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Original Article

Expression of Heat Shock Protein 70 (HSP70) and Astacin Genes of *Strongyloides stercoralis* as well as HSP70 and HSP17.1 Genes of *S. ratti* in Adult and Larval Stages of *S. stercoralis*

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Abstract

Background: Uncovering the roles and characteristics of pathogenesis-related molecules can help us develop novel management methods in parasitology. In this study, we studied the expression levels of *Strongyloides stercoralis* heat shock protein70 (HSP70) (*Sst-hsp-70*) and astacin (*Sst-ast*) as pathogenesis-related genes as well as the expression of *S. ratti* HSP70 and HSP17.1 (*Sra-hsp-70*, *Sra-hsp-17.1*) in the larvae and adult stages of *S. stercoralis*.

Methods: A hyperinfection isolate of *S. stercoralis* from Gilan Province, northern Iran was cultivated on nutrient agar. After a couple of days, parasites in different stages of life were collected, and total RNA was extracted. The expression levels of astacin and HSP genes were compared by real-time PCR.

Results: Statistically higher expression levels of *Sst-ast, Sst-hsp-70*, and *Sra-hsp-70* genes in L3 larvae than in adults were observed. However, the expression level of *Sra-hsp-17.1* was non-significantly lower in the larval stage than in adult worms.

Conclusion: Higher expression levels of *Sst-ast*, *Sst-hsp-70*, and *Sra-hsp-70* genes in the larval stages of *S. stercoralis* suggest the potential role of these enzymes in parasite cutaneous invasion and pathogenesis. However, higher expression of *Sra-hsp-17.1* in adult forms is probably involved in resistance and survival mechanisms. The similarity in gene expression between *S. stercoralis* and *S. ratti* can provide helpful hints to better understand strongyloidiasis from various perspectives, including pathogenesis, proper diagnosis, and targeted treatment.

Introduction

Strongyloides stercoralis, an intestinal nematode, is the causative agent of strongyloidiasis with a wide range of clinical signs ranging from asymptomatic to severe gastrointestinal symptoms, including abdominal pain, diarrhea, vomiting, nausea, hives, and respiratory symptoms (1-7).

Strongyloides has free and parasitic life cycles. Protease and peptidase enzymes have a vital role in the parasitic form as a considerable ratio of genes upregulated expression is reported during the parasitic life cycle of the *S. ratti* and *S. stercoralis* adult females (8).

Heat shock proteins (HSPs) are cell-supportive molecules and high-level expression of them have fundamental roles during stressful conditions and control apoptosis mechanisms (9). HSP in parasites is part of their parasitic program and is not due to a rise in host body temperature (10). HSP70 has a higher protective capacity than other HSPs, which makes it a good target for prototype studies (10-12). Recent studies in *Brugia malayi* show that HSP70 is a strongly immunogenic protein, and its recombinant form could be applied for vaccination purposes. However, the expression level of this protein in larvae and adult worms can affect vaccine efficacy and requires further consideration (13). Upregulated expression of various genes and protein families such as acetylcholinesterases (AChE), aspartic proteases (APs), prolyl oligopeptidase (POP), proteinase inhibitors (e.g., trypsin-inhibitors and cystatins), transthyretin-like proteins (TLP), sperm-coating proteins (e.g., SCP/TAPS), DAF-7, RIO protein kinases (riok-1 and riok-3) and galectins in the proteome and transcriptome content of female nematodes is an indicator of their role in parasitism (14-18). Identifying parasitism-related candidate genes is crucial in developing novel therapeutic methods in strongyloidiasis (19). HSP70 is an immunogenic molecule so host immune responses could be detected and is an appropriate component for developing diagnostic methods (20).

Astacin as a metalloprotease (MTP) is involved in nutrition, digestion, cuticle formation, and ecdysis of helminths and plays a role in the pathogenesis of *S. stercoralis* larvae by facilitating rapid penetration of the parasite into the extracellular matrix and its migration into tissues (19, 22-23), also the transition of the nematodes from the rhabditiform to the filariform stage (23). Considering the role of this enzyme in disrupting the host skin at the early stages of the migration phase, makes it a suitable target for vaccine development and infection control as well as gene knockout studies.

