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Characterisation of *Angiostrongylus cantonensis* Isolates from China by Sequences of Internal Transcribed Spacers of Nuclear Ribosomal DNA

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Abstract: The present study examined sequence variations in the Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA (rDNA) among *Angiostrongylus cantonensis* isolates from Shenzhen, Qingyuan, Jiangmen and Wenzhou in China. The ITS of nuclear rDNA was amplified from individual *A. cantonensis* by Polymerase Chain Reaction (PCR) and the representative amplicons were cloned and sequenced. The length of the ITS sequences was 1593-1614 bp for all Chinese *A. cantonensis* specimens and these sequences were composed of complete ITS-1 sequence of 712-720 bp, complete 5.8 S sequence of 153 bp, complete ITS-2 sequence of 633-650 bp and partial 28 S sequence of 70 bp. The intra-specifc sequence variation in *A. cantonensis* was 0.1-1.0% for ITS-1 and 0.0-1.3% for ITS-2 whereas sequence comparison revealed that the inter-specifc sequence differences were higher: 15.0-34.6% for ITS-1 and 22.7-24.2% for ITS-2 between *A. cantonensis* and other *Angiostrongylus* sp. The results showed that the ITS sequences were conserved among the *A. cantonensis* isolates however, they were quite different from that of other *Angiostrongylus* species. Therefore, ITS sequences could provide useful genetic markers for the specific identification and genetic characterization of *Angiostrongylus* sp.

Key words: *Angiostrongylus cantonensis*, Internal Transcribed Spacer (ITS), ribosomal DNA (rDNA), Polymerase Chain Reaction (PCR), sequence analysis, China

INTRODUCTION

Angiostrongylus cantonensis is a rat lungworm usually inhabiting the pulmonary arteries and right ventricle of rats. Human is a non-permissive host becoming infected with *A. cantonensis* when they ingest the infective third-stage larvae of this parasite contained in raw or undercooked food (Thiengo *et al*., 2010). The larvae penetrate into the blood vessels of the human intestinal tract and eventually reach the meninges.

Most of the worms die shortly after reaching the meninges and do not develop into adults. Infection of this parasite in humans leads to eosinophilic meningitis and eosinophilic meningoencephalitis (Bouree *et al*., 2010; Panackel *et al*., 2006; Ramirez-Avila *et al*., 2009). The disease is endemic in Asia and some Pacifc islands. Now, the parasite has been found to infect humans and other mammals across a wide and ever-increasing territory (Prociv *et al*., 2000). The *A. cantonensis* has been given greater attention in both clinical and laboratory studies. Nematodes of the genus *Angiostrongylus* are parasites of carnivores and rodents (Ubelaker, 1986). The occurrence of such parasites in humans emphasizes the importance of a precise differentiation among different *Angiostrongylus* species.

The specific identification of larvae and adult worms among *Angiostrongylus* sp. based on morphological characters is difficult due to vague and similar descriptions in size and body shapes among different species (Robles *et al*., 2008; Ubelaker, 1986). Consequently, molecular methods would provide alternative approaches to characterize and identify *Angiostrongylus* sp. A previous study revealed that *A. cantonensis*, *A. costaricensis* and *A. vasorum* could

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be differentiated by polymerase chain reaction-restriction fragment length polymorphism (Caldeira *et al*., 2003). The 5' end of the Small Subunit (SSU) *rRNA* gene provided a genetic marker to identify infective 3rd juvenile stage of *A. cantonensis* and distinguished it from other species (Fontanilla and Wade, 2008). The *A. vasorum* isolates were characterized using the mitochondrial COI gene and the second Internal Transcribed Spacer (ITS-2) of nuclear ribosomal DNA (rDNA) (Jefferies *et al*., 2009). Recently, phylogenetic relationship was re-constructed for *A. cantonensis*, *A. costaricensis*, *A. malaysiensis* and *A. vasorum* based on *COI* gene sequences and the results showed that the *COI* gene sequences might be a useful marker for differentiating geographical isolates of *A. cantonensis* and for uncovering cryptic species (Eamsobhana *et al*., 2010).

