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Determination of acute phase proteins after experimental *Streptococcus iniae* infection in tilapia (*Oreochromis niloticus* L.)

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Abstract: Tilapia (*Oreochromis niloticus* Linnaeus, 1758) were infected with *Streptococcus iniae* (Cat No: ATCC 29178) and the plasma changes in the acute phase protein (APP) members were examined at different time periods. A total of 72 fish with average body weights of 60 g were used. Fish in the infected group (group I) were intraperitoneally injected with *S. iniae* at 3.3×10^5 colony forming unit (CFU) in 0.1 mL level to the fish. The other group (group II) was subjected to handling stress near the infection. The control group was constituted by providing optimum conditions. On days 7, 14, and 21, blood samples were taken from the caudal veins of the fish under the appropriate anesthesia. The samples were examined on the basis of APPs, C-reactive protein (CRP), fibrinogen (FB), serum amyloid A (SAA), and transferrin (TF) and the changes in the plasma values were recorded. The internal and external symptoms seen during the examinations were detected as follows: the color concentration in 1-2 days in the infected groups, irregularity and retardation in the movements of the fish, hyperemic spots on the skin, and bloody effusion in the internal organs. In both of the infected groups, the CRP and SAA plasma values increased on days 7, 14, and 21 ($P < 0.05$). Remission was observed in the plasma TF levels in the first week; however, after the second week the levels reached normal values. Decreases in the FB levels of all of the samples were detected. Based on the data acquired in this study, it was concluded that changes in the plasma levels of the APP members can be used as a significant bioindicator in the pathogenesis of the disease in fish infections.

Key words: C-reactive protein, fibrinogen, serum amyloid A, tilapia, transferrin

Introduction

Fish are among the most important sources of protein for human consumption; thus fish farming is one of the fastest growing segments of animal production in the world (1). However, stress and infectious diseases are among the most notable constraints on the expansion of aquaculture and the realization of its full potential; thus the study of the signs and lesions induced by fish diseases helps to protect our national economy (2,3).

The acute phase response (APR) is a pervasive physiological response of the body to injury, trauma, or infection (4,5) and has been defined as "the entire array of metabolic and physiological changes which occur in response to tissue injury or infection" (5). The APR, with its changes in blood plasma composition, is thought to be beneficial to the organism by preventing microbial growth and helping to restore homeostasis.

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The APR is best characterized by dramatic changes in the concentration of a group of plasma proteins known as acute phase proteins (APPs), which are synthesized in the liver (5). Some APPs opsonize microorganisms and activate complement [serum amyloid-A (SAA) and C-reactive protein (CRP)] proteins, while others scavenge cellular remnants and free radicals, or neutralize proteolytic enzymes [fibrinogen (FB) and transferrin (TF)].

The innate arm of the immune system responds to inflammatory stimuli by the activation of phagocytes, and by altered levels of several plasma proteins. These changes in the plasma proteins have drawn attention to the importance of the innate immune response in fish. Specifically, APPs are established diagnostic tools as early indicators of inflammation and disease (6) and many are now known to play beneficial roles in mediating the complex inflammatory response and seeking to restore homeostasis (5). Some APPs to viral, bacterial, and fungal inflammatory agents were also isolated from rainbow trout (7,8) and an expression analysis of the acute phase response after infection with a gram-negative bacterium were isolated from channel catfish (9) and zebra fish (10).

However, there is no information on the progress of the APR in tilapia (*Oreochromis niloticus*). Tilapia farms in many parts of the world suffer heavy losses due to bacterial pathogens. *Streptococcus* spp. are the most serious disease problems in tilapia production, causing 80% of fish mortalities (2,11,12) and have been recently listed among the emerging problems in aquaculture (13).

S. iniae is recognized as one of the most dangerous members of this genus, causing lethal infections in both freshwater and marine fish (14). Infection by *S. iniae* generally develops into septicemia, but clinical signs, pathology, and virulence of the disease vary in different hosts (15).

S. iniae has been an important model for immunological studies (16,17). Recently, tilapia has also been recognized as a valuable model to understand immunity (12,18). Many inferences have been made on various factors (stressors), which influence mortality due to streptococcal disease caused by *S. iniae* (3,19).

