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Expression Analysis of Hsp60 Gene from Albino Northern Snakehead (*Channa argus*) after Bacterial Infections

Aiguo Zhou[1,4#], Zhuolin Sun[1#], Shulin Liu[1#], Shaolin Xie[1], Zhenlu Wang[1], Yue Zhang[2], Yanfeng Chen[3] and Jixing Zou[1*]

1 College of Marine Sciences, South China Agricultural University, Guangzhou, Guangdong, China
2 Departments of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, USA
3 School of Life Science and Engineering, Foshan University, Foshan, Guangdong, China
4 Qingyuan North River Fishery Science Institute, Qingyuan, Guangdong, China

Corresponding author: Jixing Zou

zoujixing@scau.edu.cn

College of Marine Sciences, South China Agricultural University, Guangzhou 510642, Guangdong, China

Tel: ++ 86-20-87573175

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Abstract

The great albino northern snakehead, *Channa argus*, one of the most important economical fish in China. In present study, the expression changes of *AcaHSP60* gene and *Acahsp60* protein in spleen, liver, kidney, and brain of albino *C. argus* were detected and analyzed after infected with *Edwardsiella tarda* (strain NO. DL1476) at different time points. Quantitative real-time PCR (qRT-PCR) and western blot analysis revealed tissue-specific *AcaHSP60* and *Acahsp60* expression in the control group. *AcaHSP60* mRNA levels were highly significantly increased and reached maximum in the spleen (48 h post injection), kidney (48 h post injection), and brain (24 h post injection), while it was kept at a high level and had significantly decreased in the liver post injection. These results showed that *AcaHSP60* expression was significantly tissue-specific and might be sensitive to pathogen infection in albino *C. argus*.

Keywords: Albino *Channa argus,* Heat shock protein 60*, Edwardsiella tarda* challenge, Gene expression

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Introduction

For two consecutive years, the aquaculture output of Channidae has exceeded 500,000 tons, among which more than 20% came from Guangdong [1,2]. With the development of high-density and intensive farming, seed degeneration, environment deterioration, disease outbreak, and etc, fish are extremely susceptible to the disease caused by pathogenic bacteria such as *Edwardsiella tarda*. After infection, in the body surface a lot of hyperemia, some ulceration, and anal swelling were observed. And as for the internal organs, liver and kidney displayed enlargement [3]. Infections can lead to a large- scale outbreak, causing a huge loss in the Channidae culture industry.

The albino northern snakehead fish, which is considered as an economically valuable fish and has potential use in the medical and pharmaceutical fields, were only found in Jialing River in Sichuan (105.05E, 29.58N) [4-6]. And they have to face environmental stress when introduced to a new region that was of the unique growth areas of some valuable commercial fishes [7]. Previously, we isolated two pathogens (DL1475, DL1476) identified as *E. tarda* from normal *C. argus*. Further research showed that the median lethal doses (LD_{50}s) were 7.1 × 103 cfu/g and 2.9 × 104 cfu/g for these two pathogens. In this study, *E. tarda* (DL1475) was used to infect experimental fish. Heat Shock Proteins (HSPs) are generally responsible for preventing damages to the proteins in response to the high heat. They were triggered by various environmental stress conditions such as infection, hypoxia, starvation, and water deprivation [8,9]. Meanwhile, *hsp60* plays an important role in cell protection. In addition, as a one of the most important members, *hsp60* is an immunodominant antigen in many pathogen infections. Therefore, future research may need to clarify the role of *hsp60* in autoimmune development and the role of *hsp60*-based immunotherapy [10,11].

In this backdrop, we thought that the temperature resistance of introduction and domestication, the vulnerability, and etc.

of albino *C. argus* might be closely related to the heat shock proteins [5,12]. Therefore, the effects of bacterial infection on the expression of heat shock proteins were determined. In this study, the expression of *AcaHSP60* (albino *Channa argus* heat shock protein 60) gene in different tissues (spleen, liver, kidney, and brain) was analyzed by quantitative real-time PCR and western blotting. These results will contribute to the understanding of the response mechanism of albino *C. argus* when it is introduced to the south or north of China or to other environmental stresses. Meanwhile, the results also provide a theoretical reference for the better domestication of albino *C. argus*.

