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Original Article

Identification and Characterization of a Differentially Expressed Gene (07E12) in the Infective Larvae of the Parasitic Nematode *Ascaris suum*

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Abstract

Background: Parasitic nematodes cause animal and human diseases of major socio-economic importance worldwide. The suppression of parasite development at particular developmental stages could provide an alternative approach for nematode control. In this study, *Ascaris suum* was used as a model system in the study of the differentially expressed genes in the infective L3 stage.

Methods: The gene (07E12) was screened and identified from the subtractive cDNA library for the infective larvae of *Ascaris suum* using real-time quantitative PCR. Then, the full-length cDNA of 07E12 was characterized by 3' and 5' rapid amplification of cDNA ends (RACE). The characteristics of the gene were further analyzed using bioinformatic analyses.

Results: The results showed that the gene 07E12 was differentially expressed in the third-stage larvae of *A. suum* and its expression level in the infective larvae was much higher than in other stages. It was shown that the gene 07E12 had 99% identity with the corresponding sequences of the *A. suum* whole genome shotgun sequence containing the homologous sequences with conserved sequences of Neuropeptide-Like Protein family member. Likewise, by performing BLASTN and BLASTP searches in the GenBank™, it was shown that this gene had 99 % identity with *A. suum* cre-nlp-2 protein.

Conclusion: This gene 07E12 which is differentially expressed in the third-stage larvae of *A. suum* may encode a neuropeptide-like protein family member, a very important molecule in the process of infecting a host.

Introduction

Ascaris suum infection causes major economic losses throughout the world in the pig and pork industries and farming communities due to animals' poorer food conversion and weight gains, and the need to condemn affected organs after slaughter. In China, *A. suum* is the most common parasite in pigs. Its average prevalence is approximately 30% nationwide, which translates into an estimated economic loss of hundreds of million dollars per year (1). In addition, *A. suum* can cause visceral larva migrans (VLM) in humans (2). The excessive and uncontrolled use of anthelmintic drugs has caused serious problems of resistance in nematodes and led to a greater risk of build-ups of drug residues in animal products and the environment. Therefore, there is substantial interest in the development of alternative methods of nematode control. Now that the whole genome sequences of *A. suum* are available (3), a growing number of key genes are being revealed. It was demonstrated that *Ascaris* miRNAs and endo-siRNAs are differentially expressed during gametogenesis and throughout development in the L2 larvae(4). The number and abundance of specific miRNAs increase and predominate in the larvae, while the levels of endo-siRNAs significantly decrease. This transition indicates that siRNA pathways are dominant in the germline, whereas miRNA pathways prevail in embryo and larval development.

Many genes play important roles in host invasion by infective larvae and include genes that encode surface molecules, excretory/-secretory products from nematodes, genes involved in the evasion of host responses and genes related to parasite feeding within the host, particularly infective larvae-specific genes (3, 5). In this study, *A. suum* was used as a model system in the study of the differentially expressed genes in the infective L3 stage, since the molecular mechanism underlying the invasion of the host by infective larvae of *A. suum* is not known.

Real-time quantitative PCR (RT qPCR) is a variation of the standard PCR technique used to quantify DNA or RNA in a sample. Using sequence-specific primers, the relative number of copies of a particular DNA or RNA sequence can be determined. There are numerous applications of this technique in the field of genetics: gene deletion, gene duplication, measuring the expression of genes of interest, validating micro-array experiments and monitoring biomarkers (6-11). In RT qPCR, the amount of PCR product is measured at each cycle. This ability to monitor the reaction during its exponential phase enables users to determine the initial amount of target with great precision. The use of RT qPCR has nearly replaced all other approaches (e.g. Northern blotting, RNase protection assays) (12, 13).

The rapid amplification of cDNA end (RACE) technique is a method that enables the 3' and 5' fragments of cDNA to be obtained rapidly (14). RACE is cheaper, much faster, requires only very small amounts of primary material, and provides rapid feedback on the generation of the desired product. RACE procedures have been used successfully to obtain 3' and 5' cDNA ends when some sequences from the internal portion of a gene are known (15, 16). It provides an inexpensive and powerful tool for obtaining quickly full-length cDNA when the sequence is only partially known. Starting with an mRNA mixture, gene-specific primers generated from the known regions of the gene and from non-specific anchors full-length sequences can be identified in as little as three days. RACE can also be used to identify alternative transcripts of a gene when the partial or complete sequence of only one transcript is known (17).

