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Bath vaccination of rainbow trout (*Oncorhynchus mykiss* Walbaum) against *Yersinia ruckeri*: Effects of temperature on protection and gene expression

M.K. Raida*, K. Buchmann

Faculty of Life Sciences, Department of Veterinary Pathobiology, University of Copenhagen, Frederiksberg, Denmark

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Summary Protection of rainbow trout fry following bath vaccination with a bacterin of *Y. ruckeri* O1, the bacterial pathogen causing enteric red mouth disease (ERM), was investigated at 5, 15 and 25 °C. Rainbow trout fry were acclimatised for 8 weeks at the three temperatures before vaccination. They were subsequently challenged with *Y. ruckeri* 4 and 8 weeks post-vaccination which demonstrated a significant protection of vaccinated fish kept at 15 °C. No protective effect of vaccination in rainbow trout reared at 5 and 25 °C could be recorded. Spleen tissue was sampled from vaccinated and control fish at 0, 8, 24 and 72 h post-vaccination in order to analyse gene transcript profiles using quantitative real-time RT-PCR (q-PCR). Gene expression in fish vaccinated at 15 °C (the protected fish) was up-regulated with regard to the pro-inflammatory cytokines IFN-γ, TNF-α, IL-6 and the anti-inflammatory cytokines IL-10 and TGF-β, the cell receptors TcR, CD8α, CD4, C5aR and the teleost specific immunoglobulin IgT. Passive immunisation using transfer of plasma from vaccinated fish to naïve fish conferred no protection. This indicates that humoral factors such as Ig and complement are less important in the protection induced by bath vaccination. Expression of cellular factors such as CD8α was significantly increased in the protected trout and this suggests that cellular factors including cytotoxic T-cells could play a role in immunity against *Y. ruckeri*.

Introduction

The bacterium *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 septicaemic condition with haemorrhages in the mouth, on the body surface and in internal organs [1]. *Y. ruckeri*, which belongs to the family *Enterobacteriaceae* [2,3] was initially isolated from rainbow trout, *Oncorhynchus mykiss* (Walbaum), in the Hagerman valley of Idaho, USA, in the 1950s [4] and is now widely found in all trout producing countries. Disease outbreak are mainly observed in small rainbow trout farmed in fresh water [5],

* Corresponding author.
E-mail address: mkr@life.ku.dk (M.K. Raida).

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but has also been recognised as a problem in sea water and other fish species [6].

Bacterins of formaldehyde-killed *Y. ruckeri* serogroup 1 (Hagerman strain), were the first fish vaccines to be commercialised [7] and good protective effect against ERM is obtained with vaccine administered by immersion or injection [5,8]. As ERM generally affects small fish, immersion vaccination of fish weighing 4—5 g is recommended [5,9]. Duration and efficacy of the ERM vaccine has been well characterised by classical studies in the 1980s [9] and the empirical knowledge about the bath vaccination makes it a good model-system for investigation of antibacterial immunity in rainbow trout [10]. The bacterin is taken up through the gills [11] and skin and gut epithelium [12,13]. But although, the vaccine induces effective protection, the mechanism of protection is still unknown [1,7]. In addition, the effects of temperature on induced immunity and protection against infection is largely unknown in this bath vaccination system. Therefore, we have investigated the immune reactions and protection against ERM in rainbow trout following bath vaccination at 5, 15 and 25 °C. These temperatures are spanning the physiological range for this fish species including a low sub-optimal level via the physiological optimum to the near-lethal temperature [14,15]. It is known that change of water temperature has impact on rainbow trout physiology [16—18] including their metabolic rate [16,19]. Further, immune related functions such as endocytosis [20], immune gene transcription [21] immunoglobulins concentration [21—23] and C-reactive protein [24] have been reported to be dependent on the ambient temperature. The present work contributes to our understanding of temperature effects on the development of protective immunity upon bath vaccination. We also followed the dynamic expression of a series of immune relevant genes encoding pro-inflammatory and anti-inflammatory cytokines, chemokines, immunoglobulins and cellular receptors, which reflects to which extent humoral and cellular reactions are activated in the rainbow trout's immune system following vaccination against *Y. ruckeri*.