Determination of APPs can help in monitoring the health of individual subjects, especially when several acute phase variables are combined in an index. The acute phase reaction offers a biological effect mechanism appropriate to include in future systems for assessing health in animals and human patients.

The objective of this study was to determine some APPs developing in tilapia individuals after experimentally infection with *S, iniae*, in order to better diagnose *S. iniae* infections in tilapia.

Materials and methods

Tilapia care and maintenance

Tilapia, at an average weight of 60 g and length of 14 cm, were used as experimental animals. The study was conducted at the investigation station of the General Directorate of the State Hydraulic Works (DSI), between May and October. Fish used in the experiment were obtained from the fish farm of the DSI in Adana, Turkey. A sample of fish was culture negative for *S. iniae* by standard methods (12), prior to experimentation. Fish were stocked into 1.5 × 2.5 × 1 m tanks supplied with water at 26.2 ± 0.799 °C and a daily dissolved oxygen level of 5.4 ± 0.449 mg/L. The fish were fed carp feed at 2%-3% body weight daily and kept under a light/dark photoperiod of 12:12. The fish were stocked in 3 replicates per treatment at a density of 8 fish per tank. All of the fish were acclimatized for a minimum of 1 month prior to experimentation and were free from any active infections.

Cultivation of *Streptococcus iniae*

Overnight cultures of *S. iniae* were diluted at a ratio of 1:50 in nutrition broth. Briefly, the isolate of *S. iniae*, taken from a blood agar plate, was grown in Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, MI, USA) for 24 h prior to use, and harvested in the logarithmic phase of growth. The cells were washed once in sterile THB, resuspended, and then diluted to the appropriate concentration in sterile THB. The bacterial concentration was determined using a colony forming unit (CFU) per milliliter by plating serial dilutions onto BHI agar plates.

Identification methods

Morphological characteristics of *S. iniae* were obtained by growing them on THB and brain heart infusion agar (BHIA) (Difco) plates at 30 °C, incubated for 24 h, and visualized by light microscopy.

Routine microbiological testing procedures were used to confirm the species identification of the *S. iniae* strain. The identity of the isolated strain was tested using standard biochemical identification media incubated at 25 °C for 48 h (20). Bacteriological methods for the isolation, culture, and biochemical identification of *S. iniae* were applied also as described by Bilgehan (20).

The rapid ID 32 Strep (BioMerieux, France) is a suitable alternative system for rapid identification of members of the genus *Streptococcus* and of related genera. *S. iniae*, which had been characterized previously by the catalase and esculin test, growth test in methylene blue, and sodium chloride at 6.5%, was later classified in groups using the Slidex Strepto-Kit (BioMerieux). Tilapia were identified in 40 samples by their phenotypic characteristics. They were determined using the rapid ID 32 Strep Microtest after incubation at 30 °C and readings at 4 and 24 h.

Infection procedures

An isolate of *S. iniae* (ATCC-29178), obtained from the Clinical Microbiology Laboratory of Hebrew University in Israel, was used to challenge the tilapia. Injected intraperitoneally into each fish of the tilapia groups was 0.1 mL of the bacterial suspension (typically 5 fish per group). For intraperitoneal injection, an anesthetized fish was placed supine and supported. The needle was held parallel to the fish's spine and inserted cephalad into the midline of the abdomen, just posterior to the pectoral fins, and then 3.3×10^5 CFU/0.1 mL *S. iniae* was injected intraperitoneally. The sublethal dose was used to determine disease signs and effects on APPs. The tilapia in the control groups received THB only.

Experimental design

The study was conducted in 2 sequential experiments using tilapia. In the first group (the infected group, group I), a predetermined sublethal dose of *S. iniae* was injected into 24 fish kept in the tanks (8 fish in each tank). The second group (the handling group, group II) was exposed to both *S. iniae*-infection and handling stress for 15 min additionally. The last group was the control group. The fish were sampled on days 7, 14, and 21 after the end of the challenge trial. Samples for plasma analysis were taken from the caudal vein of the infected fish and utilized to analyze CRP, SAA, FB, and TF. Samples were put on ice and randomized during the assay. The blood taking process was completed only during the first hour to keep the manipulation stress level to a minimum (21). The water temperature was 26.2 ± 0.78 °C and was observed daily for 28 days, for any abnormal clinical signs and mortalities.

