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Original Article

Transcriptome Profiling of Male Adult *Angiostrongylus cantonensis*

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Received 20 Mar 2023
Accepted 09 Jun 2023

Keywords:
Angiostrongylus cantonensis;
Helminths;
Transcriptome

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Abstract

Background: The pathogen of angiostrongyliasis is the parasite *Angiostrongylus cantonensis,* and the transcriptome profiling of the male adult was unclear. We aimed to understand how the male adults adapt, so the expression profile of *A. cantonensis* adult males was analyzed.

Methods: In order to improve the understanding of the transcriptome of adult males, RNA from three groups of male adult *A. cantonensis* was extracted and reverse transcribed to construct cDNA libraries. After sequencing, annotation of unigenes and transcripts was performed by querying the NR (Non-Redundant Protein Sequence Database), GO (Gene Ontology) and COG/KOG (Clusters of Orthologous Groups of proteins/euKaryotic Ortholog Groups) databases.

Results: For each group of adults, 43,260,894 raw reads and 43,200,341 clean reads were obtained. After successful assembly, 87,649 unigenes and 146,895 transcripts were obtained. Annotation of the unigenes and transcripts was identical and male adults expressed a series of genes encoding proteins specific to the male gender at the adult stage, such as proteins involved in energy metabolism, energy synthesis and transport. Expression of the ribosome pathway suggests a relationship with the physical activities during the adult male stage.

Conclusion: The transcriptome analysis is a good reference to understand further the expression profile of male adult *A. cantonensis*.

Introduction

Angiostrongylus cantonensis, also known as rat lung nematode, is the causative agent of angiostrongyliasis (1). In the environment, the definitive hosts for *A. cantonensis* include *Rattus rattus*, *R. norvegicus* and *Sigmodon hispidus* (2). However, humans can also be infected through accidental ingestion of *A. cantonensis*. It is noteworthy that angiostrongyliasis has become a world-wide health threat in recent years where angiostrongyliasis patients have been reported in the United States, United Kingdom, France and elsewhere (3,4). In China, two outbreaks of angiostrongyliasis were recorded in Beijing and Wenzhou (5,6).

Like most nematodes, *A. cantonensis* is a hermaphrodite. Its life cycle is comparatively complex and consists of five larvae stages (L1 to L5 respectively) and the adult stage. L3 is the highly infectious stage towards the mammalian or human host. After migration and development, adult worms reside in the lung of the definitive host, where the worm produces eggs and sperm. Male adult *A. cantonensis* is approximately 11-26 mm in length and 0.2-0.5mm wide and maintain their own morphological and structural characteristics.

The main physiological function of the male adult *A. cantonensis* is to produce sperm and mate with the female to enable the female to produce fertilized eggs and complete its life cycle. At the same time, as a parasitic nematode, male adults also have to face the unique parasitic living environment of the terminal host including the mammalian host immune system. Therefore, male adult *A. cantonensis* most likely expresses stage- and sex-related genes to survive within the host environment. Although the genome of *A. cantonensis* was sequenced (7) and a variety analyses were performed on different life stages of the worm (8-11), male adult transcriptional profile remains unclear.

To understand how the male adults adapt, the expression profile of *A. cantonensis* adult males was analyzed.

Methods

Animal

This study was strictly conducted in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (#2022030201-SGY03， Animal Ethics Committee of Huzhou University).

Material

A. cantonensis positive Sprague Dawley (SD) rats at 45dpi (day post infection) were humanely euthanized after anesthesia. Male adult *A. cantonensis* was collected from the lungs. In total, 3 biological replicates were included in this study and each rat provided one male adult *A. cantonensis*.

Total RNA extraction of adult male A. cantonensis

Each of the *A. cantonensis* adult male worms from were washed with Phosphate Buffered Saline (PBS, ThermoFisher Scientific, catalog # 10010023) 3 times, after which, 1.5 ml TRIzol (Invitrogen, catalog # 15596026) was added, worms were ground on ice and total RNA was extracted. DEPC-treated-water (20-40 µl, ThermoFisher Scientific, catalog # R0601) was used to dissolve the total RNA samples, which were then stored at -80 °C until required. The purity and concentration of the total RNA were determined using the Nanodrop and the RNA integrity was confirmed by gel electrophoresis.

