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Temporal quantification of Ts43 gene expression of *Trichinella spiralis* using real-time RT-qPCR

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ABSTRACT

Determination of the transcription level of *Trichinella spiralis* (*T. spiralis*) Ts43 gene is essential for understanding its poorly explained role in different developmental stage. Herein we report on measurement of *T. spiralis* Ts43 mRNA by adapting a relative quantitative real-time reverse transcription and polymerase chain reaction (RT-PCR). Total RNA was isolated from the samples of adult worms, newborn larvae and muscle larvae of *T. spiralis* and Ts43 mRNA was quantified by real-time RT-PCR using an internally calibrated standard curve constructed with the recombinant Ts43 and 18S rRNA plasmid. The results indicated that the Ts43 gene was expressed at any stage and the expression levels in adults and newborn larvae were lower than those in muscle larvae in general. The expression of the Ts43 gene increased from 18 days post-infection (p.i.), reached a peak at 22–30 days p.i., and then decreased to a low level by 58 days p.i. but it was still higher than that in adults and newborn larvae. The study provided the first quantitative of Ts43 mRNA expression in *T. spiralis* for further studies of Ts43 function.

1. Introduction

Trichinellosis is one of the most important parasitic zoonotic diseases, caused by ingestion of insufficiently heated or raw meat infected with *Trichinella* muscle larvae. Digestive juices from the stomach dissolve the capsule-like cyst and release the larvae which pass into the small intestine where they invade the columnar epithelium. Then the larvae mature to adults. Female worms can produce newborn larvae before expulsion by the host immune system. The migratory phase of infection begins when these newborn larvae are passed into tissue by the circulation. Following invasion they undergo development to mature muscle larvae. Severe cases of trichinellosis can occur in humans and animals, resulting primarily from sequelae associated with the muscle phase, and the disease is endemic in several parts of the word (Jasmer, 1995; Pozio, 2001; Uparanukraw and Morakote, 1997; Mitreva and Jasmer, 2006).

T. spiralis is an intracellular parasite of mammalian skeletal muscle. Infection of the host muscle cell leads to the development of an intimate host/parasite complex called the nurse cell. The process of nurse cell formation is complex and has been the subject of extensive investigation (Kuratli et al., 1999; Wu et al., 2006). Infection with the nematode alters the pattern of gene expression of the host cell in a process thought to represent muscle cell de-differentiation. It has been hypothesized that excrete-secrete (ES) proteins secreted by *T. spiralis* into the cytoplasm of the infected skeletal muscle are involved in the mechanisms of muscle cell de-differentiation and in nurse cell formation. The 43-kDa glycoprotein may play a common role in the life cycle of *T. spiralis* and the Ts43 gene probably has not been considered as a factor involved in the formation of the nurse cell in infected muscle (Vassilatis et al.,1996b). The Ts43 gene has been cloned and the nurse cell cytoplasm and the muscle cell derived nuclei in immunocytolocalization studies in 35-day-old infected muscle cells (Vassilatis et al., 1992). The authors (Vassilatis et al., 1996b) showed the timing of synthesis and secretion of the 43 kDa glycoprotein and its temporal correlation to the changes of the infected host cell in *T. spiralis* infection. The expression level of the Ts43 gene was compared in different stages by semi-quantitative polymerase chain reaction (PCR) (Wu et al., 2002) and a relative quantification of Ts43 expression in *T. spiralis* has not been carried out.

Although a function for Ts43 gene is still a matter of discussion, determination of the Ts43 gene expression in different life stages is an important factor in understanding the role of the Ts43 gene in *T. spiralis* infections. In this study, Ts43 mRNA was determined by real-time quantitative RT-PCR in adult worms, newborn larvae and muscle larvae of *T. spiralis*, and the expression level of the Ts43 gene was demonstrated. Knowledge of Ts43 gene that is expressed by the various stages of *T. spiralis* offers a potential resource to guide future investigations relevant to biology of *T. spiralis* as well as Ts43 function.