Materials and methods

Fish and rearing conditions

Juvenile rainbow trout (Skinderup strain), hatched and reared under pathogen-free conditions (Danish Centre for Wild Salmon, Randers, Denmark), were brought to the experimental university facility when reaching a total 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 fry were maintained (at a 12 h light and 12 h dark cycle) in aerated (100% oxygen saturation) tap water (15 °C) in 120 L aquaria with bio-filters (Eheim, Germany) and fed commercial trout feed (BioMar, Denmark), 2% biomass per day. The water temperature was then slowly changed (1 °C per day) until the fish were adapted to constant water temperatures at 5, 15 and 25 °C. Fish were kept at these temperatures for 8 weeks before vaccination.

Vaccination

Vaccination trials were conducted using a total of 1200 rainbow trout (400 fish per temperature). Experiments were conducted in duplicate. Half of the fish (200) in each temperature group (5, 15 and 25 °C) were bath-vaccinated with *Y. ruckeri* (serotype O1) bacterin (Pharmaq AS, Norway) diluted 1:10 in water for 10 min in aerated (100% oxygen saturation) tap water. The other half of the fish (control groups comprising 200 fish each) were sham-vaccinated with sterile water. Five immunised and five control fish were sampled at each temperature at 0, 8, 24 and 72 h post-vaccination (hpv). Fish were killed by an overdose of tricaine—methane—sulfonate (MS-222, Sigma—Aldrich, Denmark) (100 mg/L). The spleens were sampled aseptically, immediately transferred to RNA-later (Sigma—Aldrich, Denmark), pre-stored at 4 °C (24 h) and subsequently stored at −20 °C until isolation of RNA. When comparing groups for immunological parameters the vaccinated and control groups sampled at the same time points were compared.

Bacterial strain and challenge dose

Y. ruckeri serovar O1 strain 392/2003, grown in 10 mL LB-medium at 20 °C for 36 h isolated from diseased rainbow trout in Spain [25] was used for the challenge experiments. The bacteria were enumerated by counting colony forming units (CFU) on blood agar (LB media (tryptone 10 g, yeast-extract 5 g, NaCl 5 g, H_2O 1 L, pH 7.4 and with 5% bovine blood)).

The LD_{50} dose used for challenge was determined at 15 °C both for intra-peritoneal injection and bath infection. In brief, 10 fish for each dose were challenged with 10-fold dilution series of doses ranging from 5×10^3 to 5×10^6 CFU/fish for intra-peritoneal challenge and from 1×10^6 to 1×10^8 CFU mL for bath challenge. The LD_{50} used was 5×10^5 CFU/fish for i.p. challenge, and 1×10^7 CFU/mL *Y. ruckeri* in tank water for bath challenge.

Isolation of pathogens in fish head kidney following challenge

Head kidney samples from all fish that died each day were cultured on blood agar plates in an attempt to re-isolate *Y. ruckeri* and confirm the infection. Mortalities were only considered to be due to *Y. ruckeri* if the bacterium was recovered as pure culture from the head kidney.

Isolation of *Y. ruckeri* in blood following challenge

Blood was sampled from *vena caudalis* using heparinised syringes from five naïve fish infected i.p. by a uniform dose [26]. Samples were taken 0, 1, 2, 3, 4 and 6 days post-challenge at 5, 15 and 25 °C and a volume of 10 μL blood from each sample was plated on blood agar in a 10-fold dilution series in triplicate.

Challenge experiment

Intra-peritoneal (i.p.) challenge was conducted at 4 and 8 weeks post-vaccination using live *Y. ruckeri* as described