Sequencing of adult male A. cantonensis's RNA

To obtain mRNA for sequencing, mRNA in the total RNA of the replicate samples was

enriched using Oligo (DT) magnetic beads to discard rRNA. Then, RNA lysate was added to the total mRNA, which was then fragmented and amplified with random primers to synthesize the first cDNA strand (ThermoFisher Scientific, catalog #K1651). The second cDNA strand was synthesized with the first cDNA strand as the template to establish the cDNA library (ThermoFisher Scientific, catalog #A48571). The library was sequenced using the Illumina HiSeq TM 2000 platform.

Annotation of sequence reads and expression profiling

The total sequencing reads were subjected to filtering to obtain clean reads using the pipeline, which is briefly described as follows: first the joint sequence of the reading section was removed, then the reading section with N ratio greater than 10% and/or Q ≤ 5 and/or alkali base ＞50% were removed. Here, the assembly of transcriptome data adopts Trinity method.

The obtained unigenes and transcripts were annotated with Gene Ontology (GO). UniGene and transcript were compared with NR (NCBI Non-Redundant Protein Sequence Database) and COG/ KOG (e-value < 0.000 01, Clusters of Orthologous Groups of proteins/euKaryotic Ortholog Groups) databases by blastx.

Results

Sequencing results and data assembly of male adult A. cantonensis

RNA sequencing of the replicate worms yielded 129,782,682 raw reads and after filtering, 129,601,024 clean reads were obtained. On average, each group produced 43,260,894 raw reads and 43,200,341 clean reads (Table 1).

Table 1: Information on the RNA sequencing output

Sample_ID		*Total_Reads*	*Total_Bases*	*Error%*	*Q20%*	*Q30%*	*GC%*
MA_1	Raw data	43070334	6503620434	0.0241	98.25	95.23	45.57
MA_2		44800672	6764901472	0.0238	98.35	95.49	46.01
MA_3		41911676	6286751400	0.0245	98.08	94.76	45.43
MA_1	Clean data	43005692	6426204972	0.0237	98.5	95.52	45.49
MA_2		44740012	6687300072	0.0235	98.58	95.76	45.93
MA_3		41855320	6218215012	0.0241	98.32	95.05	45.34

Reads annotation of male adult A. cantonensis

After filtering, 87,649 unigenes and 146,895 transcripts were obtained and annotated using the nr，Swissprot，String，COG，KOG，NOG (Non-supervised Orthologous Groups)，GO databases. The distribution was as follows: 65.39%（57,312/87,649）unigenes were 201-400 bp，13.50%（11,836/87,649）were 401-600 bp while 46.66%（68,534/146,895）of transcripts were in the range of 201-400 bp, 13.2%（19,394/146,895) were 401-600bp in length (Table 2).

Table 2: Unigenes and transcripts obtained after annotation

Variable	*Unigene*	*Transcripts*
Total sequence num	87649	146895
Total sequence base	52459376	137685397
Percent GC	43.23	42.92
Largest	33896	33896
Smallest	201	177
Average	598.52	937.3
N50	932	1867
N90	248	325

Annotation of male adult A. cantonensis unigenes

The top 4 ranking unigene-related species as annotated based on the non-redundant (nr) database were *Ancylostoma ceylanicum* with 4036 related unigenes, *Dictyocaulus viviparus* with 1769 homologous unigenes, *Haemonchus contortus* (1557 unigenes) and *A. cantonensis* with 1228 related unigenes. The top 5 functional groups that consist of the annotated unigenes based on GO classification were as follows: GO:0005623 contained 7417 unigenes annotated with functions related to cellular component; GO:0044464 also associated with cellular components contained 7412 unigenes; 7135 unigenes were associated with roles in biological process (GO:000998; GO:0043226-related 6645 unigenes also coding for functions related to cellular component; GO:0005488 associated with molecular functions that contained 6404 unigenes.

