Humoral and non-specific immune responses in rainbow trout (*Oncorhynchus mykiss*) naturally exposed to and immunized with *Streptococcus iniae*

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Abstract:

In this study, immune responses against *Streptococcus iniae* infections in rainbow trout (*Oncorhynchus mykiss*) were analyzed. Anti-streptococcal antibody (ASA) levels, respiratory burst activity (RBA), phagocytic activity (PA) and phagocytic index (P index) were measured in kidney macrophages. Fish were allocated to three experimental groups (n = 50 per group): survivors of natural S. iniae infections, non-exposed healthy fish immunized by immersion with formalin-killed S. iniae bacterin and a control group of non-exposed healthy fish. Blood sera and kidney samples were collected from each group (n = 10 fish) at days 0, 15, 30, 45 and 65 post-immunization (p.i.). Analysis of ASA levels, RBA, PA and P index in survivors of Streptococcus infections revealed optical density (OD) values of 0.082-0.091, 0.089-0.091, 53-55 and 1.8-1.9 respectively. In the immunized non-exposed group, the ASA peak antibody level occurred at day 15 (mean OD=0.122) and declined significantly by day 65 ($p<0.05$). Similarly, the mean PA and P index values of macrophages peaked at day 15 p.i. (71 and 3.2, respectively) and declined by day 65 ($p<0.05$). Therefore, the acquired immune status of survivors of natural S. iniae infection was not altered by immunization.

Introduction

Streptococcus iniae causes serious infections and significant losses to the rainbow trout (*Oncorhynchus mykiss*) farming industry (Perera et al., 1994, Stoffregen et al., 1996, Eldar and Ghittino, 1999). The use of antibiotics has raised concerns about antibiotic residues in fish, development of antibiotic resistance and pollution of the environment. Therefore, the development of vaccines is considered essential to control this pathogen. Control of Streptococcus has increasingly focused on the use of vaccination as well as health management. Several attempts have been made to develop appropriate vaccination programs against fish Streptococcus. However, considerable variability in the degree of protection has been observed depending factors such as the fish and bacterial species as well as the route of administration. Information about immune responses in rainbow trout against streptococcal disease are scarce. Both

humoral and cellular immune responses in trout are enhanced following immunization with *S. iniae* formalin-killed cells (FKC) with or without extracellular products (ECP) administered via the intraperitoneal (i.p.) and as dip routes although i.p. administration generated a greater response (Soltani et al., 2007a, b).

A previous study by Soltani et al. (2007a), measured lysozyme activity, serum bacterial killing activity and the population of immunocompetent cells at 3, 6, 9, 12, 15 and 18 weeks p.i. with *S. iniae* FKC with or without ECP by i.p, immersion and oral routes. It was shown that the lysozyme activity and the bacterial killing capacity of sera increased during the initial period p.i. and subsequently declined. Moreover, while, leukocyte and lymphocyte populations in immunized fish were generally higher than in the control group, heterophil and monocyte counts varied during the sampling periods (Soltani et al., 2007a). Macrophages are considered to be the predominant phagocytic cell type in the fish head kidney. Stimulation of the phagocyte cell membrane, with accompanying activation of the membrane-associated NADPH-oxidase, initiates increased oxygen consumption and the production of reactive oxygen intermediates with microbicidal activity in a process known as respiratory burst activity (RBA). Vaccines and probiotics have been shown to increase respiratory burst and serum lysozyme activities in rainbow trout (Kim and Austin., 2006).

The aim of this study was to analyze the specific and non-specific immune responses of rainbow trout following immersion vaccination against *S. iniae*. Furthermore, the efficacy of natural immunity in animals surviving *S. iniae* infections and the requirement for further immunization were investigated.

Materials and Methods

Bacteria and soluble antigen preparation: *S. iniae* was isolated from diseased rainbow trout obtained from Fars province, Iran, and identified both biochemically and molecularly (Akhlaghi and Keshavarzi, 2002, Mata et al., 2004, Soltani et al., 2005). *S. iniae* bacteria were cultured in tryptic soy broth to a density of approximately 10^{10} viable cells/ml. The suspension was diluted with formalin to a final concentration of 0.3% (v/v) and stored overnight at 4ºC. Bacteria were diluted (×10) to a density of 10^9 cells/ml killed cells immediately prior to immunization. Live bacteria washed in phosphate buffered saline (PBS) were disrupted by sonication, centrifuged at 1500 ×g and filtered (0.2 µm) to prepare soluble antigen.

