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Innate immune response in rainbow trout (*Oncorhynchus mykiss*) against primary and secondary infections with *Yersinia ruckeri* O1

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ABSTRACT

Response mechanisms in teleosts against bacterial pathogens have been widely studied following injection procedures applying preparations of killed bacteria. In contrast, investigations on immune reactions in fish which have survived a primary infection and subsequently have been challenged are few or lacking. However, knowledge on these factors during infection and re-infection could provide the basis for development of improved vaccines. The innate immune response in rainbow trout (*Oncorhynchus mykiss*) against *Yersinia ruckeri* O1 has been studied following a primary intra-peritoneal injection with 5×10^5 CFU *Y. ruckeri*, and after bacterial clearance a secondary infection 35 days later. The number of pathogens in the liver was measured with a *Y. ruckeri* specific 16S ribosomal RNA quantitative real-time RT-PCR (q-PCR) during the course of infection. The bacterial counts peaked on day 3 during the primary infection and were significantly lower during the re-infection. Re-challenged fish showed a highly increased survival when compared to the naïve fish receiving a primary infection indicating development of adaptive immunity in the fish against this bacterial pathogen. We investigated the gene expression of innate immune factors in the liver during infections in order to elucidate molecules involved in survival of hosts before adaptive immunity was mounted. Transcription of mRNA was measured in liver samples taken 8 h, 1, 3, 7, 14 and 28 d post-infection using q-PCR. The investigation focused on genes encoding toll-like receptor 5 (TLR5), the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, the acute phase proteins (APPs) serum amyloid protein a (SAA), trout C polysaccharide binding protein, a CRP/SAP like pentraxin, precerebellin, transferrin, hepcidin and finally the complement factors C3, C5 and factor B. Infection elicited significantly increased gene expression of all the cytokines (IL-6 > 1000-fold), some acute phase proteins (SAA > 3000-fold) and down-regulation of complement factors (C3, C5 and factor B). SAA expression was significantly earlier activated during the re-infection when compared to the primary infection. The pattern of gene activation suggested that the innate response was based on pathogen binding to toll-like receptors, production of cytokines and subsequent release of APPs. In general, both the innate immune response and the amount of *Y. ruckeri* measured in the liver during the re-infection was much lower compared to the first infection, probably reflecting development of adaptive immunity.

1. Introduction

The enterobacterium *Yersinia ruckeri* is the causative agent of yersiniosis or enteric red mouth disease (ERM) leading to significant economic losses in salmonid aquaculture worldwide. Infection may result in a septicemic condition with hemorrhages in the oral cavity, on the body surface and in the internal organs [1]. Knowledge about the immune defence of rainbow trout against *Y. ruckeri* is important in terms of control and prevention [1]. It has been established that i.p. injection of *Y. ruckeri* in rainbow trout elicits inflammatory reactions whereby macrophages initiate the immune response partly based on a pronounced attraction of neutrophils [2,3]. A few studies have measured rainbow trout immunoregulatory gene expression in response to *Y. ruckeri* infection but focused merely on toll-like receptor 3 (TLR3), CXCd and IL-1β [4,5]. Initial reactivity to bacterial pathogens depends mainly on innate immunity mechanisms. Adaptive immune responses against *Y. ruckeri* bacterins have been studied by a number of authors [6–8] but the role of innate immune factors during the progress of *Y. ruckeri* infection in the rainbow trout is unknown. Further, the knowledge on interactions between this bacterium and its host has been obtained mainly from vaccination studies. However, it is important for future improval of vaccines to address basic immunity mechanisms conferring protection to a

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host surviving a primary infection. The present work elucidates the transcriptional activity of a range of genes involved in the innate defence and correlates this parameter with the progress of the amount of *Y. ruckeri* in the liver tissue during infection. Based on previous studies on molecular interactions between bacterial pathogens and mammalian hosts the present work has focused on a toll-like receptor binding bacterial ligands and the subsequent induction of gene expression of pro-inflammatory cytokines which in turn induces expression of complement proteins and acute phase proteins (APPs). Thus, it is generally known that ligands binding to TLRs lead to activation of the transcription factor NFκB which then translocate to the nucleus to induce new gene transcription of pro-inflammatory cytokines [9–13] and it has been shown that also fish may use TLR-like motifs for recognition of antigens [4,14,15]. Acute phase proteins and complement factors are important effector molecules in the innate immune response against pathogens [16,17]. These molecules are synthesized by hepatocytes and their production is induced by the macrophage activating factor, and a combination of tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), IL-6 [18] released by macrophages in the presence of pathogens such as bacteria.

In the present paper we used i.p. infection to ensure an identical pathogen dose in all animals, and we analyzed the expression in liver of various innate immune factors including TLR5 (sensing presence of bacterial flagellin), cytokines (activating liver cells) and a series of acute phase reactants. Further, the amount of *Y. ruckeri* present in the liver during infection was concomitantly monitored in order to elucidate the association between these immune factors and clearance of *Y. ruckeri*.