2. Materials and methods

2.1. Parasites

The parasites used were *T. spiralis* (isolate code: ISS3; original host: domestic pig of Poland). The healthy adult Chinese Kunming mice (purchased from the Experimental Animal Center of Har-bin Veterinary

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Research Institute, Harbin, China) were orally infected with 300 larvae, and adult parasites were harvested from the intestines at 7 days p.i. The adult worms were extensively washed in RPMI 1640 medium (Gibco, USA) containing 200 units/ml penicillin and 200 μg/ml streptomycin, and then cultured in RPMI containing 10% FCS (fetal calf serum, Sigma, Louis, USA) in 5% CO_2 at 37 °C for 24 h. The newborn larvae were collected from the cultured medium according to the method described elsewhere (Uparanukraw and Morakote, 1997).

The muscle larvae (14, 18, 22, 26, 30, 34, 38, 48, 58-day-old) were isolated from infected mice by the standard pepsin hydrochloride digestion method. The skeletal muscles of mice of above-days p.i. were minced into small pieces. The minced muscle tissues were incubated in artificial gastric juice with agitating at 37 °C for 10 s, which resulted in mixed suspension of larvae and host cell debris. And then the mix continued to be digested in 1% pepsin with 1% HCl for 3 h at 37 °C and was collected after appropriate filtration and differential sinking. The larvae were recovered by a modified Baerman apparatus and washed in 0.85% NaCl and their numbers were determined.

All the samples were washed finally with DEPC-treated water 3 times, avoiding contamination of the host tissue debris, and then frozen at −80 °C for RNA preparation.

2.2. RNA extraction

Total RNA was extracted from the samples of adult worms, newborn larvae and muscle larvae of *T. spiralis* from Swine isolates using a TRIZOL RNA extraction kit (Invitrogen, Vienna, Austria) according to manufacturer's instructions. The extracted RNA was dissolved in DEPC-treated water and stored in aliquots at −80 °C. To eliminate genomic DNA contamination, the RNA extracts were treated with DNase I and the mixture was incubated at room temperature for 15 min followed by heating for 15 min at 65 °C to inactivate the DNase I. The concentration and purity of RNA samples were determined by spectrophotometer (BioPhotometer Eppendorf, Germany) and quantified by measuring the absorbance at OD_{260}.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Oligonucleotide primers were designed based on sequences from the GenBank database. The forward primer of the Ts43 gene was 5'-acatttttcttagtgctttctgggt-3' and the reverse primer was 5'-accattctg-tatcatctgtagcagttct-3' (GenBank accession number M95499). Based on the sequence of 18S rRNA of *T. spiralis*, a pair of primer of 18S rRNA (5'-aggttcgaaggcgatcagatac-3' and 5'-ctgctcgccgagtcttaaatg-3'; GenBank accession number U60231) was developed. Amplification of the fragments of 99 bp Ts43 and 92 bp 18S rRNA gene was carried out by PCR using the above primers deduced from *T. spiralis*.

The RNA from each sample was reverse-transcribed to cDNA using Reverse Transcription System (Promega, Madison, USA). The RT started with incubation at 70 °C for 5 min of a mixture containing 5 μl of total RNA (2 μg), 4 μl of 2.5 mM dNTP, 4 μl of oligo$(dT)_{18}$ primers (20 pmol/L) and 7 μl of DEPC-treated sterile water. The mixture was then quenched on ice briefly and added to RT reaction buffer containing 40 U of RNasin Ribonuclease inhibitor and 200 U of M-MLV reverse transcriptase. The RT reaction was carried out at 37 °C for 1 h and then 70 °C for 15 min. The cDNA product in a final volume of 25 μl was stored at −20 °C until use.

The PCR reaction mixture contained 1 μl of the above cDNA, 2 μl of PCR buffer (10×), 2 μl of dNTP (2.5 mM), 0.5 μl of each primer (25 pmol/L), 0.5 μl of Ex*Taq* DNA polymerases (5 U/μl, Takara, Shiga, Japan) and 13.5 μl of sterile water to produce the final volume of 20 μl. PCR reaction was conducted under the following conditions: an initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 94 °C for 10 s, annealing at 60 °C for 40 s and elongation at 72 °C for 30 s, and a final extension at 72 °C for 10 min (Mini Cycler® Peltier Thermal cycler, MJ Research, Waltham, USA). The PCR products were analyzed by electrophoresis on 1% agarose gel.