On the basis of our previous study (18), in the present study the differentially expressed gene 07E12 was identified and characterized from the subtractive cDNA library for the in-

fective larvae of *A. suum* using real-time PCR and rapid amplification of cDNA ends (RACE).

Materials and Methods

Production of Different Developmental Stages of A. suum

A. suum was used as a model system in the study of the differentially expressed genes in the infective L3 stage, from which point on the infective larvae develop to infective stages in eggs (19). Pigs are infected by ingesting eggs containing infective larvae that then hatch, penetrate the cecum (20) and migrate to the livers, lungs and, finally, to the small intestine before developing into adults (21). The methods of producing different developmental stages of *A. suum* were as follows. Adult worms (males and females) of *A. suum* were collected from the small intestines of swine from an abattoir in Longyan, China. Infective eggs and infective L3s of *A. suum* were produced according to previously described methods (22). In brief, eggs from the uteri of adult females of *A. suum* were collected and incubated at 28 °C for 28 days to allow them to develop into infective eggs (containing infective L3s). To obtain pure infective L3s, 7.5% v/v sodium hypochlorite was used to treat the larvated eggs at 37 °C overnight, after which the eggs were shaken with glass-beads. Then, the exsheathed L3s and shells were separated by density gradient centrifugation using lymphocyte separating medium (LSM) (22). Following the experimental infection with infective *Ascaris* eggs (23), of helminth-free pigs (8-12 weeks of age) provided by the Experimental Animal Center of Longyan University, L3s from livers and in lungs, as well as L4s in intestines, were isolated according to an established method. All parasite materials were snap-frozen in liquid nitrogen prior to storage at -70 °C.

RNA Isolation and Real-time quantitative PCR

Samples from adult females and males and different larval stages or eggs of *A. suum* were frozen in liquid nitrogen and then ground into a fine powder. The total RNA was subsequently extracted using RNAisoplus (TaKaRa, D9108A) reagent according to the manufacture's instructions and dissolved in diethypyrocarbonate-treated water. The cDNAs used for real-time PCR were synthesized from total RNA using PrimeScript™ RT reagent Kit (Perfect Real-Time) (TaKaRa, DRR037S). The reaction mixture contained 2 μL 5 × PrimeScript Buffer, 0.5 μL PrimeScript RT Enzyme Mix I, 0.5 μL Random 6 mers (100 μM), 0.5 μL Oligo dT Primer (50 μM), 1 μL Total RNA and nuclease-free water in a final volume of 10 μL. Amplification was carried out by subjecting the samples to the following conditions: 37 °C for 15 min, followed by 85 °C for 5 s.

Based on the known 07E12 EST sequence (GeneBank accession no. ES290999) from the subtractive cDNA library for the infective larvae of *A. suum* (18), we designed a set of primers (F: 5'- CGGCACTCTTTCATTACT -3' and R: 5'- CGGGTCGGATCCAACTCT -3') according to the parameters required for the RT qPCR applications. The actin gene was used as a reference. PCR products were then quantified continuously with the Mastercycler ep realplex 2 System (Eppendorf, German) using SYBR® Premix Ex Taq™ (Perfect Real-Time) (TaKaRa, DRR041A) according to the manufacturer's instructions. The reaction mixture contained 12.5 μL SYBR® Premix Ex Taq™ (2 ×), 0.5 μL PCR Forward Primer (10 μM), 0.5 μL PCR Reverse Primer (10 μM), 2 μL cDNA and nuclease-free water in a final volume of 25 μL. The PCR amplification profile was 95 °C for 10 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Expression level was calculated using the $2^{-\Delta\Delta Ct}$ method (24) and presented in relation to gene expression in different stages of *A. suum*. Each expression assay was performed twice.