Method

Measurement of APPs in plasma

Plasma FB was analyzed using the Clauss method on a STA-SYSTEM analyzer (American Bioproducts Company, Parsippany, NJ, USA) and multifibrin was measured using a fibrin timer (Pathteq, Marburg, Germany). Blood samples taken in sodium citrate tubes were centrifuged at 2500 rpm for 15 min and the blood samples were separated. Control plasma N (code no. ORKE 65) and control plasma P (code no. OUPZ 21) were used for internal control quantities and C.V. was determined as 1.8% for this method.

Serum SAA was measured using the N-high sensitivity CRP with latex-enhanced immunonephelometric assay on a BN II analyzer (Dade Behring, Milan, Italy). The assay was carried out by and immune reaction with covalently coagulated specific anticores consisting of cell particles shaped like an envelope. A Cobas Integra autoanalyzer (Roche Diagnostics, Mannheim, Germany) was used for measurement. This assay has a detection limit of 6.8 mg/L and coefficient of variation is between 3%-7%.

Statistical analyses

The data obtained from the fish at the end of the study were determined comparatively using Duncan's multiple range test. Differences were considered significant at $P < 0.05$ or $P > 0.05$.

Results

The first experiment (infected group (group I), which involved the application of a 17% sublethal dose of *S. iniae* (3.3×10^5 CFU/0.1 mL) to tilapia intraperitoneally, resulted in the death of all infected tilapia within 24 h. In group II, only 21% of the fish died (out of 24 fish). Mortality and disease signs were recorded on a daily checklist.

The clinical signs and postmortem examinations showed darkening of the skin, lethargy in the fish, and immobility at the pool edges. These were the first signs observed in the challenged tilapia within 2-3 days. Exophthalmia was not observed in the infected fish. Histological analysis revealed lesions in the intestinal area, spleen, posterior kidney, and brain. Hemorrhages were observed on the skin, especially at the base of the fins and tail (Figure 1). Aside from this, hyperemia of the skin and bloody liquid accumulation in the body cavity were observed (Figure 2).

Identification

S. iniae was obtained from all of the infected tilapia, and specific *S. iniae* colonies on the bacterial cultures reproduced on THB and BHIA were defined by observation of gram-positive cocci. Pure colonies of *S. iniae* from all of the tissues with histological evidence of infection were determined using routine bacteriological culture methods. The cocci were isolated on THB and BHIA as gray, translucent, circular, slightly convex, pin-headed colonies, showing strong beta hemolysis.

The results of the biochemical characterizations of the isolates with the API rapid ID 32 Strep system and conventional biochemical tests are summarized in Table 1.

Table 1. Biochemical features of isolated *S. iniae* from the full automatic Rapid ID32 Strep identification system (see ref. 15).

Acid production from sugar	***S. iniae***
Arabinose	–
Glucose	+
Lactose	–
Mannitol	+
Sorbitol	–
Trehalose	+
Oxidase	–
Biochemical characters	***S. iniae***
Pyruvate	
H_2S	ND
H_2S	ND
Esculin hydrolysis	–
Hippurate	
Voges-Proskauer	–
Arginine dihydrolase (ADH)	+

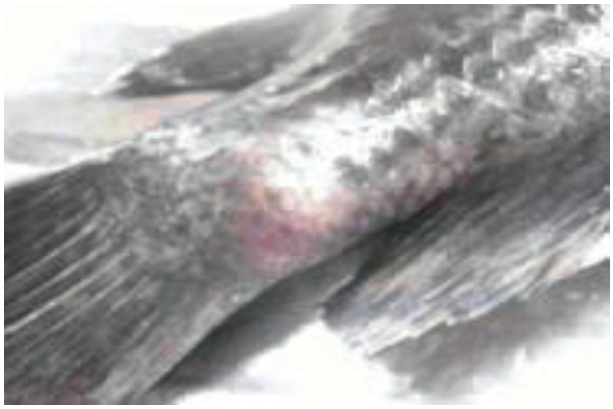

Figure 1. Tail region lesions of the tilapia infected experimentally (original).

