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Research Article

Comparative Analysis of Heat Shock Protein Genes Hsp70 and Hsp90 Expression by Live and Killed *Edwardsiella tarda* Challenge in Rohu, *Labeo rohita*

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Abstract

An inclusive variety of biotic and abiotic pressures fuels specific intracellular stress responses characterized by a family of unique proteins referred to as heat shock proteins (HSP). The present study is an expression profiling of two significant heat shock protein genes, Hsp70 and Hsp90, during live (lethal, sublethal) and killed *Edwardsiella tarda* challenge in rohu, *Labeo rohita*. Thirty rohu juveniles were challenged with the lethal dose (LD_{50}) of *E. tarda* 3.5×10^7 CFU, the sublethal dose of *E. tarda* 3.5×10^6 CFU, and formalin-killed *E. tarda* 3.5×10^7 CFU per 20g of body weight of fish, intraperitoneally. The genes' (Hsp70 and Hsp90) expressions were studied in the liver, kidney, spleen, and gill tissues of rohu at different periods: 0, 3, 6, 12, 24, 48, and 72 hours post-challenge by qPCR. The Hsp gene modulation was more significant in the liver and spleen than in kidney and gill tissues. Among the three treatments, the killed *E. tarda* showed more expression of Hsp70 production followed by the lethal dose. High Hsp90 expression was observed in fishes challenged with live *E. tarda* than killed bacteria. Further, the highest activity of glutathione peroxidase and myeloperoxidase was found in all the treatments. The study indicated the critical role of Hsp70 and Hsp90 in live infection compared to killed bacterial administration.

Keywords: Heat Shock Proteins; HSP 70; HSP 90; *Edwardsiella tarda*; *Labeo rohita*

Abbreviations

LD_{50}: Lethal Dose 50; HSP: Heat Shock Proteins; DNA: Deoxyribonucleic Acid; cDNA: Complementary DNA; Fig.: Figure; Min: Minutes; µl: Microlitre(10^{-6}l); bp: Base Pair; h: Hours; mRNA: Messenger RNA; PCR: Polymerase Chain Reaction; qPCR: Quantitative Polymerase Chain Reaction; RNA: Ribonucleic Acid; S: Seconds; Cq: Quantification Cycle

Introduction

Being a poikilothermic aquatic animal, minor changes in the environment may lead to stress in fish. Fishes are often exposed to various environmental stressors, such as pathogens, toxic gases, trauma, temperature fluctuation, and hypoxia. The fish uses various mechanisms to maintain the body's homeostasis, such as the production of heat shock proteins whose modulation is more during stressful conditions [1]. These highly conserved proteins present in all organisms were first discovered in the salivary glands of *Drosophila* [2]. The heat shock proteins were initially thought to be released only during heat stress, and it was later identified that various kinds of stress might result in its production, such as the infection by a pathogen [3]. It acts as a chaperone since it functions to refold the misfolded proteins. The heat shock proteins can be broadly classified into different families as follows Hsp110, Hsp90, Hsp70, Hsp60 (smaller HSPs), and low molecular weight Hsps depending on their molecular weights [4].

HSP70 family consists of highly conserved proteins in almost all organisms [5]. This family consists of constitutive (Hsp70) and inducible forms [6]. Hsp70 plays a vital role in the translocation of newly formed proteins from the cytoplasm to the nucleus, and it prevents the aggregation of the proteins [7]. It also influences major histocompatibility complex (MHC) dependant antigen processing and TLR signaling [8]. Hsp70 present in the protein complex of centrosome along with tubulin and elongation factor plays an essential role in the assembly and disassembly of this protein complex during cell division [9]. During thermal stress, Hsp70 plays a critical role in the thermo-tolerance process [10], and the expression of Hsp70 is increased by the adrenaline hormone [11].

Hsp90 is also induced by stress, and this protein is nearly abundant at average temperature in all the tested eukaryotes. It helps translocate bacterial toxins and Toll-like receptors (TLR) [12]. Hsp90 interacts dynamically with a diverse set of inherently unstable client proteins during physiological processes, such as kinases and transcription factors, in a highly selected manner [13]. Different co-chaperones help in the function of Hsp90 and help them to organize into discrete subunits. In a defined order, these subunits will function in the folding pathway of proteins [14]. The presence of an Hsp90 inhibitor increases the levels of Hsp70 [15]. In the fishes exposed to different stressors such as seawater challenge, handling, and heat shock, there was no correlation between Hsp90 mRNA and cortisol, a stress hormone [16].

The investigation of the relationship between heat shock protein and diseases of aquatic organisms is relatively recent. The first report indicating that the Hsp genes in fish show a response to bacterial infection was given by Forsyth., *et al*. on the bacterial kidney disease in *Oncorhynchus kisutch* [17]. Heat shock protein genes influence fish physiology in various ways, including stress physiology, immunology, environmental physiology, stress tolerance, and acclimatization [4]. The elevation of Hsp70 gene expression during disease conditions may be due to the inflammatory pathology or the ionic imbalance. After exposure to microbial and environmental stressors, Hsp70 can generate a balanced cytokine expression profile [18].