Gene expression study in parasitic *S. stercoralis* can be challenging due to their complex life cycles and the difficulty in obtaining sufficient amounts of specific life stages for analysis. However, high genetic homology of the genome sequence of four *Strongyloides* species, including *S. ratti, S. stercoralis, S. venezuelensis*, and *S. papillosus*, as well as their similarity in parasitic and free-living adult stages (24, 25), provides a chance to use other species in the research studies instead of *S. stercoralis.*

Therefore, we studied HSP70 (*Sst-hsp-70*) and astacin (*Sst-ast*) expression levels of the larval and adult stages of *S. stercoralis*. We also determined the expression levels of *S. ratti* HSP70 and HSP17.1 (*Sra-hsp-70*, *Sra-hsp-17.1*) in *S. stercoralis* and compared with expression in *S. ratti* as an available experimental model to determine whether they could be substituted with *S. stercoralis.*

Materials and Methods

Ethics statement and informed consent

This study received ethical approvals from the Ethics Committees of Iran University of Medical Sciences (IUMS), Tehran, Iran (Code no: IR.IUMS.REC. 1396.9411554004). The patients or their legal guardians provided written informed consent to participate in this study.

Sample collection and parasitological evaluation

A hyperinfection isolate of *S. stercoralis* from Gilan province (north of Iran) was cultivated on nutrient agar. After 2–3 days of incubation at room temperature in 28-30 ˚C, the plates were examined for the presence of larvae and adult worms or their tracks by light microscopy. To separate the adult worms, after 48 hours, the surface of the agar plate was washed out with lukewarm phosphate buffer saline (PBS) solution pH 7.2, supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin to decrease possible bacterial contamination (26). The filariform larvae (L3 forms) were isolated from cultures after 72 to 96 hours using the Baermann technique (27, 28).

RNA isolation and real-time PCR

Total RNA was extracted from the adult and L3 larvae using TRIzol reagent (GeneAll, South Korea) according to the manufacturer protocol (27). To determine the extracted RNA quantity, the product's optical absorption was evaluated at 260 and 280 nm using NanoDrop (Thermo Scientific, USA), and the ratio was considered. We also electrophoresed the purified RNA on 1.5% agarose to determine the quality of the extract. Complementary DNAs (cDNAs) were synthesized using 1µg of purified RNA according to the manufacturer's instructions (Biofact, South Korea) and used in real-time PCR.

The relative expression level of four genes, *Sst-ast* and *Sst-hsp-70*, *Sra-hsp-70* and *Sra-hsp-17.1*, was determined by real-time PCR using Rotor-Gene Q 6000 (Qiagen, USA) with SYBR Green PCR master mix (Takara, Japan). *Sra-gapdh gene* was applied as a housekeeping gene, and its expression level was considered for data normalization (29). The designed specific primers (Table 1) were blasted in NCBI to control for the nonspecific binding. Moreover, after optimization, a serial dilution was prepared for each product of the real-time PCR (10^{-4}-10^{-9}) and used for efficiency analysis of the primers.

All experiments were carried out in a duplicate manner at least in two separate runs. Reactions were performed in 20 µL volumes containing 10 picomoles of each primer and 1 µL of cDNA (30).

The thermo-cycling conditions were defined as follows: 95 °C for 15 min (hold), 40 cycles of 95 °C for 20 seconds (denaturation), and 60 °C for 40 seconds (annealing and extension). Finally, melting curve analysis from 60 °C to 99 °C was used to ensure consistency and specificity of the amplified product.

Table 1: Primers used in real-time PCR

Primer name	*Primer Sequence (5'–3')*	*Length (bp)*
Sra-gapdh[1]	F: GTACCACTAACTGTTTAGCTCC R: GCACCTCTTCCATCTCTCC	154
Sst-hsp-70[2]	F: TGGAATTGAAACAGCCGGAG R: TCATAGCACGTTCACCTTCG	146
Sra-hsp-70[3]	F: ACGTGCTCTTCGTCGTTTG R: AGCACGTGTGATGTTGGTGT	130
Sra-hsp-17.1[4]	F: ATGGAACCATGTCCAGAGGT R: ACGGACGAAACTTCTTTGGA	170
Sst-ast[5]	F: ATGACGCTAAAAGGCTTAACATG R: ACTCCTGTAAACATTCTTGGGC	130

[1] *Strongyloides ratti*-glyceraldehyde-3-phosphate dehydrogenase

[2] *Strongyloides stercoralis*-heat shock protein-70

[3] *Strongyloides ratti*-heat shock protein-70

[4] *Strongyloides ratti*-heat shock protein-17.1

[5] *Strongyloides stercoralis*-astacin

Statistical analysis

Normalization of gene expression was performed using *S. ratti* GAPDH (*Sra-gapdh*) as the housekeeping gene. So, the relative expression of the genes under study was calculated and compared using the $2^{-\Delta\Delta CT}$ formula. The calculated data was analyzed and presented by GraphPad Prism software (Version 6.01, CA, USA), and *P*-values lower than 0.05 were considered statistically significant. Normalization analysis by D'Agostino & Pearson omnibus normality test did not show a normal distribution of data, so we used the nonparametric Mann-Whitney test for data analysis.