Materials and Methods

Animals

A fish population of albino *C. argus* (length 15.28-17.32 cm, weight 55.92-70.29 g) was sampled from Jialing River, Neijiang city, Sichuan province, China (105.05E, 29.58N). They were acclimatized in an aquarium (100 × 80 × 60 cm^3) at 26 ± 1°C under a 12 h:12 h (light: dark cycle) for 15 days before bacterial infection experiment. Twelve fish of each group are infected with *E. tarda* (DL1475) (kept by Dr. Chen Yanfeng, School of Life Science and Engineering, Foshan University). Briefly, the bacteria were incubated overnight at 37°C to mid-logarithmic stage, collected by centrifugation, and re-suspended to a final concentration of 2.58 × 106 colony forming units ml^{-1} (CFU/mL) in the sterile Phosphate Buffered Saline (PBS). The level of *E. tarda* was 7.1 × 103 CFU g^{-1} per fish (half lethal concentration) [12]. A total of 100 µL of live *E. tarda* was injected to each fish intraperitoneally. Similarly, 100 µL PBS were injected to control fish. Six fish from each group were randomly chosen and sampled at 0, 6, 12, 24, 36, 48, and 72 h post-injection. They were then anesthetized with a lethal dose of MS-222 anesthetic (300 ppm) (Pharmaq Ltd., UK). The spleen, liver, kidney, and brain tissues of each fish were dissected, flash-frozen in liquid nitrogen, and stored at -80°C until RNA and protein extraction.

Prediction of protein characteristics and subcellular location

SOPMA software was used to predict secondary structure, SWISS-Model was used to predict three-dimensional structure of *AcaHSP60.* PSORT II was used to analyze the subcellular localization of *HSP60*-encoded proteins [13].

Reverse transcriptional cDNA and primer design

Total RNA was extracted from the tissues of albino C. argus using TRIzol reagent and treated with RNase-free DNase I (both from TaKaRa, Japan). RNA quality was assessed by electrophoresis on a 1.5% agarose gel. The concentration and purity were determined at optical density (OD)260/(OD)280 with a Nanodrop ND-2000 spectrophotometer (Thermo Electron Corporation, USA). cDNA synthesis was carried out using PrimeScript ® RT reagent Kit with gDNA Eraser (TaKaRa) according to the manufacturer's instructions. The cDNA products were stored at -20°C for later use.

The qRT PCR primers *AcaHSP60* and *β-actin* were designed in Table 1. And the primers specificities were examined by conventional PCR and melting curve analyses. *β-actin* was used as an internal control.

Table 1 Primers used to amplify the cDNA of *HSP60* and *β-actin* (Jia et al. 2010) in the albino *C. argus* by qRT PCR.

Gene name	Primers	Sequence (5'-3')
AcaHSP60	qPCRAcaHSP60F	5'-CAACCAGCACCGCAAACCT-3'
	qPCRAcaHSP60R	5'-ACCACCTGAAGCCCAACCT-3'
β-actin	qPCRactinF	5'-CACTGTGCCCATCTACGAG-3'
	qPCRactinR	5'-CCATCTCCTGCTCGAAGTC-3'

Relative qRT-PCR and Western blotting

AcaHSP60 gene expression levels were analyzed in multiple tissues (spleen, liver, kidney, and brain) under the *E. tarda* treatment with fluorescent qPCR using Bestar® SybrGreen qPCR mastermix (DBI, Germany). The PCR reaction mixture consisted of 10 µL Bestar® SybrGreen qPCR mastermix, 0.2 µL of each of the gene-specific primers of *qPCRAcaHSP60F* and *qPCRAcaHSP60R* (10 µM), 0.04 µL 50× ROX, 4.56 µL double-distilled water (ddH_2O), and 5 µL cDNA template in a total volume of 20 µL. Real-time quantitative PCR was performed on an iQ5 Real-time PCR instrument (Bio-Rad, USA). The RT-PCR was amplified using the following scheme following Bio-Rad's recommedation: incubation for 2 min at 95°C, followed by 45 cycles of 10 s at 95°C, 34 s at 60°C for optimized temperature, and 30 s at 72°C. The melting curve temperature ranged from 60°C to 98°C, and analysis was performed to confirm the presence of a single applicant. Relative expression of albino *C. argus AcaHSP60* mRNA was determined using the 2(−ΔΔCt) method [14]. The amplification efficiency (E) values of *qPCRactinF/qPCRactinR* and *qPCR AcaHSP60F/qPCRAcaHSP60R* were 99.1% and 95.9%, which were calculated when the curves of Ct (Log (cDNA dilution)) were generated. The value indicated an n-fold difference of the reference sample relative to the blank group (the calibrator).