The Internal Transcribed Spacer (ITS-1, ITS-2) sequences have been proven to provide useful genetic markers for the accurate identification of a number of parasite groups (Li *et al*., 2006; Zhu *et al*., 2007; Lin *et al*., 2008). However, prior to the present study, there had been no reports characterizing *A. cantonensis* isolates from China using ITS sequences.

Therefore, the objectives of the present study were to characterize the rDNA region spanning the ITS-1, 5.8 S gene and the ITS-2 of *A. cantonensis* isolates from China and to determine the intra-specific variation within *A. cantonensis* and the inter-specific difference among *Angiostrongylus* sp.

MATERIALS AND METHODS

Parasites and DNA extraction: Adult *A. cantonensis* samples were collected from the lungs of infected rats from different geographical localities in China. Details of each sample used in the study are shown in Table 1. All samples were fixed in 70% molecular grade ethanol and stored at -20°C. Total genomic DNA was extracted from individual worms by treatment with sodium dodecyl sulphate/proteinase K, column-purified (Wizard® SV Genomic DNA Purification System, Promega, Madison) and then eluted into 50 μL H_2O according to the manufacturer's recommendations (Zhu *et al*., 2007). DNA was also isolated from the lung of healthy rats using the same method. DNA samples were stored at -20°C until further use.

Enzymatic amplification: The rDNA region ITS plus primer flanking sequences was amplified by Polymerase Chain Reaction (PCR) using primers NC5 (forward; 5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (reverse; 5'-TTAGTTTCTTTTCCTCCGCT-3'). PCR reactions (25 μL) were performed in 3 mM of $MgCl_2$, 0.5 μM of each primer, 2.5 μL 10×rTaq buffer, 0.2 mM of each dNTPs, 1.25 U of rTaq DNA polymerase (TaKaRa) and 1 μL of DNA sample in a thermocycler (Biometra) under the following conditions: after an initial denaturation at 94°C for 5 min, then 94°C for 30 sec (denaturation); 50°C for 30 sec (annealing); 72°C for 1 min (extension) for 35 cycles followed by a final extension at 72°C for 5 min. Samples without genomic DNA were included in each PCR run as negative controls.

Table 1: *Angiostrongylus cantonensis* samples from China used in the present study and GenBank™ accession numbers of ITS sequences for *A. cantonensis* and other *Angiostrongylus* species

Species	Sample codes	Host	Geographical origin	GenBank™ accession No.
A. cantonensis	AcanSZ1	Rat	Shenzhen, China	HQ540542
	AcanSZ2	Rat	Shenzhen, China	HQ540543
	AcanSZ3	Rat	Shenzhen, China	HQ540544
	AcanSZ4	Rat	Shenzhen, China	HQ540545
	AcanSZ5	Rat	Shenzhen, China	HQ540546
	AcanSZ6	Rat	Shenzhen, China	HQ540547
	AcanQY1	Rat	Qingyuan, China	HQ540548
	AcanQY2	Rat	Qingyuan, China	HQ540549
	AcanJM	Rat	Jiangmen, China	HQ540550
	AcanWZ	Rat	Wenzhou, China	HQ540551
	AcanU	*Parmarion martensi*	Hawaii, USA	GU733322
	AcanB	Unknown	Brazil	GU733321
	AcanP	Unknown	Philippines	EU636008
A. vasorum	AvasG	*Canis familiaris*	Germany	GU045375
	AvasC	*Vulpes vulpes*	Canada	GU045368
A. costaricensis	AvasU	Gastropod	United Kingdom	GU733324
	AcosB	Unknown	Brazil	GU587748

Also host (rat) control-DNA samples were subjected to the same amplification procedures as for parasite DNA. Each amplicon (5 μL) was examined on agarose gels stained with ethidium bromide and photographed using a gel documentation system. The DL 2000 marker (TaKaRa) was utilized to estimate the sizes of the ITS PCR products.