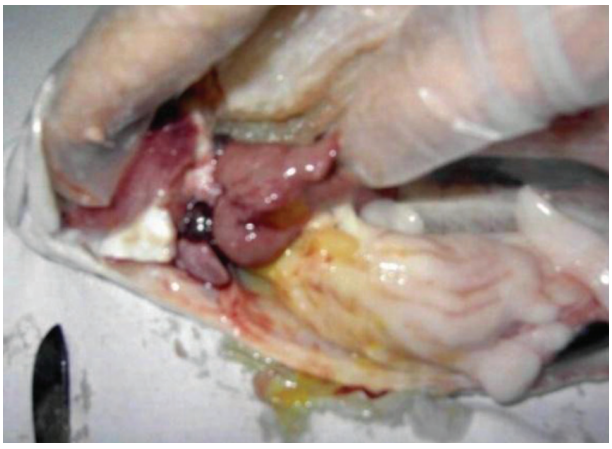

Figure 2. The tumor and inflammatory developments seen in the organs of tilapia as a consequence of experimental infection (original).

APPs in plasma

Plasma results of the infection revealed significant inflammation responses in both of the experiment groups. The mean levels were measured in tilapia given a doses of 3.3×10^5 CFU/0.1 mL. The mean levels were significantly different between the control group and those infected with these doses (Table 2).

In groups I and II, the CRP levels in normal tilapia were 2.97 mg/dL. The mean CRP levels were 3.06 mg/dL and 3.14 mg/dL at the first week (day 7) after the infection. The CRP levels increased to a maximum at 10 or 15 days after infection (Figure 3).

In groups I and II, the mean SAA levels were 2.92 and 3.07g/L (control = 2.82 mg/dL) at the first week (day 7). The SAA concentration in the blood plasma in the 2 groups showed a higher level after 2 weeks (day 14) when compared with the control group. SAA levels decreased in group II (3.05 mg/dL), whereas the SAA concentration (3.04 mg/dL) was higher at the last week (day 21) (Figure 4).

In groups I and II, median TF levels at discharge were 0.59 and 0.62 g/L (control = 0.79 g/L) at the first week. Increased TF concentrations were observed in both groups at the end of the second week (Figure 5).

After the first week in group I, the FB values were 138 g/L and 132 g/L. The levels were low (by order of 57 and 42 g/L) after days 14 and 21. Group I did not have low values in the first week, but in the second and third weeks, significantly low values were determined. In group II, while the values showed similarity, in the last stage of experiment (week 3), all of the values were lower than those in the control group (Figure 6).

Table 2. Comparative results of the 2 groups of tilapia.

	Group I	(Bacteria infection)			Group II	(Bacteria stress)		
Sampling period (day)	**0***	**7**	**14**	**21**	**0***	**7**	**14**	**21**
CRP (mg/dL)	**2.97**	**3.06**	**3.07**	**3.12**	**2.97**	**3.14**	**3.08**	**3.18**
SAA (mg/dL)	**2.82**	**2.92**	**3.00**	**3.02**	**2.82**	**3.02**	**3.09**	**3.03**
TF (d/L)	**0.78**	**0.60**	**0.75**	**0.87**	**0.78**	**0.62**	**0.78**	**0.88**
FB (g/L)	**138**	**132**	**57**	**42**	**138**	**102**	**55**	**38**

*: Control group

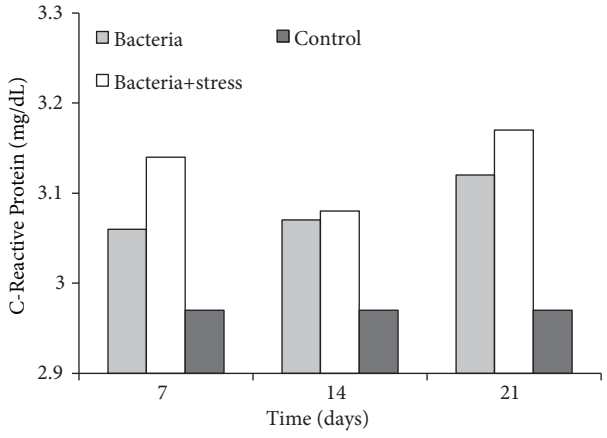

Figure 3. CRP levels in the tilapia.