Edwardsiella septicemia is an acute to chronic disease of primarily warm water fishes caused by the bacterium *E. tarda* [19]. Still, there is no practical way to control and prevent *E. tarda* infection rather than practising good hygiene habits. The complication of infection is that it becomes systemic and finally causes meningitis, septicemia, and even death [20,21]. *E. tarda* has mainly been reported in Catla (*Catla Catla*) and rohu (*L. rohita*) in the Indian subcontinent fishes [22]. Rohu is the tastiest carp among the IMC (Indian Major Carps), and it fetches high market values. Vaccination can be the best method for preventing bacterial diseases such as edwardsiellosis instead of antibiotics in fish culture facilities. While developing a live or killed vaccine using a pathogen, it is crucial to check and know the stress induced by both the vaccine types since introducing foreign antigens would cause stress to the host tissue. The vaccination strategy and the type of vaccine chosen should be based on the criteria of minimum stress inducement. Developing a killed and live vaccine against *E. tarda* for *L. rohita* is a promising field in the research area for a disease prevention strategy**.** Further, it is imperative to compare the expression of significant heat shock protein genes in *L. rohita,* induced by formalin killed, sublethal and lethal dose of *E. tarda* in its vital organs such as the gill, kidney, spleen, and liver as a measure of the induced stress to understand the background trepidation influenced by vaccination using bacterial components.

Materials and Methods

Fish and bacterial strain

Advanced fingerlings of rohu, *L. rohita* with a weight of 20±6.6g (mean±SD) used for the experimental purpose were procured from Pen, Raigad District, Maharashtra. The fishes were disinfected with five ppm $KMnO_4$ for 10 min and acclimatized in a 500L FRP tank with continuous aeration and further reared in separate tanks and fed with 0.5mm commercial dry pellets at 2% of fish's body weight. *Edwardsiella tarda* (ATCC® 15947™) used in the study was revived using brain heart infusion (BHI) broth from the cult loop, and it was incubated for 18-24 h at 28°C. The bacterial culture was then streaked on SS agar media (Salmonella-Shigella agar, Himedia, India) and incubated at 28°C for 18-24h.

Preparation of inactivated E. tarda bacterin

The strain of *E. tarda* ATCC® 15947™ in BHI broth (50 ml) containing 1.4×10^{10} CFU/ml was centrifuged at 10000×g for 10 min at 4 °C. The inactivation procedure followed was adapted from previous study [23]. The bacterial cells were pelleted and washed with PBS (0.01 M phosphate-buffered saline, pH 7.2) and treated with 0.3% formalin (V/V). After treatment, the pellet was washed

again, and suspended in 50ml PBS. The concentration was adjusted according to the requirement. The formalin-inactivation of the cells was confirmed by streaking on BHI agar.

Challenge study and sample collection

The LD_{50} dose of *E. tarda* was calculated prior to the experiment with the fishes of the same stock. Fishes were divided into groups, and different dilutions of the bacteria ranging from 10^6 to 10^9 were injected (100 µl) intraperitoneally, and the PBS injected group was taken as control. Mortality was recorded for ten days, and the mortality that occurred after 12 hr was counted; subsequently, the LD_{50} value was calculated as per the method described by [24]. A total of 30 rohu fingerlings were challenged intraperitoneally (100 µl) with the live *E. tarda* at an LD50 dose (T1) of 3.5×10^7 CFU per 20g of body weight, a sublethal dose (T2) that is log 10 lesser than the LD_{50} dose and is of 3.5×10^6 CFU per 20g of body weight and formalin-killed *E. tarda* (T3) at an LD_{50} dose of 3.5×10^7 CFU per 20g of body weight for each treatment. The kidney, liver, spleen, and gill tissues were sampled ethically at different time intervals such as 0 hr, 3 hr, 6 hr, 12 hr, 24 hr, 48 hr, and 72 hours post-challenge (hpc) in duplicates after anaesthetizing fish with clove oil. The organs were collected using sterile scissors and forceps. The collected tissues were preserved in Trizol™ reagent (Invitrogen, USA) for further analysis.