Purification, cloning, sequencing and analysis of ITS rDNA: Representative PCR products were purified using spin columns (Agarose Gel DNA Purification Kit Ver 2.0, TaKaRa) and the purified PCR products were ligated into the pGEM-T Easy plasmid vector (Promega) according to manufacturer's recommendations. The recombinant plasmid was then transformed into JM109 competent cells (Promega) and positive transformants containing recombinant plasmids were selected by PCR amplifcation. Cell cultures with confrmed recombinant plasmid were sent to Shanghai Songon Biological Engineering Biotechnology Company for sequencing. Three colonies from each sample were sequenced from both directions. Pairwise comparisons were made of the level of sequence differences according to a method reported previously (Chilton *et al*., 1995).

RESULTS AND DISCUSSION

Genomic DNA was prepared from 10 individuals of *A. cantonensis* from Shenzhen, Qingyuan, Jiangmen and

Table 2: Pairwise comparison of sequence differences (in%) in the ITS-1 (above the diagonal) and ITS-2 (below the diagonal) rDNA among *Angiostrongylus cantonensis* isolates from China and as well as other *Angiostrongylus* species

Sample codes	Acan SZ1	Acan SZ2	Acan SZ3	Acan SZ4	Acan SZ5	Acan SZ6	Acan QY1	Acan QY2	Acan JM	Acan WZ	Acan U	Acan B	Acan P	Avas G	Avas C	Avas U	Acos B
AcanSZ1	-	0.1	0.3	0.3	0.4	0.1	0.4	0.4	0.4	0.7	0.8	0.8	-	33.6	33.1	33.6	33.2
AcanSZ2	0.6	-	0.1	0.1	0.1	0.3	0.3	0.3	0.3	0.6	0.7	0.7	-	33.6	33.0	33.6	33.0
AcanSZ3	0.8	0.2	-	0.3	0.1	0.4	0.4	0.4	0.4	0.7	0.8	0.8	-	33.8	33.3	33.8	33.3
AcanSZ4	0.3	0.3	0.5	-	0.1	0.4	0.4	0.4	0.4	0.7	0.8	0.8	-	33.8	33.3	33.8	33.3
AcanSZ5	0.6	0.0	0.2	0.3	-	0.3	0.3	0.3	0.3	0.6	0.7	0.7	-	33.4	32.9	33.4	32.9
AcanSZ6	0.2	0.5	0.6	0.2	0.5	-	0.3	0.3	0.6	0.3	0.4	0.4	-	33.9	33.4	33.9	32.6
AcanQY1	0.6	0.0	0.2	0.3	0.0	0.5	-	0.3	0.6	0.3	0.4	0.4	-	33.9	33.4	33.9	32.6
AcanQY2	0.8	0.8	1.0	0.5	0.8	0.6	0.8	-	0.6	0.6	0.7	0.7	-	33.9	33.4	33.9	33.5
AcanJM	0.6	0.0	0.2	0.3	0.0	0.5	0.0	0.8	-	0.9	1.0	1.0	-	33.9	33.4	33.9	33.8
AcanWZ	0.9	1.0	1.1	0.2	1.0	0.8	1.0	0.5	1.0	-	0.7	0.7	-	34.4	33.8	34.4	32.2
AcanU	-	-	-	-	-	-	-	-	-	-	-	0.1	-	34.6	34.1	34.6	34.1
AcanB	-	-	-	-	-	-	-	-	-	-	-	-	-	34.5	33.9	34.5	33.9
AcanP	1.3	0.8	1.0	1.0	0.8	1.1	0.8	1.1	0.8	1.3	-	-	-	-	-	-	-
AvasG	22.7	22.7	22.7	22.7	22.7	22.7	22.7	22.7	22.7	23.2	-	-	22.7	-	0.1	0.1	15.0
AvasC	23.6	23.6	23.6	23.6	23.6	23.6	23.6	23.6	23.6	24.2	-	-	23.7	0.6	-	0.1	15.0
AvasU	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15.0
AcosB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