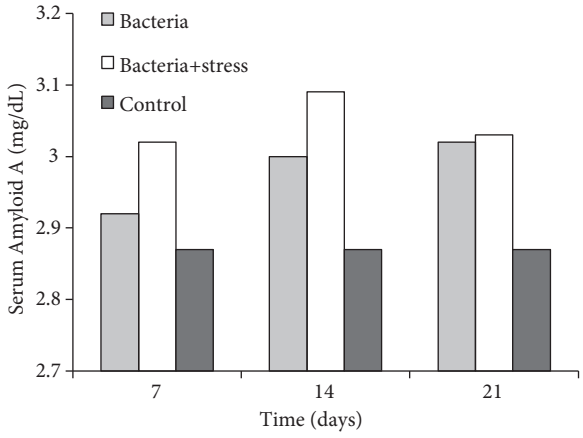

Figure 4. SAA levels in the tilapia.

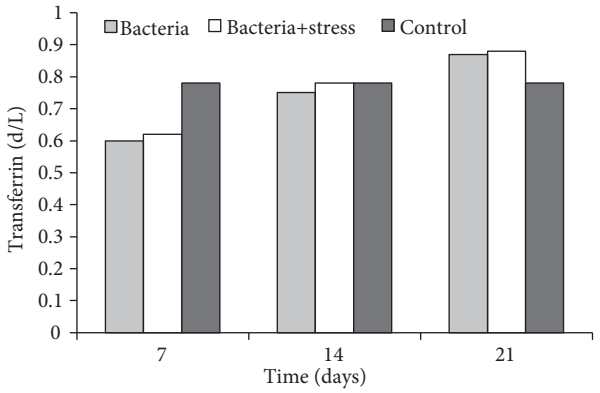

Figure 5. TF levels in the tilapia.

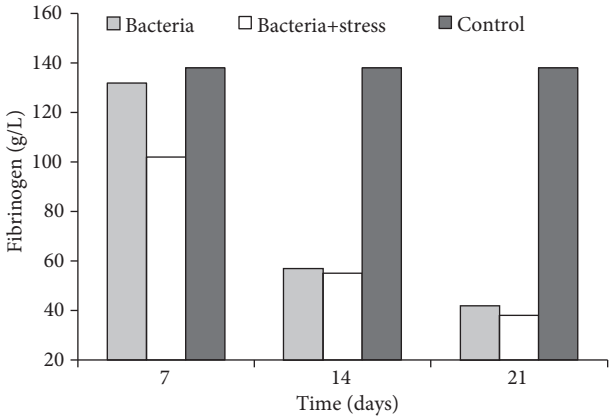

Figure 6. FB levels in the tilapia.

Discussion

Developing challenge models is one of the first steps in vaccine or pharmaceutical development for animal diseases. Fish can be infected by bath with a chosen bacteria concentration; by the introduction of diseased fish and by intracoelomic or intramuscular injection. The advantage of the first 2 challenge techniques is that they replicate the natural route of the mucosal immunity, which is involved in protecting fish from the infection. The advantage of challenging fish by an intramuscular or intracoelomic injection is that this technique is more replicable and efficient than the others (22,23). In this study, the intraperitoneal injection was chosen as challenge route for its replicability.

Russo et al. (24) developed a challenge model against *S. iniae* in rainbow sharks. For this purpose, researchers determined the lethal dose of bacteria needed to kill a predetermined percentage of fish in a fixed period of time. This species has been described as one of the most frequent in *Streptococcus* spp. infections in tilapia. In most studies, a lethal dose of bacteria/CFUs that should kill 70% of the fish population in 14 days is chosen as the target bacteria/CFU dose (23,25). In a study carried out by Bromage and Owens (26), barramundi (*Lates calcarifer*) fish experimentally was infected by *S. iniae* and morbidity and mortality rates were determined by injecting a limited number of bacteria.