RNA extraction and cDNA synthesis

According to the manufacturer's protocol, total RNA from tissue samples (kidney, liver, spleen, and gill) of different treatment groups of *L. rohita* was isolated using the TrizolTM reagent (Invitrogen, USA). Before cDNA synthesis, the total RNA isolated was treated with RNase-free DNase 1 (Fermentas, USA) to remove DNA contamination. Using the random hexamer primer, cDNA was synthesized by following the standard method.

qPCR analysis of Hsp70 and Hsp90 genes

Primer sequences of β -actin and Hsp90 were obtained from the previous study [12]. Hsp70 primers were obtained from the NCBI database (Table 1). Real-time PCR was performed in an AriaMx Realtime PCR system (Agilent, USA) using maxima R SYBR green PCR master mix (2x) (Fermentas, USA). Each reaction was prepared in triplicates to a total volume of 10 µl per reaction by adding the following components: maxima R SYBR green PCR master mix (2x)-5 µl, forward, and reverse primers (10 pM) each-1 µl, template cDNA-1 µl, nuclease-free water 2 µl. Each sample was normalized with gene-specific beta-actin primers [25]. The realtime PCR program consisted of initial denaturation at 94°C for 2 min and 40 cycles of the run at 94°C for 45 S, annealing temperature (Ta) of Hsp70 and Hsp90 were 56°C and 54°C, respectively, for the 30s, 72°C for 45 S and the final extension at 72°C for 7 min. qPCR specificity was verified by melt curve analysis at the temperature of 95° C for 10s, 65°C (1 min), and 95°C for 1 min.

Sl. No	Primer Name	Primer Sequence From 5' to 3'	Size	
1.	Hsp 70 F	TGAGAGACGCCAA-GATGGAC	104bp	KM369886.1
2.	Hsp70 R	CGTT-GAAGAAATCCTG-CAGAAGC		
3.	Hsp90F	ACCAAAGCC-GACCTCATC	121bp	[12]
4.	Hsp90R	AGAAACCCACGC-CAAACT		
5	β -actin F	TTGGCAATGAGAG-GTTCAGGT	153bp	
6.	β -actin R	TTGCCATACAG-GTCCTTACGG		

Table 1: Primers list and product length.

Relative quantification

The quantification cycle (Cq) values were calculated using Agilent aria 1.3, and the data were exported. The comparative Cq method [26] was used to calculate N-fold differential expression by calculating the average of each Cq for the triplicate samples. ΔCq value was calculated by subtracting the Cq value of the gene from its respective Cq value of normalizer, β-actin. Further, the ΔCq of the samples were subtracted from the ΔCq value of the calibrator to get the ΔΔCq. The fold difference was calculated as $2^{-\Delta\Delta Cq}$. The mean fold difference was calculated and represented as ± standard error.

Myeloperoxidase activity

The myeloperoxidase activity was determined as described and partially modified by [27]. Briefly. 15 µL of serum was diluted with 135 µL of Hanks balanced salt solution (HBSS) without Cal++

or Mg++ in a 96-well microtitre plate, in which 25 µL of 20mM 3,3,5,5-tetramethyl benzidine hydrochloride (TMB) (Genei, India) and 25 µL of 5 mM hydrogen peroxide (H_20_2) were added and incubated for 2 min. The reaction was stopped by adding 50 µL of 4M sulphuric acid and optical density was read at 450 nm by an ELISA plate reader (BIOTEK, USA).

Glutathione peroxidase activity

Glutathione peroxidase activity was assayed following the rate of NADPH oxidation at 340nm by the combined response with glutathione reductase. The specific activity was measured using the extinction coefficient of 6.22 mM/cm. [28]. Glutathione reductase activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm.

Statistical analysis

The data were analyzed using the statistical package SPSS version 22. A comparison of all the treatments was made by one-way ANOVA. Comparisons were made at 5% probability levels.

Results

Expression profiling of heat shock protein genes (Hsp70 and Hso90) was analyzed during the live and formalin-killed *E. tarda* challenge. The live challenge of *E. tarda* was done using two different treatment doses a lethal dose and a sub-lethal dose.

Hsp70 gene expression was upregulated in the liver during all the treatments, such as the lethal dose (T1), sublethal dose (T2), and formalin-killed *E.tarda* (T3). Whereas T3 showed the highest expression level of the Hsp70 gene in the liver tissue, it is significant at 6 hpc and 48 hpc (Figure 1a). In T1, it is upregulated at the initial stage (3 hpc), and the further gene expression was reduced, and moderately higher expression was observed at 24 hpc (Figure 1a). The T2 showed significantly higher Hsp70 gene expressions at 48 hpc in the liver (Figure 1a). In spleen tissue, Hsp70 gene expression was gradually increased up to 24 hpc in T1 with a slight decline at 6 hpc (Figure 1b). No significant Hsp70 gene expression was observed for T2 for all the experimental periods in the spleen. The T3 showed higher Hsp70 gene expression (7-fold) at 12 hpc in the spleen (Figure 1b). The T1 showed significantly higher Hsp70 gene expression at 24 hpc in the kidney (Figure 1c). No significant Hsp70 gene expression was observed in all the experimental periods for the T2 and T3 in the kidney tissue (Figure 1c). During all the experimental periods, no significant Hsp70 gene expression was observed in the gill for all three treatments (Figure 1d).