Western blot was performed to analyze the expression levels of *AcaHSP60* protein in the different tissues (spleen, liver, kidney, and brain) challenged with *E. tarda*. The samples were analyzed by adding anti-hsp60 monoclonal antibody (H3524, Sigma, USA), followed by anti-mouse IgG-peroxidase conjugate secondary antibody (A2304, Sigma, USA). *β-actin* was used as control. Changes in the levels of expression of the *Acahsp60* were analyzed by using the software Image Quant TL 7.0 (GE health care, USA). The western blot was done triplicate.

Statistical analysis

Expression fold change data were logarithmic transformed before performing one-way analysis of variance (one-way ANOVA) using SPSS 17.0 (SPSS Inc., USA). When overall differences were significant, Tukey's test was conducted to compare the means between individual treatments. For the western blot, the band intensity was calculated by Image Quant TL 7.0. And then the statistical analysis was performed to compare the intensity between each band under the same organs. Differences were considered significant and highly significant at $p<0.05$ and $p<0.01$, respectively.

Results

Protein structure prediction and subcellular localization

The predict secondary structure of albino *C. argus* and human *hsp60* protein sequences: α-helix was 53.57% and 52.01%, extended strand was 12% and 12.74%, β-turn was 8.17% and 8.90%, and random coil 26.26% and 26.35% were obtained for albino *C. argus* and human *hsp60* proteins, respectively. The predict three-dimensional structure of *Acahsp60* showed that the proteins were mainly composed of α-helices, and albino *C. argus* was basically similar to that of human *hsp60* [15]. The high degree of conservation suggested that there may be a close correlation between the structure and function of the protein molecule. The high degree of conservation in structures suggested that a close correlation of the *hsp60* protein functions may exist between albino *C. argus* and human. The subcellular localization of *hsp60*-encoded proteins showed that 69.6% were located in the mitochondria, 17.4% in the cytoplasm, and 13.0% in the nucleus. Comprehensive analysis suggested that most of the protein is distributed in the mitochondria.

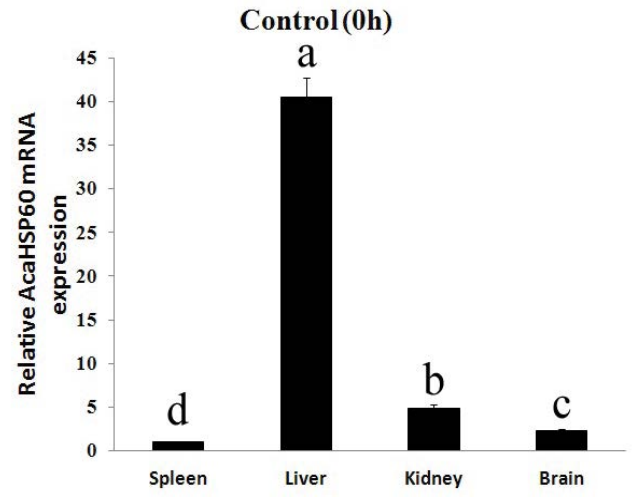

Figure 1 **Figure 1** Tissue-specific *HSP60* mRNA expression in untreated albino northern snakehead, *Channa argus*. The results were expressed as mean ± standard error (n=6, shown as bars) after normalizing the values to *β-actin* expression (internal control). The column showed the fold-change in expression relative to the spleen tissue of the healthy control (0h) fish. Bars bearing different letters were significantly different ($p < 0.05$; Tukey's test).