3' and 5' RACE

The 3' RACE System for Rapid Amplification of cDNA Ends (Takara) and 5' RACE System for Rapid Amplification of cDNA Ends (Takara) were used to perform 3' and 5' RACE for the 07E12 EST expressed differentially in the infective L3 stage of *A. suum*. For 3' RACE, first-strand cDNA was synthesized from 1 µg total RNA using the adaptor primer provided by the manufacturer. Target cDNA was then amplified by using the 3' RACE Outer Primer and a forward, gene-specific primer (5' -GAAGAACGCCGTGCGAAAA-3') based on the sequence of the 07E12 EST. PCR was performed at 94 °C for 3 min and 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, followed by an additional 10 min at 72 °C. For 5' RACE, template cDNA was synthesized from 3 µg total RNA, which was treated by CIAP and TAP and ligated with the 5' adaptor according to the manufacturer's protocol, using the random 9 primer provided by the manufacturer. For 5' RACE, target cDNA was amplified by using the 5' RACE Outer Primer and gene-specific reverse primer (5'-GGAATCCAACTCTGCCAAGC-3') according to the manufacturer's protocol. PCR was performed at 94 °C for 3 min and then 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, followed by an additional 10 min at 72 °C. The 3' and 5' RACE products were ligated into pMD20-T Vector (Takara) and then transformed into competent *E. coli* strain JM109. Finally, the DNA sequences were determined and submitted to GenBank.

Bioinformatics analyses

The sequences of 5' and 3' RACE products were edited and assembled using DNAStar. According to the assembled sequences, a set of primers was designed and PCR was performed in order to test and verify the sequences using the above protocols. Similar sequences were identified by performing online BLASTN searches (http://www.ncbi.nlm.nih.gov/BLAST). The deduced amino-acid sequence was assembled, analyzed and aligned with similar sequences using BLASTP.

Results

Analysis of 07E12 gene expression in different developmental stages of A. suum

Real-time quantitative PCR amplification was successful in all examined samples of *A. suum*.

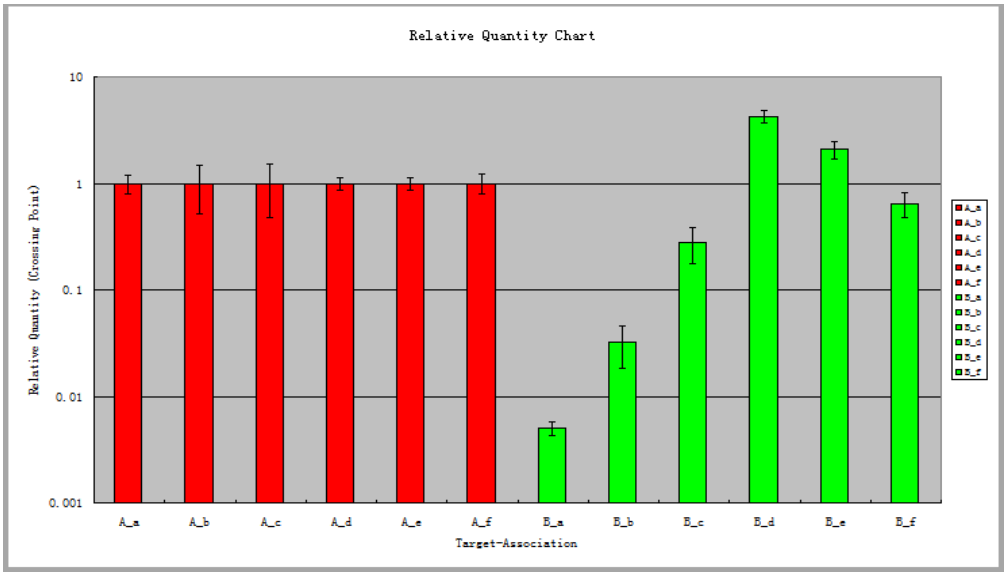

Fig. 1: Expression levels of ß-actin gene (control) and 07E12 gene (target gene) in different developmental stages of *A. suum* using real-time quantitative PCR

The expression changes in gene 07E12 in different developmental stages were evaluated. Although the control showed no distinct change in any stage, gene 07E12 was differentially expressed in the infective-stage larvae, in which the expression level is much greater than in other stages (Fig. 1).

The highest transcriptional level of 07E12 was expressed in the infective larvae, which indicates that the gene could play an important role in the development process of the infective larvae. A_a, A_b, A_c, A_d, A_e and A_f represent the expression levels of ß-actin gene in females, males, eggs, infective larvae (infective L3s), third-stage larvae (L3s) and fourth-stage larvae (L4s), respectively (black columns). B_a, B_b, B_c, B_d, B_e and B_f represent the expression level of 07E12 gene in females, males, eggs, and infective L3s, L3s and L4s, respectively (grey columns)