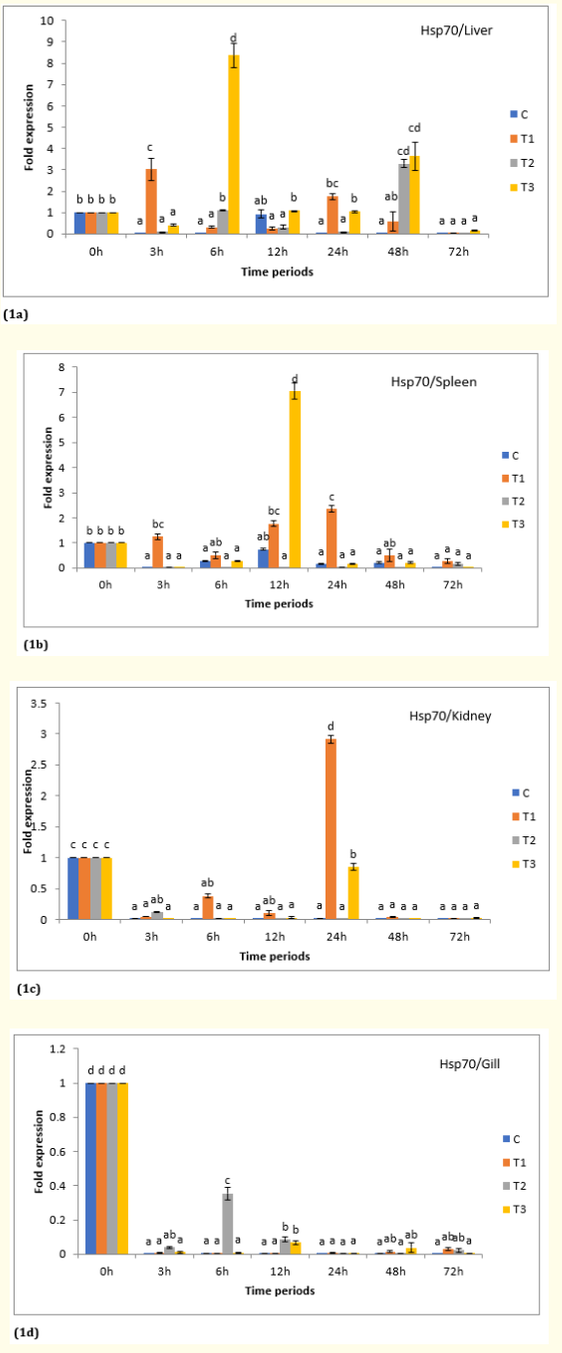
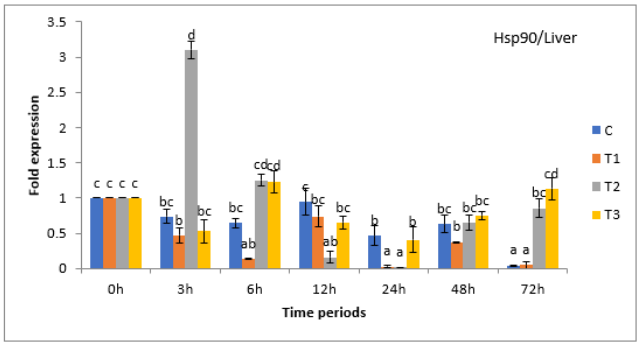
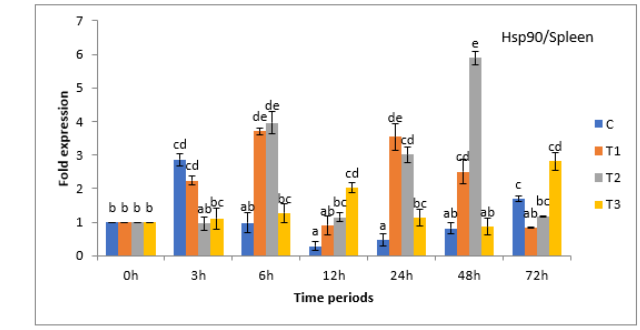


Figure 1: Expression analysis of Heat shock protein 70 (Hsp70) gene in *L. rohita* liver (1a), spleen (1b), kidney (1c), gill (1d) at different time periods (*h*, hours post-challenge) after injection of Phosphate buffered saline (C), lethal dose of *E. tarda* (T1), sublethal dose of *E. tarda* (T2), and formalin-killed *E.tarda* (T3). Fold expression was calculated as $2^{-\Delta\Delta C}$T; Control (0 h post-challenge) was taken as the calibrator for the treatment groups. Significant differences between different time periods are indicated by different letters (a, b, c and d) over the bars.