Evaluation of immune responses: Fifty healthy rainbow trout (40±2 g) were allocated for immunization (Group 1). These fish were survivors of natural infections from a farm with a prevalence of *S. iniae* of 5% in cultured kidney samples. The fish were maintained in a raceway in the same infected farm with an average water temperature of 17ºC. A further 100 healthy rainbow trout (46±3.35 g) were selected from a trout recirculation system farm without any signs of streptococcosis and allocated to two groups (n = 50 per group): Group 2, immunized and Group 3, control. Cultures from random kidney samples were all negative. These fish were maintained in 300 liter recirculation tanks with an average temperature of 17ºC. Groups 1 and 2 were immersed in FKC (30 s) and returned to their maintenance conditions. Ten anesthetized fish from each group were bled and head kidneys were removed aseptically at the beginning of the experiment and subsequently from 10 further animals after intervals of 15, 30, 45 and 65 days post-immunization (p.i.).

Detection of anti-*S. iniae* antibodies (ASA) by enzyme linked immunosorbent assay (*ELISA*): The protocol used for ELISA detection of ASA was adapted from the procedures described by Akhlaghi et al., (1996) with some modifications. Briefly, polystyrene 96-well plates (ICN Flow, Linbro, Sydney, Australia) were coated with 100 µl/well sonicated *S. iniae* and kept overnight in refrigerator (4°C). The antigen was then removed and remaining free binding sites were blocked with 100 µl/well 1% (w/v) gelatin (Oxoid) in PBS + 0.05% Tween 20 (PBST) for 30 min at room temperature (RT). Plates

were then washed five times in distilled water + 0.05% Tween 20 (DWT) using a Titertek® microplate washer 120 (ICN Flow laboratories). Serially diluted serum samples were added (100 µl/well) in duplicate and incubated for 90 min at RT. After washing with DWT, rabbit anti-rainbow trout antibodies (diluted 1:200, Shiraz branch- vaccine and serum research institute) were added, incubated and washed as in the previous stage. This was followed by incubation with swine anti-rabbit-HRP (diluted® 1:1000, Dako Patts) using the previously described conditions. After washing, 100 µl per well of 2,2'-azino-bis-(3-ethyl benzene thiazoline-6 sulphonic acid) diammonium salt (ABTS) in 100 mM citrate phosphate, pH 4.2, 2.5 mM hydrogen peroxide was added. The reaction was stopped after incubation for 20 min at RT by the addition of 50 µl 0.015 M sodium azide in 0.1 M citric acid and the OD was measured at a wavelength of 490 nm using an EL 309 automated microplate reader (Bio-Tek).

Collection of macrophages from head kidneys: Head kidney macrophages, collected according to the procedures described by Kim and Austin (2006) with some modifications, were used for analysis of RBA and PA. Briefly, head kidneys were processed individually by disruption across a nylon mesh (100 µm) with L-15 medium containing 2% (v/v) fetal calf serum (FCS), 100 µl/ml gentamycin (Sigma) and 10 µl/ml heparin (Sigma). The resulting suspensions were layered onto a 34 to 51% (v/v) Percoll (Sigma) gradient diluted in Hank's Balanced Salt Solution (HBSS, Sigma) before tubes were centrifuged at 400 ×g for 25 min at 4°C. The band of cells located at the 34% to 51% interface was collected and washed twice with HBSS. The cell density was adjusted to 10^6 cells/ml in L-15 medium supplemented with 0.1% (v/v) FCS and 100 µl/ml gentamycin. Viability was evaluated by the trypan blue exclusion method.

Macrphage production of reactive oxygen species (respiratory burst activity, RBA): Superoxide anion production by head kidney macrophages was determined by the reduction of nitroblue tetrazolium (NBT, Sigma) following a previously described method (Chung and Secombs, 1988).

Phagocytic activity: Phagocytic activity of head kidney macrophages was evaluated using a previously described method (Kim and Austin, 2006) with some modifications. One ml of the macrophage cell suspension (10^6 cells/ml) obtained from each individual fish was allowed to adhere onto a methanol-cleaned glass slide for 1 h at 18°C in a humid chamber. Non-adherent cells were removed by washing with HBSS before adding 1.0 ml autoclaved congo red-colored yeast cells (10^8 cells/ml). Phagocytosis was allowed to proceed for 1 h. Air-dried slides were fixed in absolute methanol for 3 min and stained by Giemsa's method for 15 min. Approximately 200 cells were counted randomly and PA was expressed as:

PA = number of phagocytosing cells/number of total cells × 100

The phagocytic index was determined by the number of yeast cells phagocytosed per macrophage cell.

Statistical analysis: Comparisons of results between different treated groups were carried out using one-way analysis of variance (ANOVA, SPSS-Wins Software). A value of $p<0.05$ was considered statistically significant.

