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Differential immune response of rainbow trout (*Oncorhynchus mykiss*) at early developmental stages (larvae and fry) against the bacterial pathogen *Yersinia ruckeri*

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ABSTRACT

Innate immune factors play a crucial role in survival of young fish especially during early stages of life when adaptive immunity is not fully developed. In the present study, we investigated the immune response of rainbow trout (*Oncorhynchus mykiss*) larvae and fry at an early stage of development. We exposed 17 and 87° days post hatch larvae and fry (152 and 1118 degree days post hatch; avg. wt. 70 and 770 mg, respectively) to the bacterial pathogen, *Yersinia ruckeri* for 4 h by bath challenge. Samples were taken at 4, 24, 72 and 96 h post exposure for qPCR and immunohistochemical analyses to elucidate the immune response mounted by these young fish. Larvae showed no mortality although infected larvae at 48 h post exposure showed hyperaemia in the mouth region and inflammation on the dorsal side of the body. Gene expression studies showed an up-regulation of iNOS and IL-22 in infected larvae 24 h post exposure but most of the investigated genes did not show any difference between infected and uninfected larvae. Immunohistochemical studies demonstrated a high expression of IgT molecules in gills and CD8 positive cells in thymus of both infected and uninfected larvae. Infection of rainbow trout fry with *Y. ruckeri*, in contrast, induced a cumulative mortality of 74%. A high expression of cytokines (IL-1β, TNF-α, IL-22, IL-8 and IL-10), acute phase proteins (SAA, hepcidin, transferrin and precerebellin), complement factors (C3, C5 and factor B), antimicrobial peptide (cathelicidin-2) and iNOS was found in infected fry when compared to the uninfected control. IgT molecules and mannose binding lectins in gills of both infected and uninfected fry were detected by immunohistochemistry. The study indicated that early life stages (yolk-sac larvae), merely up-regulate a few genes and suggests a limited capacity of larvae to mount an immune response by gene regulation at the transcriptional level. Based on the observed clearance of bacteria and lack of mortality it could be speculated that larvae may be covered by protective shield of different immune factors providing protection against broad range of pathogens. However, the increased susceptibility of older fry suggests that *Y. ruckeri* may utilize some of the immune elements to enter the naive fish. The up-regulation of iNOS and IL-22 in the infected larvae implicates an important role of these molecules in immune response at early developmental stages. A dense covering of surfaces of gill filaments by IgT antibody in the young fish suggest a role of this antibody as innate immune factor at early developmental stages.

1. Introduction

Under natural conditions fish larvae are hatched out into a hostile aquatic environment containing numerous pathogens and it is crucial for these early developmental stages to possess protective immune mechanisms to cope with this challenge. At the time of hatching the immune system of the fish larva is still in a developing stage and lack the functionality found in adults (Ellis, 1988; Zapata et al., 1990). At this stage of life, they may be protected by both innate and adaptive immune substances transferred from the female spawner to eggs during vitellogenesis (Magnadottir et al., 2005) but innate immune factors are considered to play a crucial role in survival of these young larvae (Mulero et al., 2008). Complement, lysozyme, antimicrobial peptides and MHC I and CD8 (Fischer et al., 2005) together with a humoral response to T-independent and T-dependent antigens occur in young fish (Lam et al., 2004; Tatner, 1986). However, the association between activation of immune relevant genes and the protection offered against *Yersinia ruckeri* in rainbow trout at early developmental stages is unknown. The present study was undertaken to investigate the immune response of rainbow trout at two developmental stages (yolk-sac larvae and developing fry) and to elucidate the factors responsible for protection against the bacterial pathogen *Y. ruckeri*.

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2. Materials and methods

2.1. Fish larvae (experiment 1)

The trout eggs were obtained from Fousing Trout Farm, Jutland, Denmark. Two of the aquaria were hatched in a pathogen-free rearing facility (Salmon Hatchery, Bornholm, Denmark). Eight days post hatch (8 dph) yolk-sac larvae were transported from hatchery and maintained in the fish keeping facility at the University of Copenhagen, Faculty of Life Sciences, Denmark. Yolk-sac larvae were reared in the laboratory facility at 8–9 °C until the yolk-sac was absorbed, after which larvae were used for the experiment. After 17 days post hatch (17 dph; 152 degree days post hatch: average weight 70 mg), they were distributed into four different aerated aquaria (density of 100 larvae/aquarium) at a temperature of 12–13 °C. Then, larvae in two aquaria were immersed into a solution of *Y. ruckeri* (1.0×10^8 CFU/ml) for 4 h whereafter they were removed and placed into fresh water. The remaining two aquaria without any bacterial inoculation served as controls. Clinical signs and mortality (if any) was recorded. Samples were taken for real time quantitative PCR (RT qPCR) and immunohistochemistry at 4, 24, 72 and 96 h post infection. For qPCR, whole larvae were preserved in RNAlater (Sigma–Aldrich, Denmark) after making a minor incision in the abdominal region to facilitate the penetration of RNAlater. Similarly, for immunohistochemistry whole larvae were preserved in 4% buffered formalin for 24 h and then transferred to 70% ethanol (Sigma–Aldrich, Denmark) and stored at 4 °C until processing. All treatment groups were duplicated.

This experiment was repeated with different batch of larvae (15 dph; 192 degree days post hatch; average weight 77 mg). A total of 50 larvae were distributed in aquarium and exposed to *Y. ruckeri* (5.5×10^7 CFU/ml) for 4 h whereafter water was replaced. All treatment groups were duplicated and experiment was conducted at 15–16 °C. The larvae were observed for mortality and clinical signs until day 14 post exposure but no samples were taken for qPCR and immunohistochemistry.