Results

Gene expression analysis

The adult and larval stages of the parasites were purified from culture media and used for RNA extraction and cDNA synthesis. Electrophoresis of RNA showed three RNA bands confirming the acceptable quality of the purified RNA.

Expression of astacin and HSP70 genes in adult and larval stages of S. stercoralis

The expression level of the astacin and HSP genes was compared in the adult and larval stages of *S. stercoralis* stages by real-time PCR. Fig. 1A shows that the *Sst-ast* demonstrated a 3.5-fold higher expression level in the L3 stage than the adult forms (*P*=0.032). Fig. 1B demonstrates that the *Sst-hsp-70* gene had a 2.9-fold higher expression in the L3 stage compared to the adult forms (*P*=0.029).

Expression of HSP70 and HSP17.1 of Strongyloides ratti in adult and larval stages of S. stercoralis

The comparison expression levels of the HSP70 and HSP17.1 of *S. ratti* in the adult and larval stages of *S. stercoralis* by real-time PCR showed a 4-fold higher expression of the *Sra-hsp-70* gene in the L3 stage (*P*=0.007) (Fig. 1C) and a 0.8-fold lower expression of the *Sra-hsp-17.1* in the L3 stage (*P*= 0.14) (Fig. 1D) than the adult forms.

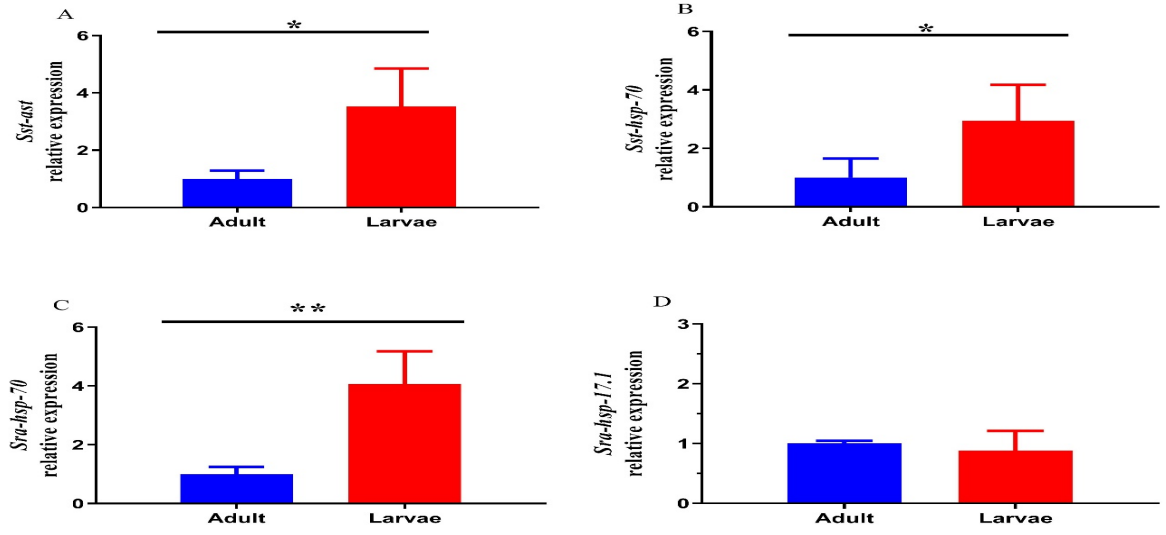

Fig. 1: Comparison of the expression level of HSP and astacin genes of *S. stercoralis* and HSP70 and HSP17.1 of *S. ratti* in adult and L3 larval stages of *S. stercoralis*. Expression of genes including A) *Sst-ast*; B) *Sst-hsp-70*; C) *Sra-hsp-70*; D) *Sra-hsp-17.1* was measured in adult and larval stages of *S. stercoralis* by real-time PCR. The mean (±SD) of the relative expression level from each gene was compared using a nonparametric Mann-Whitney test (*P<0.05, **P<0.01)

