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Changes in transferrin gene expression in sea bass (*Dicentrarchus labrax*) challenged with *Vibrio anguillarum*

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Abstract: *Vibrio anguillarum* expresses several virulence factors and causes hemorrhagic septicemia accompanied by serious losses in marine fish. Transferrin is a glycoprotein, also known as a multitasking protein, which is mainly synthesized by the liver. It has a fundamental role in the immune system. In the present study, the transferrin gene expression of sea bass (*Dicentrarchus labrax*) was investigated during an experimental infection with *V. anguillarum*. Fish samples were examined by hematological and serological methods as well as real-time polymerase chain reaction. The infection was performed via water. The sampled fish displayed vibriosis infection symptoms, both internally and externally. The transferrin saturation in the diseased fish serum decreased dramatically and the transferrin gene expression increased during the first 2 days; however, it decreased in the subsequent days.

Key words: Transferrin, *Vibrio anguillarum*, Q-PCR, iron, siderophore

1. Introduction

Aquaculture is an important economical asset in Turkey, which was the leading European producer of sea bass in 2008 (1). *Listonella anguillarum* (basonym: *Vibrio anguillarum*), a pathogen of sea bass (*Dicentrarchus labrax*), causes hemorrhagic septicemia accompanied by serious losses (2,3).

V. anguillarum is a gram-negative, polarly flagellated, comma-shaped rod bacterium that expresses several virulence factors. The most researched virulence factor, pJM1 plasmid and its genes, is related to its ability to scavenge the iron contained in host tissues. The main mechanisms of iron acquisition are based on the synthesis of siderophores (4–9).

Iron is an essential element for the metabolism of organisms, but is toxic when it is found free in the blood. Fish growth can be influenced by adding dietary levels of iron in addition to that already present in the water (10). It has also been shown that iron availability in the gastrointestinal tract might play a significant role in the growth and multiplication of *V. anguillarum* and *Photobacterium damselae* subsp. *piscicida* (11–13).

Transferrin is a glycoprotein that is mainly synthesized by the liver and its role includes the storage, use, and transport of ferrous iron through the blood. It is known as a multitasking protein. It also has antibacterial activity through the binding of free iron present in the blood (14,15). However, *V. anguillarum* can utilize iron ions sequestered from transferrin by the bacterial siderophore (2,4,5,16).

The transferrin response in sea bass infected with *P. damselae* subsp. *piscicida* was recently studied (13). In that experiment, the transferrin gene was mainly found to be expressed in the liver and brain. These authors found that the transferrin expression was increased in the brain tissue, but in the liver, its expression was decreased during infection. In the present study, the transferrin gene expression in the liver as an immune reaction in fish was investigated in sea bass during an experimental infection with *V. anguillarum* for 7 days. Fish samples were examined by hematological and serological methods as well as real-time polymerase chain reaction (Q-PCR) for gene expression.

2. Materials and methods

2.1. Fish

Sea bass, 30–40 g in weight, were kept in 200–L fiberglass tanks containing filtered, aerated, and circulated artificial

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sea water (30 ppt, 22–24 °C) at the experimental laboratory of the Fisheries Faculty, İstanbul University, in İstanbul, Turkey. The fish were separated into 4 groups of 25 fish; 2 tanks were designated as control and 2 tanks were designated as experimental. There was no water flow or feeding during the experiment.

2.2. Bacterial samples

A *Vibrio anguillarum* strain isolated in the laboratory was used for the experimental infection. This strain was isolated from diseased cultured sea bass and characterized by colony morphology, Gram staining, growth on thiosulfate bile salt sucrose (TCBS) agar (Merck) and *V. anguillarum* medium (VAM) (17), biochemical properties (API 20E, bioMérieux), and agglutination (BIONOR Mono-Va) (12,18).