2.2. Fish fry (experiment 2)

Eighty-seven days post hatch (87 dph; 1118 degree days post hatch; avg. wt. 770 mg) rainbow trout fry (from the same batch of larvae) were distributed in four 10 L capacity aquaria at a density of 100 fry/aquarium. Two of the aquaria were exposed to *Y. ruckeri* (6.0×10^8 CFU/ml) for 4 h at 14–15 °C and subsequently replaced with new fresh water. Other two aquaria served as uninfected control. Samples (whole fry with incision) were taken for qPCR and immunohistochemistry at different time points i.e. 4, 24, 72, 96 h and 25 days post infection. Clinical signs and mortality was recorded. Samples were preserved in similar manner as mentioned in Section 2.1.

2.3. Bacteria

Y. ruckeri serotype O1 biotype 1 was used for the challenge experiment. The bacteria were grown in LB-medium (tryptone 10 g, yeast extract 5 g, NaCl 5 g, H_2O 1000 ml, pH 7.4) by incubating the inoculated broth on the shaker at 22 °C for 48 h. The bacteria were enumerated by counting colony forming units (CFU) from serial dilutions on blood agar plates (supplemented with 5% bovine blood) after 48 h culture at 22 °C.

2.4. RNA extraction and reverse transcription

Larvae were removed from RNAlater and cut into small pieces followed by sonication (Artek Sonic Dismembrator Model 300, Virginia, USA) on ice in 700 μl of lysis buffer containing 2-mercaptoethanol (Sigma–Aldrich Cat. No. RTN350, Denmark). Similarly whole fry was removed from RNAlater whereupon all the internal organs were recovered using a scalpel and sonicated in 700 μl of lysis buffer containing 2-mercaptoethanol. Thus from rainbow trout fry (experiment 2), only internal organs (spleen, liver, kidney, gills, intestine, etc.) were included for RNA extraction to avoid a large volume of muscle tissues. From both experiments (1 and 2), total RNA was extracted from 350 μl of sonicated extract from individual fish using GenElute™ total RNA kit (Sigma–Aldrich Cat. No. RTN350, Denmark). The extracted RNA was subsequently treated with DNase (DNase I, Fermentas Cat. No EN0521, Denmark) to remove genomic DNA contamination. RNA concentration and purity was measured spectrophotometrically on a NanoDrop reader (Saveen & Werner ApS, Denmark) and the integrity and purity of RNA was examined by running in 1.5% agarose gel. The RNA was stored at −80 °C. RNA was reversely transcribed using random hexamers (TaqMan® Reverse Transcription, Applied Biosystems Cat. No. 4311235, Denmark). Reactions were performed in a T3 Thermocycler (Biometra, Germany) with 20 μl reaction volume containing 400 ng of RNA. The reactions were carried out under the following conditions: 25 °C for 10 min, 37 °C for 60 min and 95 °C for 5 min. A volume of 20 μl of resulting cDNA was diluted ten times by adding 180 μl of RNase free water (Invitrogen Cat. No 10977, Denmark) and stored in −20 °C until further use.

2.5. Real time quantitative PCR (RT qPCR)

Real time quantitative PCR assays were performed using a Stratagene Mx3005P (AH Diagnostics, Aarhus, Denmark) real time PCR machine. The cDNA was used as a template for qPCR reactions with primer and probe designed for particular genes (Table 1). Reactions were run in readymade master mix (Brillaint® II QPCR master mix, Stratagene, US) with 5.5 μm $MgCl_2$ concentration. In order to verify that only one product was produced and no primer dimer was formed all qPCR assays were assessed by SYBR Green qPCR assay and subsequent melting curve analysis. A 12.5 μl setup were used: 6.25 μl of Brillaint® II QPCR master mix (Agilent stratagene, US), 0.5 μl forward primer (10 μM), 0.5 μl of reverse primer (10 μM), 0.5 μl TaqMan probe (5 μM), 2.25 μl DNase/RNase free H_2O and 2.5 μl of cDNA template. The cycling conditions were 95 °C for 15 min followed by 45 cycles of 95 °C for 30 s and 60 °C for 30 s. Control (without template) and reverse transcriptase minus (RT minus) negative control were run in each assay. RT qPCR was used to monitor the changes in expression of different immune relevant genes following infection with *Y. ruckeri*. Regulation of following genes were examined: pro-inflammatory cytokines (IL-1β and TNF-α), regulatory cytokines (TGF-β and IL-10), IL-22, chemokine (IL-8), immunoglobulins (IgM and IgT), T-cell marker (CD8), acute phase proteins (hepcidin, serum amyloid A (SAA), serum amyloid P (SAP), transferrin and precerebellin), complement factors (C3, C5 and factor B), antimicrobial peptides (defensin-1 and cathelicidin-2), antimicrobial enzyme lysozyme and inducible nitric oxide synthase (iNOS). A qPCR assay was designed to quantify the amount of *Y. ruckeri* by analysing 16S ribosomal RNA (rRNA) of *Y. ruckeri* (Raida and Buchmann, 2009). The infection level in fish tissue was expressed as the negative value of ΔCt (Ct value−Ct value ELF 1α).

2.6. Immunohistochemistry

Whole fish larvae were fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 24 h at 4 °C and then transferred to 70% ethanol and stored at 4 °C until further use. Whole fish larvae were mounted in paraffin (experiment 1) but due to the larger size of fish fry in the second experiment, only organs which could be