AcaHSP60 gene expression analysis

The qRT-PCR showed that the *AcaHSP60* gene was specifically expressed in spleen, kidney, brain, and liver in control group. Compared to the spleen, the liver had the highest expression level (40.54-fold) ($p<0.01$), followed by the kidney (4.93-fold) and brain (2.30-fold) ($p<0.05$) (Figure 1).

After *E. tarda* infected albino *Channa argus* , *AcaHSP60* mRNA levels in brain were significantly decreased after 6 h and 12 h post injection (0.45-fold and 0.43-fold, respectively) ($p<0.01$) and reached a maximum value at 24 h post injection (2.26-fold) ($p<0.01$). It then showed a downward trend before it reached to maximum again at 72 h (Figure 2a). In spleen and kidney, *AcaHSP60* showed a significantly increased and reached a

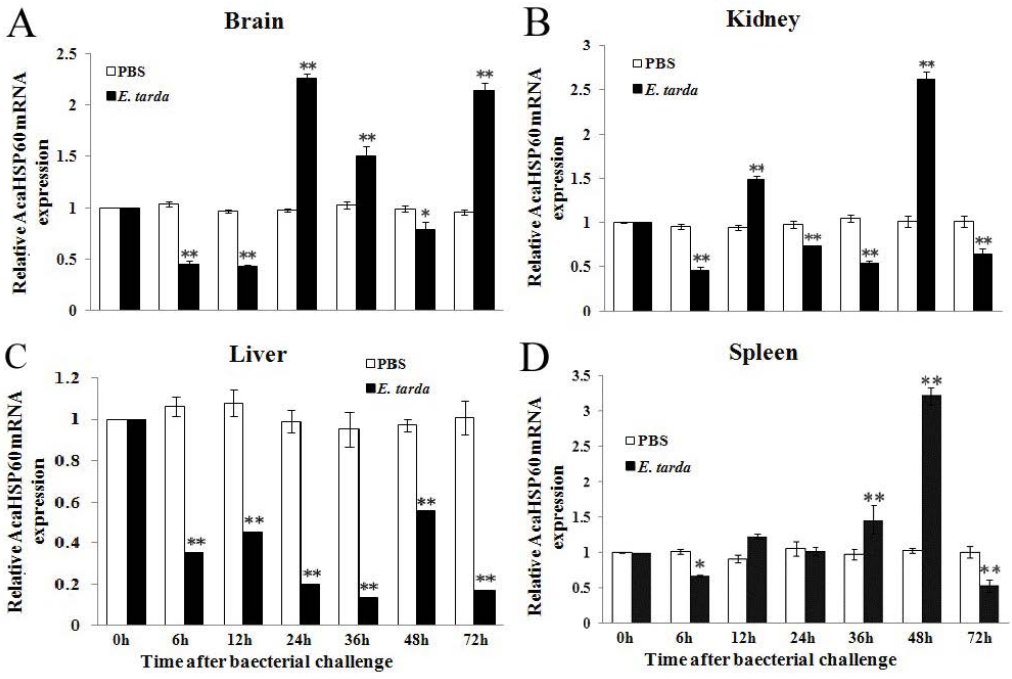

Figure 2 **Figure 2** *HSP60* mRNA expressions in a) Brain b) Kidney c) Liver and d) Spleen relative to *β*-actin analyzed by real-time PCR in albino northern snakehead, *Channa argus* after *E. tarda* challenge. The graph represented as mean ± standard error (n=6). * represents $p<0.05$ ** represents $p<0.01$.

maximum after 48 h post injection (3.21-fold for Figure 2b, and 2.61-fold respectively for Figure 2c) (p<0.01). Then it showed a significant decrease (0.53-fold for Figure 2b and 0.64-fold for Figure 2c) (p<0.01) (Figures 2b and 2d). Compared to brain, spleen, and kidney, liver had been in a state of high level of expression, while showed a most significant decrease after injection (0.35-fold at 6 h) (p<0.01) (Figure 1, Figure 2c). The overall trends were consistent with the tissues of spleen and kidney after injection (Figure 2).

Compared with control group (0 h), the *AcaHSP60* protein expression in brain was significantly increased at 24 h post injection (p<0.01). Two deeper protein bands (12 h and 48 h) was found in kidney, and two deeper bands (36 h and 48 h) were found in spleen, while the protein expression showed a significantly decreased level in the liver (p<0.01), which showed a basically similarity with the result from qRT-PCR (Figure 3).