The full-length cDNA of 07E12 sequence analysis

The 3' and 5' RACE products for 07E12 were amplified and characterized. Sequencing revealed that the 3' RACE sequence was 699 bp in length, while the 5' RACE sequence was 539 bp long. The 3' and 5' RACE sequences were edited and assembled, giving a full-length for gene 07E12 of 719 bp. The primers were designed (F: 5'- AGTGTTCCATTTGAATGTGT -3' and R: 5'- GCATGGCATAAACAAAATTCTG -3') according to the assembled sequence and the full-length cDNA was verified (Fig. 2). The full-length cDNA has an open reading frame (ORF) of 558 bp and similar sequences were identified by performing BLASTN searches in the GenBank™. This gene has 99 % identity with *A. suum* whole genome shotgun sequence (accession no. AEUI02000055, ANBK01004475 and AMPH01002048), but was found to be an uncharacterized gene and could encode Neuropeptide-Like Protein family member (nlp-2). The full-length nucleotide sequence was deposited in the GenBank™ database and assigned the accession no. JZ107277. The deduced amino acid sequence from the ORF of gene 07E12 had a predicted molecular mass of 20.68 kDa and 185 amino acids. Similar sequences were identified by performing BLASTP searches in the GenBank™. A multiple sequence alignment of the deduced amino acids of gene 07E12 ORF with other relevant sequences is shown in Fig. 3. The translated ORF of *A. suum* gene 07E12 shared 99% identity with *A. suum* cre-nlp-2 protein and has homologous conserved sequences of Neuropeptide-Like Protein family member (SMAMGRLGLRP and SIALGRSGFRP). In addition, the translated ORF of gene 07E12 has 55%, 54%, 54% and 48% identities with the putative 19.60 kDa cre-nlp-2 protein of *Caenorhabditis remanei*, 19.40 kDa nlp-2 protein of *C.elegans*, 19.39 kDa cbn-nlp-2 protein of *C. brenneri,* and 19.20 kDa cbr-nlp-2 protein of *C. briggsae*, respectively.

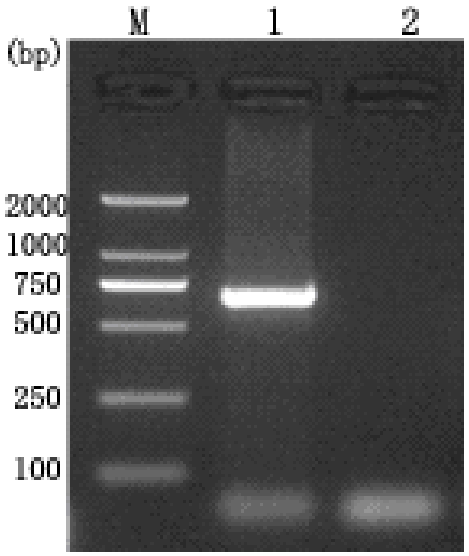

Fig. 2: Verification result of the assembled full-length cDNA. Lane M represents the DL 2000 DNA size marker (Takara). Lane 1 represents the overlapping 3' end cDNA and 5' end cDNA. Lane 2 is the negative control

Fig. 3: Alignment of deduced amino acid sequences of 07E12 ORF differentially expressed in *A. suum* infective larvae compared with those of related sequences available in the GenBank™. Number 1 represents the 07E12 protein, numbers 2 to 6 represent 20.76 kDa cre-nlp-2 of *A. suum* (ERG86101), the putative 19.60 kDa cre-nlp-2 protein of *C. remanei* (EFO96454), 19.40 kDa nlp-2 protein of *C.elegans* (CCD71462), 19.39 kDa cbn-nlp-2 protein of *C. brenneri* (EGT30199), and 19.20 kDa Cbr-nlp-2 protein of *C. briggsae* (CBG02131), respectively. Underlines sequences represent homologous conserved sequences of Neuropeptide-Like Protein family member

Discussion

The present study demonstrates that the gene 07E12 has its highest expression level in the infective larvae and 99% identity with *A. suum* whole genome shotgun sequence; it is also a previously uncharacterized gene in *A. suum*. This gene had a similarity of 99% with *A. suum* cre-nlp-2 protein and with conserved sequences of the Neuropeptide-Like Protein family member (SMAMGRLGLRP and SIALGRSGFRP) detected by performing

BLASTP searches in the GenBank™. Hence, it is possible that gene 07E12 encodes a Neuropeptide-Like Protein family member in the infective larvae of *A. suum*.