2.4. Construction of recombinant plasmid

In order to eliminate artifacts that could arise during the PCR process, ten PCR reactions were set up for each sample together with controls (no cDNA). The representative PCR products were purified with an E.Z.N.A® Gel Extraction Kit (Omega Bio-tech, Doraville, USA) and were ligated into a pMD18-T Vector (Takara, Kyoto, Japan) according to manufacturer's guidelines. The ligated vectors were transformed into competent cells of *Escherichia coli* DH5α and the clones were identified by digesting the plasmid DNA with *Eco*RI (Takara, Shiga, Japan). The positive plasmids were sequenced and their nucleotide sequences were analyzed using DNASTAR software (DNASTAR, Inc.).

2.5. Quantitative real-time RT-PCR

The quantification of Ts43 gene transcripts was carried out by real-time amplification of Ts43 gene and an endogenous housekeeping gene 18S rRNA as control from the above-prepared cDNA using the real-time detection system (ABI 7500 FAST, Applied Biosystems) and Taqman fluorescence probes (Ts43 probe: 5'-FAM-tgcacaactgtttgcaaattcatgcagc-TAMRA-3'; 18S rRNA probe: 5'-FAM-ccaaccagcgattcgccgaagt-TAMRA-3'). The total volume of reaction was 20 μl and contained 0.5 μl of fluorescence probe, 0.5 μl of forward and reverse primers each for Ts43 or 18 S rRNA, 2 μl of PCR buffer (10×), 2 μl of dNTP Mix (10 mM), 0.5 μl of Ex*Taq* DNA polymerases (5 U/μl, Takara, Shiga, Japan), Mg^{2+} (25 mM) 3.5 μl, 1 μl of plasmid DNA or cDNA template and 9.5 μl of sterile water. The PCR protocol consisted of an initial denaturation at 95 °C for 2 min, 40 cycles of denaturation at 94 °C for 10 s, annealing at 60 °C for 40 s and elongation at 72 °C for 20 s, after 36 cycles, an extension reaction was performed at 72 °C for 8 min.

Amount of Ts43 mRNA was calculated using a relative standard curve method (Ginzinger, 2002; Cui et al., 2005; Ning et al., 2005) and transcript levels were represented as the copy number of target gene/10^7 18S rRNA copies (Wu et al., 2006). Briefly, the amplified fragments of both Ts43 and 18S rRNA genes were cloned and the standard curves of the cycle threshold (Ct) values were obtained from serial dilutions from 10^3 to 10^7copies of the purified recombinant plasmid. For each experimental sample, the amount of mRNA of each target gene and 18S rRNA was determined from the respective standard curves, and the quantity of Ts43 mRNA was divided by that of 18S rRNA mRNA to obtain a normalized value for Ts43 gene expression.

All samples were measured in triplicate. Data were analyzed by SPSS software (Statistical Package for the Social Sciences, version 10.0 for Windows; SPSS Inc, Chicago, IL). An independent sample T-test was used to analyze differences in mRNA expression between different stages. Differences with $p<0.01$ were considered to be statistically significant in this study.

3. Results

3.1. The extraction of RNA and the amplification of the target gene

The A_{260}/A_{280} ratio of total RNA extracted was between 1.8 and 2.0 for all samples. The RT-PCR products were analyzed by gel electrophoresis. No primer dimers were visible and the amplified Ts43 and 18S rRNA gene fragments with the expected size of 99 bp and 92 bp by real-time RT-PCR were specific as confirmed by gel electrophoresis (Fig. 1) and direct sequencing (data not shown). The recombinant plasmid DNA was digested with *Eco*RI and a size-specific band was obtained respectively. When the recombinant plasmid DNA was used as the template for subsequent real-time PCR, the amplified products were identical to those by RT-PCR.