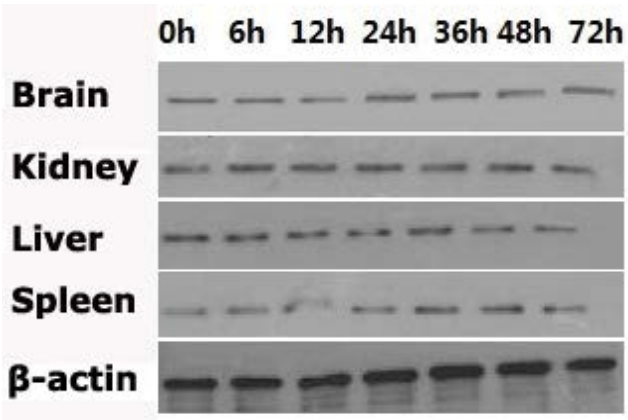

Figure 3 **Figure 3** Immunoblot analysis of AcaHsp60 and β-actin proteins in albino *C. argus* after *E. tarda* challenge (0, 6, 12, 24, 36, 48 and 72 h).

Discussion

The great northern snakehead *Channa argus*, has been widely farmed and deeply loved by consumers in Asia. The albino has a higher content of protein, amino acid, and trace element. It also has lower content of fat, better water holding capacity, and tenderness [16]. Hence the development and utilization of its germplasm resource has great aquaculture prospects [6,4]. *HSP60*, an important stress-response gene, caused a strong inflammatory response in the innate immune system to dangerous or pathogenic signals when the organism was stressed, such as temperature increases, bacterial infection, heavy metal levels, and any sudden changes in the cellular environments [5,17-21]. It has been showed that the *HSP60* activates immune-related factors IL-12, IL-15, B7, IL-8, MLP, MZP, and NO to resist invasion of pathogens [22,23]. In this study, the full-length cDNA sequence of *HSP60* gene in albino northern snakehead was cloned for the first time. Prediction of the spatial structure revealed that the *HSP60* protein contained three typical functional regions, including a *HSP60* characteristic sequence, C-terminal typical GGM repeat motif and an ATP binding domain. And it also showed that its three-dimensional structure was similar to that of human *HSP60*. Studies have shown that *HSP60* was present in the organelles of mitochondria and chloroplasts of all prokaryotic and eukaryotic organisms [24-25], and our prediction got the similar results with them. *E. tarda* easily induces the rot disease of the Channidae, especially for the imported varieties of albino northern snakehead [26]. Study found that the *HSP60* was expressed in all the examined tissues of *Oncorhynchus mykiss*, and the expression levels in the liver were obviously higher than that of other tissues [27], which were consistent with that of our study. Meanwhile, some studies indicated that the high expression was a protective effect on the cells [28]. As for the *E. tarda* infected the albino northern snakehead, the expression of *AcaHSP60* in liver was keep a relatively high level before and after injection, which may be closely related to the function of the liver as an important organ of life activities and its function. The expression of spleen and kidney tissues reached the maximum value after 48 h post injection, and then showed a decreasing trend. This may explain that the two lymphoid organs have important functions in implementing immune responses. In the brain, the *AcaHSP60* showed a very significant decline and then showed a very significant increase. The western blot results were basically consistent with the RT-PCR result. There was few report about expression changes of *HSP60* upon *E. tarda* infection; however, did A. hydrophila infection to the grass carp, and they found that *HSP60* expression was upregulated in most tissues. A. Hydrophila infection to the kidney and spleen of Mandarin fish and found *HSP60* expression was increased in both kidney and spleen at 12 h. These results are different with our study, but they also showed that *HSP60* significantly changed upon stimulation of bacteria [7,29].

In short, it appears *AcaHSP60* gene could likely be highly conserved between human being and fish because of its similarity to each other and its presence in same components of the cell, and expressed in all the detected organs of albino northern snakehead, indicates that these organs resisted when responding to external stress. The results lay a theoretical foundation for further research on the *AcaHSP60* protein *in vitro* expression, physiological function verification, and genetic regulation mechanism.

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