Neuropeptides are the types of endogenous actively substances that exist in organisms' nerve tissue and participates in nervous system functions, which act as chemical signals in the nervous system to modulate behavior (25). Neuropeptides are also predicted to be in non-neuronal tissues, including intestine, somatic gonad, muscle, and hypodermis (26). Secretion of neuropeptides from these tissues is likely to have an endocrine role. These genes have been divided into three main categories: the ins genes, which encode most of the insulin-like peptides, the flp genes, which encode the FMRFamide-related peptides, and the nlp genes, which encode non-insulin, non-FMRFamide- related peptides.

Many of the isolated or predicted peptides in parasitic nematodes, such as *A. suum*, *Haemonchus contortus*, *Ancylostoma caninum*, *Heterodera glycines* and *Meloidogyne arenaria*, are identical or highly similar to *C. elegans* peptides, suggesting that the function of these peptides is similar across species (25). Comparison of *A. suum* neuropeptide sequences with those found in *C. elegans* revealed an interesting combination of conserved and divergent peptide sequences within the superfamily of RFamide-related peptides (27). Neuropeptide-like protein (nlp) genes - encode peptides distinct from the FaRP family could be responsible for the majority of neuropeptide signaling in this animal. 32 nlp genes were identified in *C. elegans* and defined the genes at least 11 families of putative neuropeptides with unique motifs; similar expressed sequence tags were identified in other invertebrate species for all 11 families (26, 27). Six of these families are defined by putative bioactive motifs (FAFA, GGxY-amide, MRxamide, LQFamide, LxDxamide, and GGARAF); the remaining five families are related to allatostatin, myomodulin, buccalin_drosulfakinin, orcokinin, and APGWamide neuropeptides (MGL_Famide, FRPamide, MSFamide, GFxGF, and YGG-Wamide families, respectively). Of these nlp genes, nlp-2 belongs to FRPamide family with nlp-22 and nlp-23. The nlps are a diverse group of neuropeptides that have little similarity among each other and many of the nlps genes are expressed in chemosensory neurons, which mediate responses to the environment, and the HSN neuron, which regulates egg-laying in *C. elegans* (27). The predicted nlp-1 and nlp-2 neuropeptides have a certain similarity with buccalin and myomodulin, respectively. Putative neuropeptides containing an FRPG are also found within ESTs from *Toxocara canis*, which is similar to nlp-2 (28).

It has been suggested that neuropeptides in nematodes play widespread and varied roles in the nervous system via cellular expression patterns and genetic analysis. Because the anatomical comparison of *C. elegans* with *A. suum* shows strikingly similarities, it may be the case that these nematodes adapt to their very different environments by varying the location and function of intracellular messengers rather than evolving an entirely novel neural structure (29). *A. suum* already has the preliminary nervous system and the nlp genes could play an important role in the process. Some nlp genes encode FMRFamide-related peptides can influence tension and contractility of neuromuscular strips isolated from *A. suum* body wall (30). In the present study, the gene (07E12) encode cre-nlp-2 protein may be resistant to animal immune response since the gene was previously screened from the subtractive cDNA library for the infective larvae of *A. suum* (18) and the previous RNAi assay showed that the RNAi effect was evident after soaking 96h in RNA-treated worms by RT-PCR detecting (31).

Neuropeptides are integral to behavior and nervous systems in animals. However, the functional studies to individual neuropeptide gene have been still less reported thus far, especially in parasitic nematode. In the present study, the gene encode nlp protein was identified, but the specific function about the gene

is still not clear, although it also was found in other species. Yew (29) has made to catalogue and map known and predicted peptide sequences in *A. suum* by combining peptide gene information with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and MALDI-based Fourier transform (MALDI-FT) mass spectrometry and the results showed that the expression profile of 41 peptides has been mapped in the major nervous structures of *A. suum*. Seventeen of these peptides are considered to be novel in *A. suum*, and their expression has not been previously reported. Some insulin-like peptides and the FMRFamide-related peptides have been more studied and identified in *C. elegans* and other species including *A. suum,* comparing to the non-insulin, non-FMRFamiderelated peptides (nlp) thus far. Further characterization of the nlp genes is likely to provide a greater understanding of mechanisms involved in neuropeptide function in development and behavior.

Conclusion

The present study identified a gene differentially expressed in the infective larvae of *A. suum* by using real-time quantitative PCR and RACE. This gene may encode a neuropeptide-like protein family member, a very important molecule in the process of infecting a host. Further studies of the expression of this gene *in vitro* are warranted to elucidate its biological functions during the process of development or infection in the infective larvae of *A. suum*.

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