2.3. Production of siderophore

Chrome azurol S (CAS) (19) agar was used to determine the siderophore production of the isolated *V. anguillarum* strain. *Bacillus cereus* was also used as a positive control. Petri dishes were prepared with the CAS agar. After becoming solid, bacterial strains were spotted on the agar. At the end of the incubation time (24–48 h), siderophore producing strains showed yellow halos around the colonies.

2.4. Bacterial challenge and determination of the LD_{50}

V. anguillarum bacteria were prepared in nutrient broth (Merck) and incubated for 24 h at 24 °C in a shaking incubator. Bacteria were collected by centrifugation and washed with sterile phosphate-buffered saline (Sigma). Bacterial density was determined by spectrophotometer measurement of the optical density (OD_{600} = 1 × 10^9 bacteria mL^{-1}) (20). Initially, the determination of the median lethal dose (LD_{50}) was performed by a preliminary study (8 tanks with 160 fish). Four different dose regimes (10^3 to 10^6 mL^{-1}) were used to arrive at the LD_{50}. At the end of the study, 10^5 bacteria mL^{-1} was chosen for the main experimental infection because this dose was appropriate for monitoring of the disease progression.

After the determination of the $LD_{50,}$ the main infection was performed via water, with final dose of *V. anguillarum* at 10^5 mL^{-1} in 200 L of water. The infected fish were kept under observation for 7 days.

2.5. Sampled fish

Infected and control sea bass were anesthetized with 2-phenoxyethanol (Sigma) each day throughout the study period (Table 1). After day 2 of the experiment, fish deaths began, and after day 6, all of the infected fish died due to acute infection. The dead fish were collected and counted, but not examined further. Two fish samples were taken daily from the experimentally infected and control tanks, but after day 2, as the fish deaths began, the number of samples from the infected tanks increased. Following dissection, samples for RNA isolation and bacterial identification were aseptically taken from the liver. The bacteria samples were inoculated on tryptic soy agar (Merck) + 2% NaCl, TCBS, and VAM, and the biochemical characteristics of bacteria able to grow on that medium were investigated.

Table 1. Daily sampled and dead fish (collected from the infected fish group) number during infection with *V. anguillarum*.

Days postinfection	Infected fish sample	Dead fish
Day 1	2	0
Day 2	2	2
Day 3	3	2
Day 4	5	6
Day 5	3	6
Day 6	8	6
Day 7	0	5

2.6. Serological samples

Blood samples were drawn from the caudal vena of live fish. Erythrocyte and leukocyte numbers were determined as previously described (10,21). Serum samples were collected from the blood and kept at –20 °C until used. The liquid ferrozine method (UIBC Kit, BT Products) was used to determine the transferrin saturation. The serum iron level was measured only on days 3 and 6 of the infection using an autoanalyzer. As it was required to have at least a 1–mL sample for serum iron determination, it was possible to obtain this quantity by collecting serum from 3 to 4 fish.

2.7. Total RNA extraction and cDNA synthesis

Liver tissue samples of 10–30 mg were immediately used for RNA extraction. Total RNA was extracted using an Ultraclean Tissue RNA Isolation Kit (MO BIO) following the manufacturer's instructions and was examined by gel electrophoresis (1% agarose and 7% formaldehyde gels in Tris-acetate EDTA, 5 V/cm and 45 min) for structural damage (22). The isolated RNA was directly used in quantitative (Q) PCR as a negative control to test for DNA contamination. First-strand cDNA was synthesized from the total RNA (1 µL) using random hexamers and a Transcriptor High Fidelity cDNA Synthesis Kit (Roche) according to the kit's manual.

2.8. Quantitative PCR

The 29 expressed sequence tags (ESTs) of sea bass transferrin found by Chini et al. (23) were compared with the transferrin gene sequences of other marine fish species (red coral, black coral, etc.) for homology, which were obtained from sequences currently available in GenBank, retrieved using the BLAST program (http://www.ncbi.nlm.nih.gov). The sequence with GenBank accession no. CX535550 EST, having 815 base pairs, was chosen and

PCR primer sets were designed with Primer 3 software (http://frodo.wi.mit.edu) for the Q-PCR assays (Table 2).