COG database annotation of unigenes showed the top 5 most highly related functional items were: [R] General function (739 unigenes), [T] Signal transduction mechanisms (630 unigenes), [O] Post-translational modification, protein turnover, chaperones (512 unigenes), [J] Translation, ribosomal structure and biogenesis (471 unigenes), [C] Energy production and conversion (369 unigenes). KOG annotation of unigenes are: [T] Signal transduction mechanisms (related with 1321 unigenes),[R] General function (970 unigenes), [O] Post-translational modification, protein turnover, chaperones (889 unigenes), [K] Transcription-related with 542 unigenes, [J] Translation, ribosomal structure and biogenesis (502 unigenes) (Fig. 1).

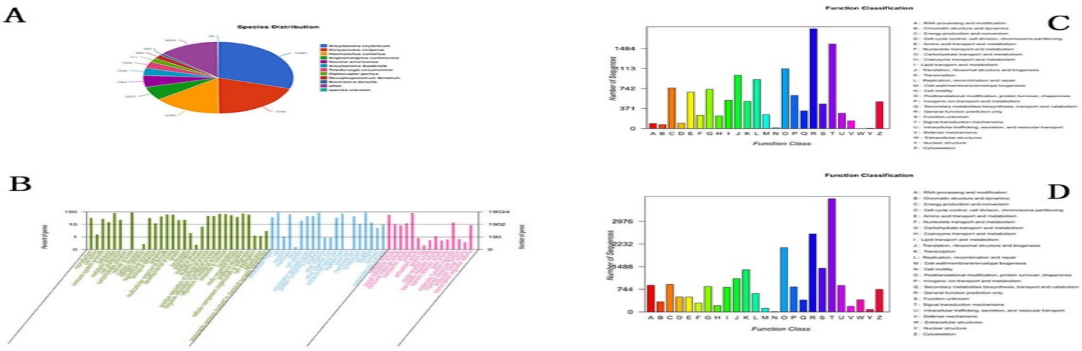

Fig. 1: Unigenes Annotations of A. cantonensis provided by NR, GO and COG/KOG

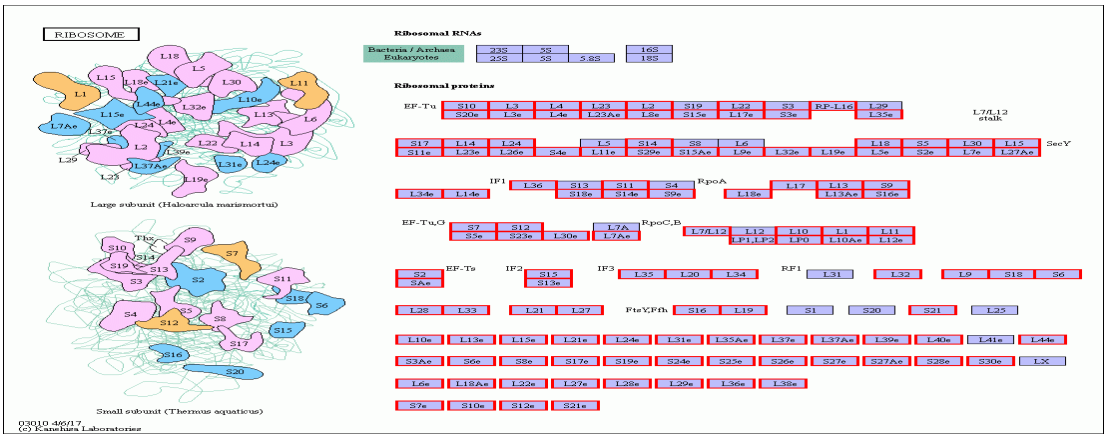

Fig. 2: Ribosome pathway, unigenes analyse result by querying KEGG (Encyclopedia of Genes and Genomes) database