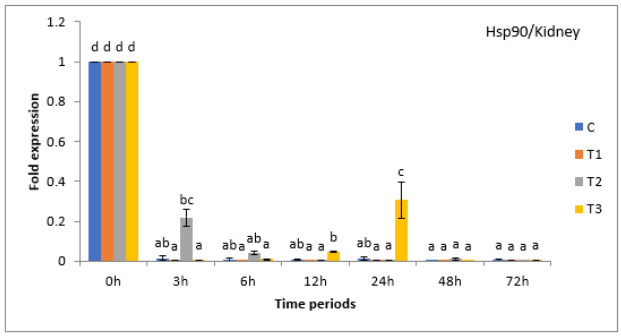
The Hsp90 gene expression was upregulated in the T2 in the liver. It showed initial high expression at 3 hpc, and expression gradually decreased later. Whereas the T3 showed no significant Hsp90 gene expression at the initial stages, the liver showed mild expression at 6 hpc and 72 hpc (Figure 2a). The T1 showed no significant Hsp90 gene expression in all the experimental periods in the liver (Figure 2a). In the spleen, the T1 showed a gradual increase in Hsp90 gene expression during 3 to 6 hpc, and then the expression was reduced at 12 hpc; however, the expression was higher at 24 hpc and gradually decreased during 48 to 72 hpc (Figure 2b). The T2 showed the maximum Hsp90 gene expression at 48 hpc in the spleen. The T3 showed a moderate fold change at 12 hpc and 72 hpc in the spleen (Figure 2b). The T1 and T2 showed no significant Hsp90 gene expression than control in the kidney (Figure 2c). The T3 showed no significant Hsp90 gene expression though a mild change was observed at 24 hpc in the kidney (Figure 2c). During all the experimental periods, no significant Hsp90 gene expression was observed in the gill for all three treatments (Figure 2d).



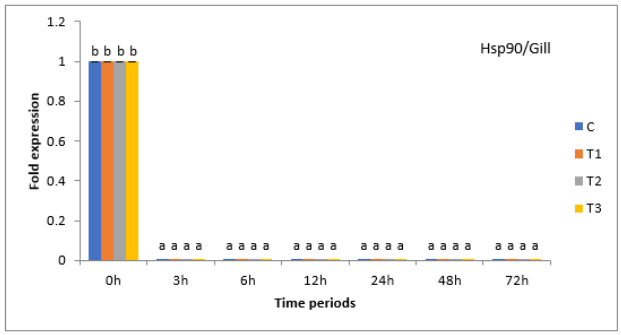
(2a)



(2b)



(2c)



(2d)

Figure 2: Expression of Heat shock protein 90 (Hsp90) gene in *L. rohita* liver (2a), spleen (2b), kidney (2c), and gill (2d) at different time periods (*h*, hours post-challenge) after injection of Phosphate buffered saline (C), lethal dose of *E. tarda* (T1), sublethal dose of *E. tarda* (T2), and formalin-killed *E. tarda* (T3). Fold expression was calculated as $2^{-\Delta\Delta C}T$; Control (0 h post-challenge) was taken as the calibrator for the treatment groups. Significant differences between different time periods are indicated by different letters (a, b, c, d and e) over the bars.

Among the three treatments, the formalin-killed *E. tarda* showed more expression of Hsp70 production followed by sublethal and lethal doses. High Hsp90 expression was observed in fishes challenged with live *E. tarda* than killed bacteria. Among all the sampled organs, the liver and spleen were found to play a significant role in producing both Hsp70 and Hsp90 genes. Simultaneously, both the Hsp70 and Hsp90 gene expressions were almost insignificant in the gill and kidney tissues. Interestingly, the overall high Hsp70 gene expression was found to occur at 6-24 hpc, and the overall high Hsp90 gene expression was found to occur at

3 and 48 hpc. Among the genes, Hsp90 and Hsp70 high fold change was observed for Hsp70 than Hsp90 gene, accounting for around 8 fold increment (Figure 1a).

The significantly ($p<0.05$) high myeloperoxidase activity was noticed in all the treatment groups (T1-T3) when compared with control on 6 and 48 hpc. Further, the highest activity in myeloperoxidase was noted with T2 and T3 at 48 and 6 hpc, respectively (Figure 3). A significantly ($p<0.05$) high glutathione peroxidase activity was noticed in all the treatment groups (T1-T3) when compared with control on 3 and 72 hpc. Further, the highest activity in glutathione peroxidase was noted with T1, T3, and T2 at 3 and 72 hpc, respectively, with significantly less activity at 48 hpc for all three treatments (Figure 4).

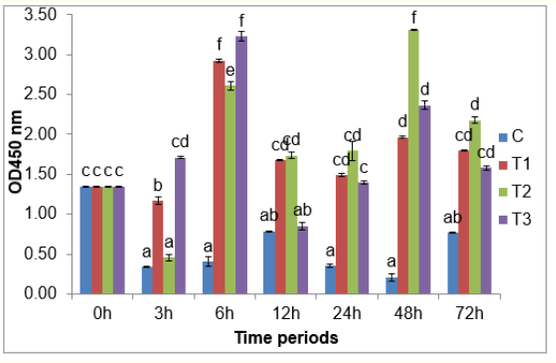


Figure 3: Myeloperoxidase activity of *L. rohita* at different time periods after injection of (C) Phosphate buffered saline as control, lethal dose of *E. tarda* (T1), sublethal dose of *E. tarda* (T2), formalin-killed *E.tarda* (T3). Significant differences between different time periods are indicated by different letters (a, b, c, d, e and f) over the bars.

