensis*, either between or within groups, or among populations, suggested that the population structure of *A. cantonensis* in Thailand has been affected by population subdivision. The very low gene flow among the populations, high genetic diversity among populations, and a lack of genetic difference within populations, suggested that the *A. cantonensis* population may have created distinct gene frequencies and local fixation of genetic variations within their own populations (inbreeding).

The population structures of parasites are fundamentally related to their hosts. The *A. cantonensis* population in this study appeared to be increasing in genetic structure (pattern of individual genetic makeup within a population) because of local fixation of genetic variations. The sedentary nature of the definitive host, or the high morbidity of all infected hosts (both intermediate and definitive), and the large numbers of specific obligate hosts (broad spectrum of specific hosts) are factors enhancing genetic structure (Nadler, 1995). Rats and snails, which are the definitive and intermediate hosts of

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**Fig 1—Eight different geographical localities in Thailand where *Ac. fulica* samples were collected. CM, Chiang Mai; NK, Nong Khai; KK, Khon Kaen; CB, Chanthaburi; KB, Kanchanaburi; LB, Lop Buri; PK, Prachuap Khiri Khan; and NR, Narathiwat.**
Fig 2–RAPD band patterns of 8 population of *A. cantonensis* collected from KB (lane 1), NK (lane 2), NR (lane 3), CM (lane 4), LB (lane 5), PK (lane 6), KK (lane 7), and CB (lane 8) generated by OPB-20. Primer M = 100 bp DNA ladder. Abbreviations are listed in legend of Fig 1.

Fig 3–Genetic relationship among 8 *A. cantonensis* populations in Thailand. The cluster diagram was constructed by neighbor-joining method. The scale bar indicates genetic distance, based on RAPD polymorphism.

**ACKNOWLEDGEMENTS**

We thank the National Research Council of Thailand for financial support, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University for providing facilities to complete the study, Mr Paul Adams, Office of Research Services, Faculty of Tropical Medicine, Mahidol University for correcting the English of the manuscript.

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