Table 3: The top 10 most-related unigenes annotated by ORF Finder

Seq_id	*Protein_id*	*Pfam_id*	*Domain*	*DomainDescription*	*DomainE-Value*
TRINITY_DN78_c0_g2	TRINITY_DN78_c0_g2_i10|m.21	PF01496.16	V_ATPase_I	V-type ATPase 116kDa subunit family	0
TRINITY_DN9006_c0_g1	TRINITY_DN9006_c0_g1_i1|m.103	PF00311.14	PEPcase	Phosphoenolpyruvate carboxylase	0
TRINITY_DN17851_c0_g1	TRINITY_DN17851_c0_g1_i1|m.215	PF00305.16	Lipoxygenase	Lipoxygenase	0
TRINITY_DN42022_c0_g1	TRINITY_DN42022_c0_g1_i3|m.260	PF01044.16	Vinculin	Vinculin family	0
TRINITY_DN1707_c0_g1	TRINITY_DN1707_c0_g1_i8|m.1351	PF00063.18	Myosin_head	Myosin head (motor domain)	1.50E-295
TRINITY_DN2743_c0_g1	TRINITY_DN2743_c0_g1_i5|m.1201	PF05693.10	Glycogen_syn	Glycogen synthase	5.10E-290
TRINITY_DN59450_c0_g1	TRINITY_DN59450_c0_g1_i1|m.69	PF01496.16	V_ATPase_I	V-type ATPase 116kDa subunit family	6.80E-290
TRINITY_DN5363_c0_g1	TRINITY_DN5363_c0_g1_i5|m.634	PF02460.15	Patched	Patched family	1.10E-275
TRINITY_DN5820_c0_g1	TRINITY_DN5820_c0_g1_i1|m.1613	PF00012.17	HSP70	Hsp70 protein	8.90E-274
TRINITY_DN946_c0_g1	TRINITY_DN946_c0_g1_i2|m.2219	PF00821.15	PEPCK	Phosphoenolpyruvate carboxykinase	8.80E-270

A. Unigenes annotated using the nr database to the species level; B. Unigenes annotation using the GO database; C. Annotation of unigenes as queried against the COG database; D. Unigenes annotation based on the KOG database (Table 3).

The unigenes were analyzed using the KEGG (Encyclopedia of Genes and Genomes) database and the most related pathway was the ribosome pathway involving 1556 unigenes as shown in Fig. 2.

Annotations of male adult A. cantonensis transcripts

When the transcripts were submitted to the nr database, the most related species were *Ancylostoma ceylanicum* (10,981 transcripts that were homologous), *Dictyocaulus viviparous* (7,299 transcripts) and *Haemonchus contortus* (5380 transcripts). The top 5 most rated GO annotation of transcripts at the secondary level were: GO: 0005623 (related to 17,814 transcripts), GO:0044464 (17,802 transcripts), GO:0009987 (17,125 transcripts), GO:0043226 (16,336 transcripts) and GO:0005488 (15,246 transcripts).

The transcripts were also queried against the COG and KOG databases. The query against COG resulted in the following 5 most related items: [R] General function prediction only (1,851 transcripts), [T] Signal transduction mechanisms (1,569 transcripts), [O] Post-translational modification, protein turnover, chaperones (1,109 transcripts), [J] Translation, ribosomal structure and biogenesis (989 transcripts). Meanwhile, the top 5 most related KOG queried outcomes were: [T] Signal transduction mechanisms (3,718 transcripts), [R] General function prediction only (2,565 transcripts), [O] Post-translational modification, protein turnover, chaperones (2,113 transcripts), [S] Function unknown (involving 1,437 transcripts), [K] Transcription (1,387 transcripts) (Fig. 3).