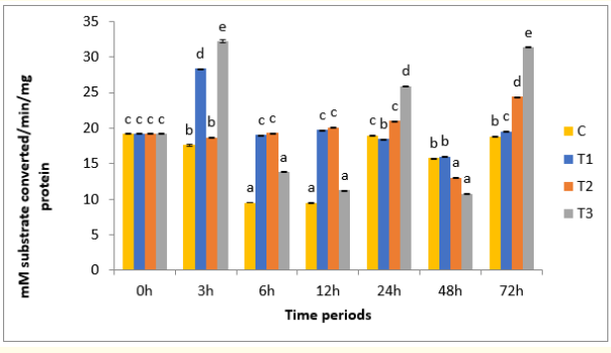


Figure 4: Glutathione peroxidase activity of *L. rohita* at different time periods after injection of (C) Phosphate buffered saline, lethal dose of *E. tarda* (T1), sublethal dose of *E. tarda* (T2), formalin-killed *E.tarda* (T3). Significant differences between different time periods are indicated by different letters (a, b, c, d, and e) over the bars.

Discussion

Heat shock proteins are a unique kind of proteins found in almost all higher organisms known to perform certain critical biological functions such as protein structural modifications, protein degradation, assembly of some complexes, thermotolerance, and buffering the expression of some mutations. Another essential function of Hsp is the regulation of the physiology of the animal in response to stress [29]. The present study was taken up to explore the role of Hsp during *E. tarda* infection in *L. rohita*. Hsp, an indicator of the stress response, may also have a specific function in pathogen stress. Differential expression pattern of Hsp during bacterial infection was observed by Yang., *et al*. [30]. Also, there is a study targetting Hsp response during *A. hydrophila* infection in rohu. Das., *et al*. have studied the expression of many Hsp genes during *A. hydrophila* infection [12]. However, a study on Hsp expression in the live bacterial vs killed bacterial challenge has not yet been undertaken. In the current study, we have analyzed Hsp response in *L. rohita* during artificial infection with an intracellular pathogen, i.e., *E. tarda*, concerning live vs killed bacteria. The study's ultimate aim was to identify the role of Hsp (stress) under killed bacterial induction, which would help develop a killed bacterial vaccine in the future. In the current study, expression of two heat shock proteins such as Hsp70 and Hsp90, were profiled for *E. tarda* infected *L. rohita* during different periods such as 3, 6, 12, 24, 48, and 72 hpc for various treatments such as the lethal dose of *E. tarda*, the sublethal dose of *E. tarda*, and the formalin-killed *E. tarda*.

Highly conserved Hsp70 is a group of proteins found in all studied cells, except for one hydra species, *Hydra oligactis* [31]. It is also the most widely studied heat shock protein in fish during infections. Hsp70 was denoted as a cellular thermometer by Sanders and Martin [32] because of its increased expression against the damage caused by many stressors, including pathogenic organisms. In two species, such as buffalo sculpin (*Enophrys bison*) and English sole (*Parophrys vetulus*), exposed to different temperatures, synthesis of Hsp70 was observed by Dietz and Somero [33]. In the current experiment for all three treatments, the Hsp70 expression showed a varying profile in all the four tested tissues, such as the kidney, liver, spleen, and gill. Nevertheless, Hsp70 specifically showed the highest expression in the liver tissue, suggesting its vital role in Hsp70 production during bacterial infection, especially *E. tarda.* A similar result was also obtained by Forsyth., *et al*. [17]. Nakano., *et al*. also observed higher Hsp70 expression in the liver of Japanese flounder during *E. tarda* infection [34]. Except for the gill, all the other three tissues, such as the kidney, liver, and spleen, showed good expression of Hsp70 for *E. tarda* challenge. As *E. tarda* causes

systemic infection, tissues like the liver, kidney, and spleen showed higher Hsp70 expression. A similar outcome was observed in a study by Nasrullah., *et al*. [35], wherein the upregulation of Hsp70 in the liver, kidney, and spleen of *Clarius gariepinus* during *A. hydrophila* infection was noticed.