Results

Anti-*S. iniae* antibodies: The highest OD for ASA of survivors of streptoccoccosis on day 0 was 0.089. This value ranged from OD 0.084 to 0.091 during the period from 15 to 65 p.i., which was not significantly different from that of day 0 ($P<0.05$). However, significant differences were observed in comparison with non-immunized control fish. In the non-exposed immunized rainbow trout, ASA was significantly higher at day 15 (mean OD = 0.122±0.006) and subsequently declined gradually to the control level (OD = 0.049-0.051) (Fig. 1).

Respiratory burst activity: Respiratory burst activity of head kidney macrophages in immunized rainbow trout streptococcosis survivors ranged from

Table 1: Respiratory burst activity in fish following natural exposure or immersion immunization with *S. iniae* (mean optical density ± standard deviation). Identical superscript letters indicate no significant differences between groups.

Days after immunization	Mean OD of naturally exposed (survivors) immunized fish	Mean OD of immunized non-exposed fish	Mean OD of non-immunized, non-exposed fish (control)
0	$0.089^{a} \pm 0.014$	$0.082^{d} \pm 0.012$	$0.078^{d} \pm 0.012$
15	$0.091^{a} \pm 0.009$	$0.124^{b} \pm 0.017$	$0.079^{d} \pm 0.006$
30	$0.089^{a} \pm 0.011$	$0.095^{c} \pm 0.009$	$0.074^{d} \pm 0.007$
45	$0.091^{a} \pm 0.013$	$0.088^{a} \pm 0.011$	$0.078^{d} \pm 0.011$
65	$0.091^{a} \pm 0.012$	$0.073^{c} \pm 0.008$	$0.079^{d} \pm 0.013$

Table 2: Phagocytic activity of streptococcosis survivors and non-exposed fish vaccinated with S. iniae bacteria by immersion. Identical superscript letters indicate no significant differences between groups.

Days after immunization	Mean OD of naturally exposed (survivors) immunized fish	Mean OD of immunized non-exposed fish	Mean OD of non-immunized, non-exposed fish (control)
0	$0.089^{a} \pm 0.014$	$0.082^{d} \pm 0.012$	$0.078^{d} \pm 0.012$
15	$0.091^{a} \pm 0.009$	$0.124^{b} \pm 0.017$	$0.079^{d} \pm 0.006$
30	$0.089^{a} \pm 0.011$	$0.095^{c} \pm 0.009$	$0.074^{d} \pm 0.007$
45	$0.091^{a} \pm 0.013$	$0.088^{a} \pm 0.011$	$0.078^{d} \pm 0.011$
65	$0.091^{a} \pm 0.012$	$0.073^{c} \pm 0.008$	$0.079^{d} \pm 0.013$

Table 3: Mean phagocytic index of fish following natural exposure or immersion vaccination with *S. iniae*. Identical superscript letters indicate no significant differences between groups.

Days after immunization	Naturally exposed (survivors) vaccinated	Non-exposed vaccinated	Non-immunized, non-exposed control
0	$1.8^{a} \pm 0.4$	$1.2^{c} \pm 0.1$	$1.3^{c} \pm 0.3$
15	$1.9^{a} \pm 0.3$	$3.2^{b} \pm 0.6$	$1.2^{c} \pm 0.1$
30	$1.8^{a} \pm 0.3$	$1.9^{a} \pm 0.4$	$1.3^{c} \pm 0.3$
45	$1.9^{a} \pm 0.2$	$1.6^{a} \pm 0.3$	$1.2^{c} \pm 0.2$
65	$1.9^{a} \pm 0.4$	$1.3^{c} \pm 0.2$	$1.2^{c} \pm 0.2$

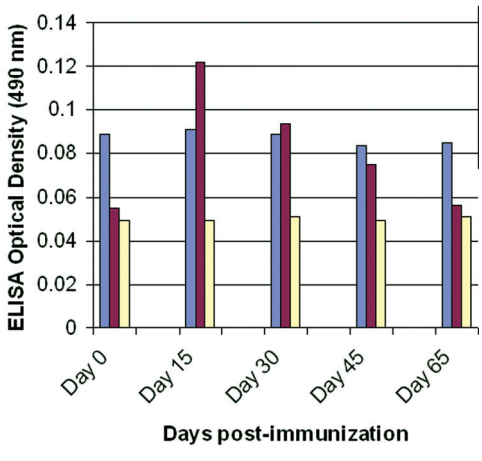

Figure 1: Humoral immune responses in *S. iniae* vaccinated rainbow trout streptococcosis survivors and previously non-exposed fish. Vaccinated survivors of natural infection Vaccinated non-exposed Control

OD 0.089 to 0.091 at day 0 to day 65 p.i. Optical densities for non-exposed immunized fish peaked at day 15 and subsequently declined (Table 1).