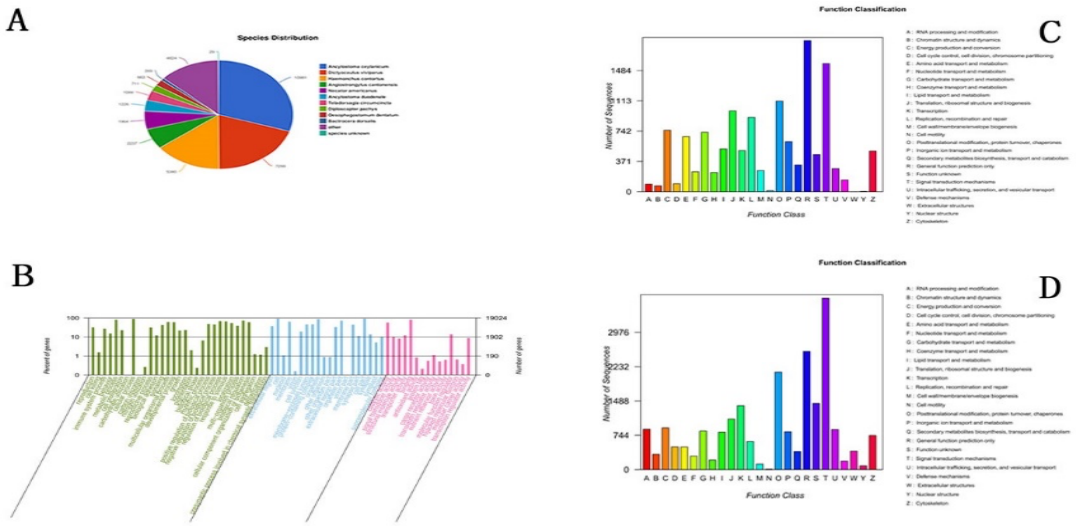

Fig. 3: Annotation of transcripts of A. cantonensis provided by the NR, GO and COG/KOG databases. A. Transcript annotation from the nr database to the species level; B. Transcript annotation according to the GO database ; C. Transcript annotation based on the COG database; D. Transcript annotation by the KOG database information.

Discussion

In recent years, next generation sequencing (NGS) represented a powerful tool on transcriptional and genome researches. In this study, we used the NGS to analyze the male adult transcriptional profile of *A. cantonensis* by querying the GO, COG/KOG and KEGG databases.

GO annotation identified the typical three functional categories: biological process，cellular component and molecular function. In total, 19,024 transcripts and 7,952 unigenes were successfully annotated using the GO database. The identity of the functional

groups for a number of transcripts and unigenes were noted to overlap and these were GO:0005623，GO:0044464，GO:0009987，GO:0043226 and GO:0005488.

When the COG/KOG databases were used for annotation, 20,974 and 10422 unigenes and transcripts were successfully annotated with the results from individual databases showing similar annotations. The top 5 most related items were [R] General function prediction only (poorly characterized), [O] Post-translational modification, protein turnover, chaperones (cellular processes and signaling), [J] Translation, ribosomal structure and biogenesis (information storage and processing), [T] Signal transduction mechanisms (cellular processes and signaling), [G] Carbohydrate transport and metabolism (metabolism).

KEGG annotation showed that 1,556 unigene were related to ko03010 which is similar to the 3 most related COG/KOG annotation of unigenes and transcripts i.e. translation, ribosomal structure and biogenesis. These results suggested that ribosome related genes and pathways might play important functions in male adult *A. cantonensis*, including robust metabolism and spermatogenesis. ORF annotation results indicated TRINITY_DN78_c0_g2 and TRINITY_DN59450_c0_g1 were related with energy metabolism, TRINITY_DN2743_c0_g1 related with energy synthesis, TRINITY_DN1707_c0_g1 is involved with material transportation, while TRINITY_DN5820_c0_g1 plays a role in host-parasite interaction.

NGS is not a common platform to analyze transcriptomes of all organisms including worms, for example, in efforts to understand gender-level differences in worms [1, 2]. By comparing the transcriptome sequences of *Brugia malayi* from different periods and different genders, a series of highly expressed genes were identified, including structural-related genes in male adults (GO:0005198), which involves high expression of sperm-related structural proteins (12). Here in this study, we also found that 785 unigenes and 1,577 transcripts were involved in this GO entry, including some structural proteins related to spermatogenesis. Others reports on the transcriptome of the free-living nematode *Caenorhabditis elegans* showed phosphorylation and dephosphorylation of some structural proteins and ubiquitin-related proteins were closely related to the physiology of male adults (13-15), which correlate well with the results from our study.

Sequencing of *A. cantonensis* has been completed (16), however, annotation of the genome is incomplete.

Conclusion

This study is the first to describe the transcriptional profile of male adult *A. cantonensis* using the next-generation sequencing platform. The outcome of this study will assist in future efforts to understand gene expression profiles of male adult *A. cantonensis*.

Acknowledgements

This research was supported by Huzhou Natural Science Foundation (Grant No. 2022GY06) and the Key Laboratory of Vector Biology and Pathogen Control of Zhejiang Province, Huzhou University (Grant No. HUZUL 202101).

Conflict of Interest

The authors declare that there is no conflict of interests.

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