2. Materials and methods

2.1. Fish and rearing conditions

Rainbow trout fry (Skinderup strain from Jutland, Denmark), hatched and reared under pathogen-free conditions (Danish Centre for Wild Salmon, Randers, Denmark), were brought to the experimental fish keeping facility at the University of Copenhagen, when reaching a body weight of 4–6 g. The pathogen-free status of the fish was confirmed upon their arrival in the laboratory by analysis for bacterial, parasitic and viral pathogens. The 600 fish were kept in three 200 l tanks with biofilters (Eheim, Germany) and maintained at a 12 h light and 12 h dark cycle in aerated (100% oxygen saturation) tap water at 13 °C. They were fed a commercial trout feed (BioMar, Denmark) (2% biomass per day).

2.2. Infection

Y. ruckeri serovar I (strain 392/2003), isolated from diseased rainbow trout in Spain [19] was used for the challenge experiments. The bacteria were grown in LB-medium (Oxoid LP0042, Tryptone 10 g, Oxoid LP0021Yeast-extract 5 g, NaCl 5 g, H_2O to 1000 ml, pH 7.4) at 20 °C for 36 h and enumerated as colony forming units (CFU) by the spread plate method on blood agar (Oxoid bloodagar base CM0055 40 g/l) with 5% bovine blood).

2.3. Challenge experiment

Primary infection trials were conducted using a total of 400 rainbow trout, half of them were used as non-infected control fish. All fish were anaesthetized by immersion in 40 mg/l tricaine methane sulfonate (MS-222, Sigma–Aldrich, Denmark). Two hundred trout were infected by intra-peritoneal injection with 5×10^5 CFU/fish in 50 μl PBS corresponding to a previously determined LD_{50} (data not shown). Two hundred non-infected control fish were injected with 50 μl sterile PBS.

Following primary challenge the fish were observed for 35 days. In the re-challenge experiment a total of 97 surviving fish from the primary challenge received an additional injection of 5×10^5 bacteria 35 days after the primary infection. When performing the re-challenge of the survivors, a group of 200 naïve fish were infected as control to confirm the virulence of the bacterial broth. Bacterial samples from the head kidney from all fish that died were cultured on blood agar plates to confirm the cause of death. Mortalities were only considered to be caused by *Y. ruckeri* if the bacteria were recovered as pure culture from the head kidney.

2.4. Sampling for gene expression studies

Five infected and five control fish were sampled at 0 h, 8 h and 1, 3, 7, 14 and 28 d after each infection. No moribund fish were sampled for gene expression experiments. Fish were killed by immersion into a solution of MS-222 (100 mg/l). Liver tissue was sampled aseptically, immediately transferred to RNA-later (Sigma–Aldrich), pre-stored for 24 h at 4 °C and subsequently stored at −20 °C until isolation of RNA. When comparing groups for immunological parameters the infected fish and non-infected control fish sampled at the same time points were compared.

2.5. Expression of Y. ruckeri specific 16S ribosomal RNA gene in the liver of rainbow trout

A primer pair and a TaqMan probe were designed in an unconserved regions of the *Y. ruckeri* partial 16S ribosomal RNA gene (genbank: X75275), which provides a specific amplification of *Y. ruckeri* strains only [20]. Forward primer: 5′GCGAGGAGGAAGGGTTAAGTG3′. Reverse primer: 5′GTTAGCCGGTGCTTCTTCTG3′ and the probe: 5′AATAGCACTGAACATTGACGTTACTCG3′.

2.6. Isolation of total RNA and cDNA synthesis

Homogenisation of tissue was done by sonication on ice (Sonicator Ultrasonic Liquid Processor Model XL 2020, heat Systems, New York, USA) and subsequently total RNA was isolated using GenElute™ total RNA kit (Sigma–Aldrich). Removal of genomic DNA was conducted with Deoxyribonuclease I (Sigma–Aldrich). RNA quantity was checked by OD260/280 measurements (SmartSpec ™ 3000, BIO-RAD, USA). cDNA synthesis was performed on 400 ng total RNA in a 20 μl setup using TaqMan® reverse transcription reagents following the manufacturer's instructions (Applied Biosystems, USA). Random hexamer primers were used in the reverse transcription reactions. RT-reactions lacking reverse transcriptase (RT minus) but not RNA were also performed to verify that the samples did not contain genomic DNA. The synthesized cDNA samples were diluted 1:10 in MilliQ H_2O and stored at −20 °C.

2.7. Gene expression analysis

Liver samples were analyzed using qPCR as described below for expression of genes encoding cytokines (IL-1β, IL-6 and TNF-α), TLR5, the APPs SAA, CRP/SAP like pentraxin, Trout C-polysaccharide binding protein (TPBP), transferrin, precerebellin like protein (precerebellin), hepcidin and the complement factors C3, C5 and factor B. qPCR assays were performed using a Stratagene MX3000PTM real-time PCR system. Based on available GenBank (NCBI) sequences primers and dual-labelled TaqMan® probes conjugated with 5′ HEX, FAM or CY 5 and a 3′ BHQ1 or BHQ2 were designed using Primer3 software (http://frodo.wi.mit.edu/). Primers