When compared with all the four tissues, the gill showed minimal expression for Hsp70 at all hpc periods, which can be correlated with the results of Dietz and Somero [33], in which the Hsp70 concentrations in the gill of the sanddab (*Citharichthys stigmaeus*) were much lower during various temperature exposures. The vital role of other tissues such as the liver and kidney can be predicted against *E. tarda* infection using Hsp70 expression in the points till 48 hpc and 24 hpc. This result can be correlated with the results obtained by Forsyth., *et al*. [17], wherein the increased expression of Hsp70 in the liver and kidney tissues of Coho salmon, *Oncorhynchus kisutch*, during *Renibacterium salmoninarum* infection was noted, however at 63 days post-exposure. In the present study, when the Hsp70 gene expression was compared, the kidney showed a low expression (2.8-fold) and lay next to the liver (8.4-fold) and spleen (7-fold), which contradicts the results obtained by Wang., *et al*. [36] in that the expression of ScHSP70a is higher in kidney than liver and spleen (i.e., liver and spleen almost had same expression level) in the mandarin fish, *Siniperca chuatsi* under normal physiological conditions.

The group injected with the formalin-killed *E. tarda* showed the highest Hsp70 expression in the present study. In a similar study by [23], the formalin-inactivated *E. tarda* bacterin showed the highest Hsp70 expression with the increasing bacterin load (i.e., the maximum Hsp70 expression at 10^9 CFU ml^{-1}), suggesting the need to optimize the concentration of vaccine in a manner that it does not exceed the phagocytic capacity of host or cause stress to fish. In the present study, next to formalin-killed *E. tarda*, the lethal dose of *E. tarda* showed a higher Hsp70 expression, which was pronounced in all the three tissues except the gill; inducement can be due to the stress caused by live infection of *E. tarda* just as what happened with acute infection of *Vibrio anguillarum* in rainbow trout [37]. Since Hsp70 plays a significant role in folding proteins, increased expression of Hsp indicates dynamic and triggered activities due to the introduction of a foreign antigen into the cells, leading to the formation and translocation of the new protein. It can be interpreted that the host body is trying to compensate for the stress caused by the different treatments of *E. tarda;* as suggested by Sung and MacRae [38] that, microbial infection is the devastating stressor that can modulate the physiological activity of fish. This also implies that the host body is undoubtedly having some reaction against the treatment, and this reaction is more in the case of formalin-killed *E. tarda* than the lethal dose of *E. tarda* because the fishes might have been exhausted due to the lethal *E. tarda* dose. Hsp70 was also over-expressed in rainbow trout after acute *Vibrio anguillarum* challenge [37]. In the present study, Hsp70 was upregulated at an earlier stage, i.e., 6 hpc (8.4 fold) and 12 hpc (7 fold), in the liver and spleen, respectively. The expression of Hsp70 in the liver was observed to fluctuate during the different post-challenge periods. A similar result was observed by Peng., *et al*. [39] in which the SpHsp70 expression in the liver was upregulated at 6 hpc (3.9 fold); however, the spleen showed a different period of upregulation, i.e., 120 hpc (2.5 fold), after *Streptococcus agalactiae* challenge in Ya-Fish (*Schizothorax sprenanti*). Further, the Hsp70 expression pattern in the liver is inconsistent during different experimental periods with two independent bacterial challenges using *Streptococcus agalactiae* and *Aeromonas hydrophila* in Ya-Fish [39].

In the present study, Hsp70 is observed to be a key player in stress in the liver, spleen, and kidney. Overall, many cascade activities must have been undergone in the liver, where Hsp70 played a key role. Lower expression of Hsp70 in gill maybe because of the route of administration of the bacteria. In the present study route of administration was intraperitoneal, and gill tissue reacts better to local infection than systemic [40]. The overall elevated expression of Hsp70 in vital organ indicate a protective response to bacterial infection.

Dietz and Somero [41] observed enhanced synthesis of Hsp90 in the brain of two species, such as *Gillichthys mirabilis* and *Gillichthys seta*, held at elevated temperatures. Hsp90 seemed to be involved in bacterial immunity, and increased expression was also found during viral infections in fish [42,43]. During bacterial infections, the role of this protein was uncertain and mostly related to the particular environment. In the kidney and gill, the fold change of expression was meagre. However, Xie., *et al*. [40] noticed upregulation of Hsp90 in the gill during *Flavobacterium columnare* challenge. These results further confirm that gill tissue reacts better to local infection than systemic. The present experiment

found that Hsp90 is mainly expressed in the liver and spleen during bacterial infection. However, Li., *et al*. reported the Hsp90A expression during salinity stress in different tissues, including the liver, kidney, and gill in silvery pomfret (*Pampus argenteus)* [44].

In the liver, Hsp90 is shown to be down-regulated at all the time points with the lethal dose *E. tarda* treatment; however, in the *E. tarda*-induced group (sublethal), it was observed to increase during the early time point (3 h), and then it was noticed to be reducing. In the spleen, the Hsp90 expression pattern was irregular and not conclusive. This type of fluctuation in expression pattern was also noticed in previous studies. For example, increased expression of the Hsp90 was found at 3, 12, and 24 hpc, no change after 24 hpc, and down-regulation at 48 hpc in gills of black tiger shrimp *Penaeus monodon* when challenged with *Vibrio harveyi* [45]. In the *E. tarda* infected *L. rohita*, Hsp90 was almost negligibly expressed in the kidney and gill. This contradicts the results of Dietz and Somero, in which the expression of Hsp90 in gill tissue of English sole was found to be high at elevated temperatures [33]. Compared to all the treatments, a rising level of Hsp90 was observed in the live bacterial groups. Lethal and sublethal groups show higher expression in the spleen than the formalin-killed *E. tarda* group. A sublethal dose of *E. tarda* induces the highest Hsp90 at 48 hpc (5.8-fold), followed by the lethal dose.