A serial dilution from 10^7 to 10^3 copies of the target cDNA sequences was used to obtain the calibration curves. The Roche Light Cycler DNA Master SYBR Green I Kit and Roche Light Cycler 2.0 (Roche) were utilized for all of the reactions. Reaction mixes contained 25 ng of template DNA, 0.5 μM of each primer, and 2.5 μM $MgCl_2$. The following thermocycling program was applied: 95 °C, 10 min; 45 cycles of 10 s at 95 °C, 5 s at 55 °C, and 15 s at 72 °C. A melting curve analysis was performed from 55 °C to 95 °C to determine if only 1 amplified product was generated during Q-PCR. The Q-PCR runs were analyzed using Roche Light Cycler Software 4.05. The efficiencies were between 1.8 and 2.0, and the correlation factors (r^2) were not lower than 0.97 in all reactions. For the statistical analyses, one-way ANOVA with Tukey's post hoc test was used.

3. Results

3.1. Bacterial results

The sampled fish displayed hemorrhagic responses on the body surface, mouth, and gills. Internally, hemorrhages were found on the surface of the liver, spleen, and visceral organs. An accumulation of clear yellowish fluid and a white opaque mucoid substance were found in the intestine. Reisolated bacteria from the sampled fish were identified as *V. anguillarum* by phenotypic and biochemical characteristics. The API 20E profile number of these bacteria was 3007524. Identification was confirmed with the positive result of a BIONOR Mono-Va agglutination kit. Furthermore, the strain produced a siderophore on the CAS agar.

The average numbers of erythrocytes in the infected fish were lower than in the control fish (Figure 1). In the first 3 days, the average numbers of leukocytes were higher in the infected fish and then they started decrease to lower than that of the control fish.

Bacteria were observed on the blood smears during the infection (data not shown). The transferrin saturation and iron values in the diseased fish serum decreased dramatically (Figure 2).

3.2. Q-PCR results

A statistically significant change in transferrin expression level was observed in the infected group compared to the control. Changes in the relative abundance of transferrin gene (transferrin/actin gene abundance) are given in Figure 3. The transferrin expression level dropped 2.2-fold at 1 day after infection. The transferrin expression significantly increased 3.8-fold ($P < 0.05$) relative the control 2 days after the *V. anguillarum* exposure. The transferrin mRNA relative abundances of the infected fish were higher compared to that of the control at day 3. However, after day 2, the transferrin expression level of the infected group dropped to approximately 4-fold at 4–5 days and 6-fold ($P < 0.05$) at day 6.

4. Discussion

In this study, *Vibrio anguillarum* was reisolated from the liver after experimental infection and the liver samples were taken for gene expression analysis by Q-PCR. The diseased fish were noted to have petechiae at the base of the fins and on the skin. Internally, our findings were similar to those of other studies (2,12,24,25). In the infected fish, the erythrocyte numbers as well as transferrin saturation and serum iron levels decreased. With the exception of the first 3 days, the leukocyte numbers also decreased in the infected fish. Bacterial cells were found in the blood veins and hemorrhagic areas. These findings are similar with those of other researchers (2,13,26).

The functional structure of a gene provides valuable information about the organism. When an environmental condition (chemical, physiological, or pathological) is changed, it may cause responses at the transcriptome level in the organism (27). In several studies, the expression of different genes, related to the fish immune system, was determined during infection (13,28,29).

In the present study, the transferrin gene expression increased during the first days of the infection. This may be due to the immune system, which had tried to stop iron utilization by the bacteria as an acute phase response. From day 3 onwards after infection, while the transferrin saturation decreased logarithmically, the transferrin gene expression decreased in direct proportion. During the last

Table 2. Primers of the transferrin gene EST and β-actin gene of sea bass used for Q-PCR.