Discussion

We evaluated the expression of astacin (*Sst-ast*) from metalloprotease (MTP) and HSP70 (*Sst-hsp-70*) from the heat shock proteins group in the larval and adult stages of *S. stercoralis* and found that the expression level of these genes in the larval stage was higher than the adult stages. The increase of MTP enzyme was also reported in *Ancylostoma caninum* by Zhan et al., which showed *Aca-mtp-1* is mainly expressed in the L3 stage of *A. caninum* and has a lower expression in adult worms (31). Moreover, the application of anti-MTP in western blotting of the total lysate of the adult worms confirmed its absence in the adults' excretory-secretory products. This finding suggests that the expression of MTPs is mainly restricted to L3 larvae, and its mRNA is not translated into protein, or it is probably degraded in mature worms (29). In addition, *Aca-mtp-1* is released only in response to stimuli that activate L3 to continue feeding and most likely works in infectious processes (32). *Aca-mtp-1* facilitates cutaneous penetration of the parasite into the skin and migration of L3 larvae in host tissues and can act as an enzyme and play a role in the second ecdysis during infection (31, 33). Jing et al. also used a real-time PCR technique to study the MTP and astacin in *Steinernema carpocapsa* insect nematode. They found that *Sca-ast* is up-regulated in the parasitic stages of the nematode and plays a vital role in penetrating through the skin and digesting food (22).

In addition to the increased expression level of *Sst-hsp-70* in *S. stercoralis* larval stages, we showed that the expression of the HSP70 of *S. ratti* in the larval stages of *S. stercoralis* is higher than in adults. This chaperone molecule protects the parasite in terms of stress and temperature alterations. During infection with L3 form, the temperature increases from the environment to the host body temperature, which is required to initiate the activation and feeding of L3 larvae. HSPs play important roles during parasite invasion, and their expression increases during infection initiation and at elevated temperatures (34). In 2015, Chen et al. investigated the expression patterns of HSP70 and HSP90 of *Anisakis pegreffii* by real-time PCR and western blotting. Although both proteins play essential roles in stress responses in *Anisakis*, they showed that *Ape-hsp-70* and *Ape-hsp-90* have higher expression in L4 larvae than in L3 larvae. Moreover, they showed that this parasite's *Ape-hsp-70* expression is higher than *Ape-hsp-90*. In the human body, the L3 larvae differentiate into L4 form, and the expression of both HSP proteins is increased (35). HSPs and, most importantly, HSP70s are among the dominant antigens recognized by the immune system in a wide range of parasites. Thus, in addition to their role in pathogen-host interactions, they are of immunological importance (34). Recent studies in *B. malayi* show that HSP70 is a vital immunogenic protein of this parasite, and its recombinant form could be applied for vaccination purposes. However, the level and position of the expressed protein in larvae and adult worms can affect vaccination efficacy (13).

The comparison expression levels of the HSP17.1 of *S. ratti* in the adult and larval stages of *S. stercoralis* revealed lower expression in the L3 stage than in the adult forms. Younis et al. study showed the expression level of small excretory-secretory HSPs, *Sra-hsp-17.1* and *Sra-hsp-17.2* have 6 and 9-fold higher expression in adult worms of *S. ratti*. The HSPs, as mentioned above, are similar to those of *S. stercoralis* HSPs. Increased expression of adult worm HSPs in the host intestine reflects the role of these HSPs in adapting the parasite to its new environment (36). Since HSP17s of *S. ratti* resemble the small HSP sequences of *S. stercoralis*; therefore, it can be deduced that increased expression of HSP17 in adult worms may increase humoral immune responses. Monocytes exposed to *Sra-hsp17s* release interleukin-10, which confirms the intense role of

HSP17 in host immune responses. HSP17.1 also binds to intestinal epithelial cells.

Therefore, it can be concluded that HSP17.1 may play a role in host mucosal immune response by binding to intestinal epithelial cells and the involvement of mucosal immune cells (36) and may confirm the increased expression observed in this study in adult worms.

Conclusion

Comparison of the expression level of *Sst-ast*, *Sst-hsp-70*, and *Sra-hsp-70* genes in larval and adult forms showed significantly higher expression of the mentioned genes in the larval forms of *S. stercoralis* that suggest the potential role in parasite cutaneous invasion and pathogenesis. Whereas *Sra-hsp-17.1* gene showed higher expression in the adult worms than in the larval stage, which is probably involved in resistance and survival mechanisms. Considering the lack of access to *S. stercoralis* specimens in most studies, triggers the idea that the rat model of strongyloidiasis could be used instead of *S. stercoralis* in future studies. Identifying genes that can be considered biomarkers in determining the pathogenesis and infiltration of *S. stercoralis* can be helpful in better diagnosis, targeted treatment, and appropriate prevention of strongyloidiasis.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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