The liver's Hsp90 expression was high in the initial stages and gradually reduced during the later periods. A similar pattern was noticed by Das., *et al*. and Rungrassamee., *et al*. [45], in which the expression was high in the initial periods and down-regulated at later stages [12]. Zhou., *et al*. also observed the upregulation of Hsp90 in the initial stages of a bacterial infection in *Channa argus.* Hsp90 is required for transporting bacterial toxins from the endosome to cytosol [46]. This may be the reason for the initial up-regulation because of the bacterial proliferation and transportation of bacterial toxins [47]. Das., *et al*. have quoted that the down-regulation in the later stage may occur due to reduced bacterial load in the surviving fish [12]. In the kidney, although Hsp90 was down-regulated, a mild expression was found at 24 hpc, compared to other time points in the vaccinated group and at 3 hpc in the sublethal group. This denotes that Hsp90 has a significant role in the vaccination and sublethal infection stress. This indicated an essential function of Hsp90 in mild bacterial infections when bacteria are multiplying. The present study shows that the inflammatory response during active bacterial infections is more at the early stage of infections, especially in the liver (3 hpc) and spleen (48 hpc). However, another study states that the differences in the cytokine responses to live versus heat-killed bacteria provide the transcriptional up-regulation of pro-inflammatory cytokines in response to live bacteria in the liver than spleen and kidney [48]. The rise of Hsp90 in the spleen during later hpc in the formalin-killed *E. tarda* group indicates a less inflammatory response than a live bacterial infection. The present study corresponds to the statement of Pu., *et al*. that Hsp90 could be used as a biomarker for bacterial stress [49].

Neutrophils are the primary defenders of the innate immune system. These phagocytic cells ingest bacteria into phagosomes, where most microbes are killed and digested. Neutrophils contain a rich supply of the green heme enzyme MPO (Myeloperoxidase), which constitutes a powerful antimicrobial system in combination with H_2O_2 and chloride [50]. Stosik., *et al*. observed increased MPO activity in common carp after bacterial infection [51]. Sahoo., *et al*. recorded higher MPO activity in the resistant line of *L. rohita* compared to the susceptible line after *Aeromonas* infection [52]. Behera and Swain, demonstrated increased MPO activity in *L. rohita* on immunization with H.S-layer protein of *A. hydrophila* adsorbed on nano-sized calcium phosphate particles [53]. The present result agrees with the above findings; however, the results contrast with Mohanty and Sahoo, where a significant decline in MPO activity in *L. rohita* when challenged with *E. tarda* [54]. MPO activity is an absolute indicator of oxidative stress. Under bacterial infection, many phagocytic activities are there, which theoretically increase the level of MPO activity. The present study also found elevated MPO activity in all treatment groups. However, MPO was significantly higher in the live bacterial challenge group (T1 and T2) compared to the killed *E.tarda* group (T3). This also can be correlated to the triggered expression of Hsp70 and Hsp90 in the liver and spleen, where maximum host response is expected after intraperitoneal administration of bacteria.

Glutathione peroxidase (GPx) catalyzes the reduction of various hydroperoxides (e.g., H_2O_2) via oxidation of reduced glutathione (GSH) into its disulfide form glutathione disulphide (GSSH). H_2O_2 produced during stress by other mechanism of the body will be detoxified by GPx. Zhang., *et al*. reported the highest activity of GPx at 24 hpc of the pathogen in *Venerupis philippinarum* [55]

as in the formalin killed *E.tarda* treated groups (T3). Significant elevation in all treatment groups at 6 hpc corresponds with Hsp90 and Hsp70 expression in systemic tissue in the present study. Overall it was found that Hsp70 and Hsp90 have a critical role to play during bacterial infection. The expression pattern under live vs the killed bacterial situation differed at some time points. Further study on stress-related molecules would help understand the host's response under pathogen stress. This will also be useful for developing a killed vaccine against the pathogen.

Conclusion

The study concludes that Hsp70 can be an indicator of vaccination stress (i.e., formalin-killed *E. tarda*) and Hsp90 can be an indicator of infection stress (i.e., the sublethal dose of *E. tarda*). The current research indicates that Hsp90 can be a marker for live vs killed bacterial induction, especially in delimited tissues such as the spleen and liver. However, the study needs further research and characterization of similar stress proteins and the involvement of those proteins in live vs killed bacterial administration to find the interplay and correlations.

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Conflict of Interest

The authors state that there is no conflict of interest.

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