Forward for transferrin	5'- AATGGCAGTGGATTGAGGAC -3'
Reverse for transferrin	5'- CAAGACCTCTTGCCCTTCAG -3'
Forward for β-actin	5'- TGACCCTGAAGTACCCCATC -3'
Reverse for β-actin	5'- AGGAGTAGCCACGCTCTGTC -3'

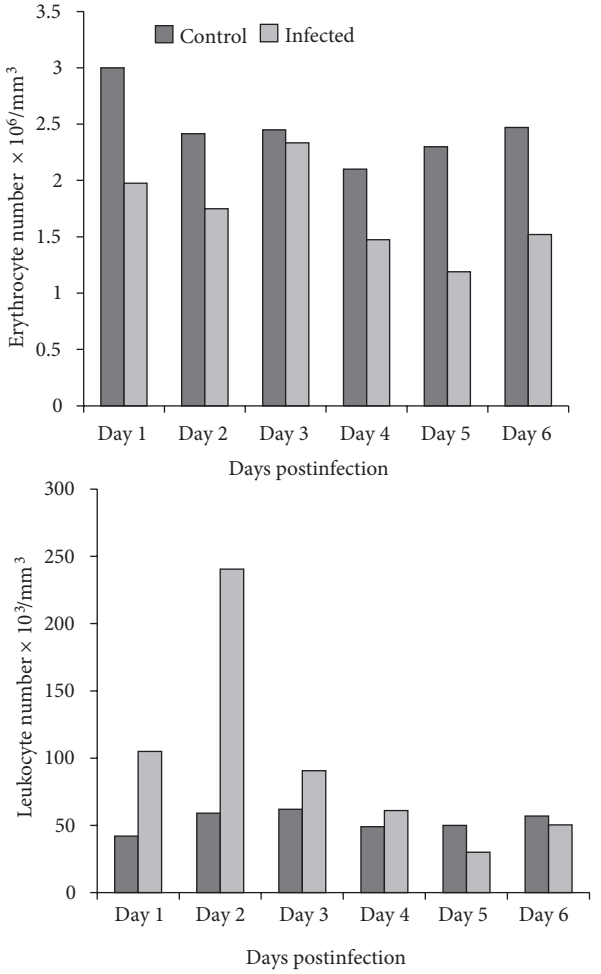

Figure 1. Erythrocyte and leukocyte average numbers of sea bass during infection with *V. anguillarum*.