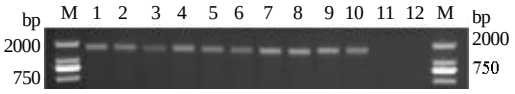

Fig. 1: Agarose gel electrophoresis of amplifed PCR products for the Internal Transcribed Spacers (ITS) of rDNA of *A. cantonensis* samples from different locations in China. Lanes 1-12 represent samples AcanSZ1, AcanSZ2, AcanSZ3, AcanSZ4, AcanSZ5, AcanSZ6, AcanQY1, AcanQY2, AcanJM, AcanWZ, host control and negative (no-DNA) control, respectively. M represents a DNA size marker (ordinate values bp)

Wenzhou in China (Table 1). As expected, a fragment of approximately 1600 bp in length was amplified from each parasite gDNA. On agarose gels, there was no size variation detected among all of the amplicons and no products were amplified from the host or no-DNA control samples (Fig. 1). Representative PCR products were purified and cloned into pGEM-T Easy plasmid vector. The positive recombinant plasmids were selected by bacterial PCR amplifcation.

The obtained rDNA sequences of *A. cantonensis* isolates were 1593-1614 bp in length and their A+T contents were 58.03-58.36%. These sequences were composed of complete ITS-1 sequence of 712-720 bp complete 5.8 S rDNA of 153 bp, complete ITS-2 sequence of 633-650 bp and partial 28 S sequence of 70 bp (GenBank Accession numbers HQ540542-HQ540551).

The lengths and sequences of 5.8 S gene for all *A. cantonensis* isolates were identical whereas there was one nucleotide difference in the 5.8 S rDNA sequences of *A. cantonensis* and *A. vasorum* available in GenBank™ (GU045375 and GU045368). The variation in length of the ITS-1 and ITS-2 sequences among *A. cantonensis* isolates was related to the insertions/deletions of nucleotides within the simple sequence repeats $(AT)_n$. In the ITS-1 rDNA, sequence variation among *A. cantonensis* from the USA (GU733322), Brazil (GU733321) and 4 *A. cantonensis* isolates from China was 0.1-1.0% (Table 2). However, the inter-specifc sequence differences were 15.0-34.6% among *A. cantonensis* and other *Angiostrongylus* species (Table 2). For instance, sequence differences in the ITS-1 between *A. cantonensis* and *A. vasorum* (GU045375, GU045368 and GU733324) were 32.9-34.6% and between *A. cantonensis* and *A. costaricensis* (GU587748) were 32.2-34.1% (Table 2).

Variation in the simple sequence repeats $(AT)_n$ of about 18 bp were detected in the ITS-1 rDNA among *A. cantonensis* samples. Compared with *A. vasorum*, *A. cantonensis* had deletions in the ITS-1 sequences. In the ITS-2 rDNA while the intra-specifc sequence variation among *A. cantonensis* from Philippines (EU636008) and 4 isolates from China was 0.0-1.3%, the inter-specifc sequence differences between *A. cantonensis* and *A. vasorum* (GU045375 and GU045368) were 22.7-24.2% (Table 2). Variation in the simple sequence repeats $(GT)_n$ of about 18 bp were detected among *A. cantonensis* for ITS-2. Compared with *A. vasorum*, the *A. cantonensis* had 2 additional $(GT)_n$ repeats in the ITS-2 sequence.

CONCLUSION

The present study characterized *A. cantonensis* isolates from Shenzhen, Qingyuan, Jiangmen and Wenzhou in China by ITS rDNA sequences. Sequence comparison revealed that the inter-specifc sequence differences among *Angiostrongylus* species were significantly higher than intra-specifc sequence variations within *A. cantonensis*. There were a number of nucleotide positions in the ITS-1 and ITS-2 sequences with no apparent intra-specifc variation but distinct differences

among *A. cantonensis*, *A. vasorum* and *A. costaricensis* which may provide useful genetic markers for the identification and differentiation of different *Angiostrongylus* species.

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