Phagocytic activity: Phagocytic activity of head kidney macrophages of immunized rainbow trout streptococcosis survivors ranged from 53 to 55 during the period to day 65, while in vaccinated non-exposed rainbow trout the highest activity was observed at day 15 and subsequently declined gradually (Table 2).

Phagocytic index: The phagocytic index of immunized rainbow trout streptococcosis survivors ranged from 1.8 to 1.9 during the period to day 65, while in vaccinated non-exposed fish the phagocytic index was measured as 3.2, 1.9 and 1.6 at days 15, 30

and 45 p.i., respectively. The phagocytic index of each group was not significantly different at day 0 compared with day 65 p.i. (Table 3).

Discussion

In this study, healthy non-exposed fish responded well to vaccination by immersion with *S. iniae*. Measurement of anti-*S. iniae* antibodies by ELISA, revealed a mean OD of 0.122 at day 15 p.i. that subsequently declined gradually (Fig. 1), which is consistent with the report of Shelby et al. (2002) who demonstrated an antibody response against *S. iniae* using ELISA. Furthermore, Barnes et al. (2002) showed antiserum isolated from fish exposed to *S. iniae* agglutinated the homologous strain but did not agglutinate other isolates. Normal trout serum did not agglutinate any isolates. It has also been reported that antibody production in red Tilapia (*Oreochromis niloticus* × *O. mossambicus*) vaccinated by immersion with formalin-killed *S. iniae* was higher one week post-immunization than in control fish (Suanyuk and Itsaro, 2011). In a previous study by Akhlaghi et al. (1996), anti-*Streptococcus sp*. antibodies were not detected by ELISA in rainbow trout immunized by immersion with FKC, although the bacteria used were subsequently characterized as *L. garvieae* (Schmidtke and Carson, 2003). It is possible that this inconsistency results from differences in the immune responses to these organisms, in addition to the use of whole bacteria cells as capture antigens in the ELISA in the previous study.

The immunity provided by anti-*S. iniae* whole sera (ASI) and heat-inactivated ASI (HIASI) is mediated by *S. iniae* specific antibodies. Heat-inactivation of complement (HIASI) further suggests that ASI antibodies play a primary role in immunity against *S. iniae* infection (Shelby et al., 2002). Similarly, significantly higher antibody titers were detected in fish immunized by immersion with *S. iniae* FKC compared with fish immunized orally with this vaccine (Soltani et al., 2007b).

The mean anti-*S. iniae* antibody levels of immunized rainbow trout streptococcosis survivors was measured in this study to be OD 0.089 at day 0 and did not change significantly at 15 days p.i., although differences were detected in comparison with the control group. The almost constant OD range from 0.084 to 0.091 during the period to day 65 p.i. resembles the humoral immune response of fish to live *S. iniae*. It is speculated that this observation may be due to the exposure of streptococcosis survivors to a serotype in the rainbow trout farm that differed from the serotype used for the ELISA. In the case of *S. iniae* vaccines, the critical importance of inclusion of both identified serotypes has been demonstrated by the observation that that vaccines formulated only with serotype I do not provide cross-protection against infection caused by serotype II (Bachrach et al., 2001).

Non-specific immunity factors such as respiratory burst activity, phagocytic activity and phagocytic index of head kidney macrophages of non-exposed trout showed a significant ($P<0.05$) response to *S. iniae* antigens 15 days p.i. (Tables 1 and 2), while significant non-specific immune responses were not detected in survivors of streptococcosis. Respiratory burst, which is a measure of the increase of oxidation level in phagocytes stimulated by foreign agents, is considered an important indicator of non-specific defense in fish (Nematolahi et al., 2005). Survivors of streptococcosis exhibited high respiratory burst activity, phagocytic activity and phagocytic index at day 0. It can be speculated that this observation is due to exposure of the non-specific immune system to bacterial products such as ECP. A report by Klesius et al. (2007) indicated that secretion or excretion of *S. agalactiae* and *S. iniae* ECP is partially responsible for recruitment of macrophages to *S. agalactiae* or *S. iniae* localized in the organs. Tissue injury or host-derived factors may also be responsible for the migration of macrophages. A similar pattern of head kidney macrophage phagocytic activity and phagocytic index was observed in rainbow trout that were naturally exposed to Streptococcus.

Streptococcal infections of fish are considered re-emerging pathologies that affect a variety of wild and cultured fish throughout the world (Bercovier et al.,

1997). In conclusion, this study indicates that non-exposed juvenile rainbow trout can be immunized effectively using the immersion route, while immunization of survivors did not alter the acquired immune status of these animals.

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