In our investigation, experimental fish were infected with 3.3×10^5/0.1 CFU/mL. However, the nonlethal dose was determined as a disease dose. Therefore, mortality due to *S. iniae* was confirmed at 17% for group I and 21% for group II, within 7 days (first week) of the bacterial challenge.

Disease signs have been reported for *S. iniae* infections of cultured tilapia (27) and experimentally injected tilapia (22). Dermal hemorrhages on the body surface (27) and around the mouth, opercula, base of fins, and anus (22) have been observed in *S. iniae* infected moribund tilapia. Perera et al. (22) also reported loss of orientation, corneal opacity, exophthalmia, and eye disfigurement.

In this study on tilapia, following the intraperitoneal challenge, the *S. iniae* bacterial antigen was observed in the spleens, kidneys, livers, and skin at all of the time points tested. The spleens and kidneys of the tilapia contained lesions, hemorrhages in the fins and tails (Figure 1), hyperemia on the skin, and bloody liquid accumulation in the body cavity (Figure 2).

These observations are often assumed to be identical to those that occur from *S. iniae* infections. In addition, disease signs may vary according to natural and artificial *S. iniae* infections and experimental routes of infection. Although some of the internal and external symptoms obtained from our study showed similarity to previously published studies, the effect mechanism of the infection and

alterations of their periods are supposed have been to be due to the dose of *S. iniae* used to infect the tilapia by different modes of infections.

In the second part of our study, differences in CRP, SAA, FB, and TF levels, which are used as a biomarker of the inflammatory response in humans and other mammals, were evaluated as a possible indicator of physiological states in fish exposed to *S. iniae* infection. The tests are important for the detection of early stages of disease in fish.

Our data on blood plasma CRP levels showed no significant differences in either group during the first week ($P < 0.05$). While SAA levels increased in the first and second weeks, levels decreased at the end of the third week. In the experimental infection model, observed FB levels were recorded as low for both groups (Table 2).

The results showed that the concentrations of these proteins in the blood changed rapidly following stress and infection. Our study suggested that these parameters have a specific importance in tilapia blood plasma.

Jensen et al. (28) examined changes in APPs in salmonids. In their study, fish were injected with live *A. salmonicida*, and then examined for altered levels of transcripts for SAA, serum amyloid P (SAP), and albumin. While SAA was undetectable in the control samples, it was detected at 48 h after the injections, and at 120 h it reached levels approximately 40 times those at 48 h.

Gerwick et al. (29) injected rainbow trout (*Oncorhynchus mykiss*) with a variety of potential inflammatory agents, and changes in the concentrations of plasma proteins were sought in polyacrylamide gels, in which plasma proteins had been electrophoresed. They showed increases at day 2 after the injections, and most within 1 week. They reported the greatest number of changes occurring after injection with a vibrio bacteria emulsified in Freund's incomplete adjuvant.

APPs have been shown to vary depending on the sex, age and size, season, water temperature, pH, toxicants, infections, and degree of stressors (30).

Bayne and Gerwick (4) identified APPs in mammals and in both teleosts and elasmobranchs, including CRP, SAP, and several components of the complement system. Others reported in teleosts included TF and thrombin. Of these, only CRP was reported to cause an increase in acute phase plasma. In trout, a precerebellin-like protein was an APP with unknown functions. It appeared that, as in mammals, hepatocytes were the prime source of APP in fish, and that proinflammatory cytokines induced transcription of their genes. It is known that the individual differences determined in plasma protein profiles among the fish change in accordance with whether the fish were exposed to genetic differences or a particular antigene before or not.. We think that this may be the reason for the differences in our study.

Conclusion

In light of the data acquired in this study, infection with *S. iniae* of tilapia is strong evidence of the changes in APPs including inflammation. The APR can be used for the assessment of general health, including starvation and growth. Proinflammatory cytokines and blood proteins of hepatic origin are potential variables for monitoring the changes induced. APPs are more useful for monitoring health than cytokines, because the latter are cleared from circulation within a few hours, whereas APP levels after a single stimulus remain unchanged for 48 h or longer. Determination of APPs can help in monitoring the health of individual subjects, especially when several acute phase variables are combined in an index. Moreover, the significance of APPs as nonspecific variables for monitoring inflammatory activity has been adopted in veterinary clinical chemistry.

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