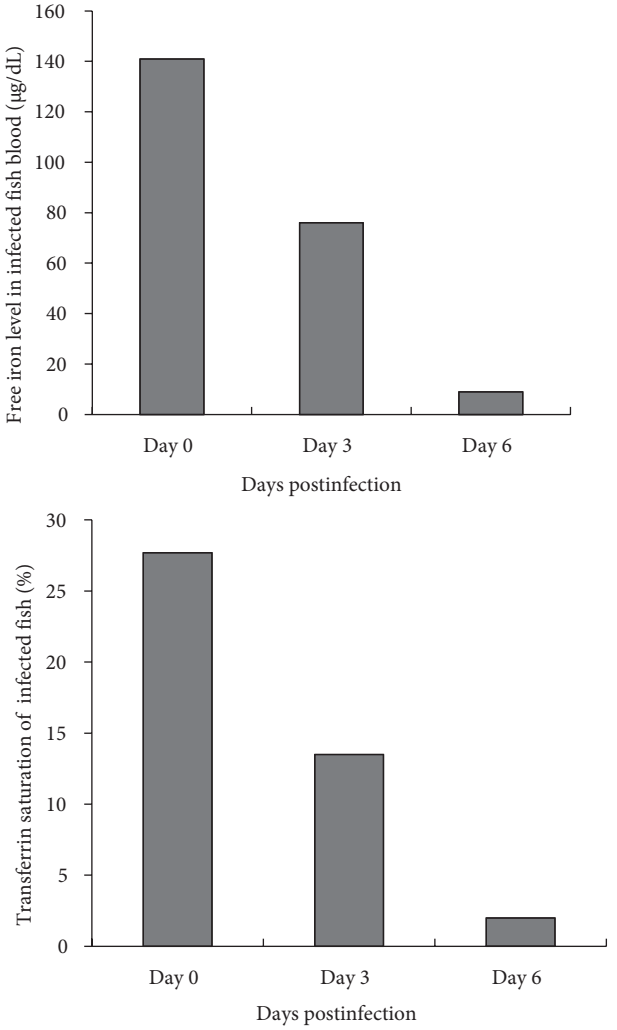

Figure 2. Free iron and transferrin saturation average levels of fish infected with *V. anguillarum*. Day 0 is noninfected fish blood.

days, since the transferrin saturation and iron levels in the blood were low, the transferrin was probably much higher than needed and its expression almost ceased. Bayne et al. (28) studied the genes involved in the acute phase response in trout infected with *V. anguillarum* intraperitoneally. In that study, specimens were collected over a 4-day period and the authors reported that the transferrin gene expression increased, contrary to our findings. However, they did not provide any information about bacterial density and utilized infection level. Neves et al. (13) investigated changes in the expression of transferrin and ferritin genes in response to experimental infection with *Photobacterium damselae* subsp. *piscicida*. In their experiment, real-time PCR was used and the transferrin genes were mainly found to be expressed in the liver. As an interesting finding, during the infection period, it was determined that transferrin was synthesized in the brain, and while its expression was increasing in the brain tissue, in the liver, which is the main site for transferrin synthesis, the expression was decreasing. In the present study, the transferrin gene expression increased during the first 2 days of infection, but in the following days, the expression decreased. Our findings, except for those of the first 2 days, correspond with those of Neves et al. (13), who investigated the gene expression in the liver of sea bass experimentally infected with *P. damselae* subsp. *piscicida*.

In conclusion, changes in the transferrin gene expression were observed during infection with *V. anguillarum*, the causative agent of vibriosis. In moribund fish, hemorrhage in the visceral organs and morbidity, as a result of acute septicemia due to weakening of the immune system, were observed. Since transferrin was not produced, it could not assist in the defense against *V. anguillarum*.

We can speculate that a halt in the gene expression may be due to the exhaustion of free iron in the environment. Further studies need to investigate how the transferrin gene expression level is changed during a chronic

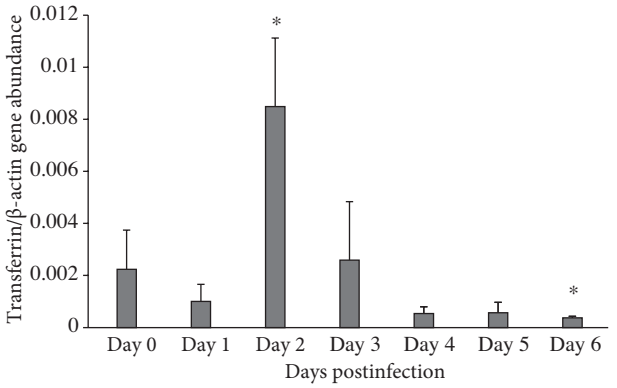

Figure 3. Y-axis values are expressed as mean transferrin/β-actin gene abundance in the liver under experimental infection with *V. anguillarum*. The day 0 value is healthy fish as a control group. Differences from the control group were considered significant at *P < 0.05. Vertical bars show standard errors.

infection and which factors affect its regulation. Using *V. anguillarum* at concentrations below the LD_{50} value would cause more chronic infection. In this study, changes in the transferrin gene expression levels in an acute infection were determined in the liver; however, the brain tissue was not used because the liver is the main site of transferrin synthesis. Neves et al. (13) published their study, which expresses the importance of transferrin synthesis in the brain, after the experiments in the present study were conducted. To understand the changes in the liver and brain tissue observed during chronic *V. anguillarum* infection, further studies are needed.

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