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Immunization of sea bream (*Sparus aurata*) juveniles against *Photobacterium damselae* subsp. *piscicida* by short bath: Effect on some pro-inflammatory molecules and the Mx gene expression

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ABSTRACT

Cytokines are a family of proteins derived from macrophages, lymphocytes, granulocytes, mast cells and epithelial cells and can be divided into interferons (IFNs), Interleukins (ILs) and Tumor Necrosis factors (TNFs) among others. The presence of cytokines in a wide number of fish species has been proved and several molecules types have been already cloned and sequenced. In this work some proinflamatory molecules and Mx gene were detected in the liver of vaccinated sea bream juveniles with an average body weight of 5 g. The method of immunization was by short bath and three different bacterins against the marine pathogen *Photobacterium damselae* subsp. *piscicida* were designed and used to immunize fish. Five genes encoding for five different molecules were analyzed by real time PCR: IL-1β, IL Ir-2, Cox-2, Mx and TNFα. Gene expression was quantified along four days after fish immunization and results were compared among groups. Results show that the heat-inactivated vaccine stimulates the up-regulation of IL-1β, IL Ir-2, Cox-2 and TNFα genes whereas the UV-light inactivated vaccine was the unique vaccine which stimulates the expression of Mx gene. The present is a novel study that shows by the first time the effect of the inactivation process of vaccines on the expression levels of genes involved in the defense against *Photobacterium damselae* subsp *piscicida*.

1. Introduction

Several fish cytokines have been cloned in recent years and researchers used the gene-expression to measure and evaluate the immune response. Although some difference with mammals does exist, it appears that fish possess most of the relevant molecules involved in immune-modulation and leucocytes communication. Pro-inflamatory cytokines such as IL-1β and TNF-α have been already identified in teleost species [1,2] Interleukins are a sub-group of cytokines, molecules involved in the intercellular communication and regulation of the immune system. These molecules tend to be either pro-inflammatory or act as antagonists to inhibit the activities of particular family members [3]. IL-1β genes have been identified in various teleost fish species including rainbow trout [1], sea bass [4], channel catfish [5], yellowfin sea bream [6] and Nile Tilapia [7]. IL-1β activates target cells by binding to IL-1 receptors on the cell surface and ultimately triggering inflammation to cope with pathogen infection. Two IL-1β receptors have been described in mammals and in various fish species including sea bream (IL-Ir1 and IL-Ir2). It has been demonstrated that IL-1β as well as TNF-α were highly increased after formaline-inactivated vaccination against *Areomonas salmonicida* [8]. The influence of Cyclooxygenase-2 (COX-2) within the immune system is via synthesis of prostaglandins (PG) that have a variety of functions, but are important in all stages of inflammation [15]. COX-2 is a pro-inflammatory enzyme closely connected to the fish innate immune response; in fact this enzyme catalyzes the reaction in cascade which leads to the conversion of the arachidonic acid into PGs. The COX-2 expression is induced by different inflammatory mediators (such as IFN-γ, TNF-α, IL-1β, growth factors, etc.) in several cellular types (monocytes, macrophages, endothelial cells, etc.) and tissues. The roles of PGs in the inflammatory response of mammals include

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vasodilation and increased vascular permeability by interaction with histamine and bradykinin, and down regulation of leukocyte functions by reduction of the respiratory burst, lymphocyte proliferation and antibody production [16].

Under the evolutionary point of view, the fish immune system it can be situated in an intermediate point between the mammals immune system and the insect's immune system. Actually in fish both components of the immunologic response (innate and specific) do co-exist but the first one is very much developed than the second and it represents the first and mayor protection against pathogens.

In human and other mammals, IFNs are the first line of defence against virus infections, Mx is a protein induced by IFN that interfere with the virus replication by inhibiting viral polymerase [9] Mx gene has been cloned in some fish species such as rainbow trout [10] Atlantic salmon [11] Atlantic halibut [12] Japanese flounder [13] puffer fish [14], sea bream [15] and Senegalese sole [16] Mx gene expression in fish has been demonstrated after dsRNA virus infection, LPS inoculation and some bacterin vaccination. In this study we wanted to analyze if our bacterin was able to enhance TNF-α, IL-1β, IL-1r2, COX-2 and Mx gene expression in vaccinated fish.

2. Materials and methods

2.1. Experimental animals

A total of 1500 sea bream juveniles with an average body weight of 5 g were kindly provided by a local farm (ADSA, Alevines y Doradas S.A.) and held at the ICCM (Instituto Canario de Ciencias Marinas, Canary Islands, Spain) facilities. Fish were randomly distributed into10 fiber glass tanks of 500 L (100 fish/tank triplicate for each treatment). All tanks were supplied with an open water system, continuous aeration and exposed to natural photoperiod (around 12 h: 12 h, L: D). Fish were fed to satiation twice a day for 7 days a week. Temperature of the water was kept at 22 °C throughout all the experimental period.

2.2. Bacteria and vaccines

The strain of Phdp we used in this work was isolated from a natural outbreak in the Canary Islands. This strain was called 94/99 since it was isolated in 1999 and it was the case number 94 and it was used to prepare bacterin to vaccine experimental fish. 94/99 bacteria culture was identified using biochemical characterization by API 20E (Biomerieux®, Spain) and by PCR analysis as previously described [17] and finally was stored lyophilized.

Lyophilized bacteria was rehydrated and initially grown in blood agar plate enriched with 2% of sodium chloride and 1% glucose in order to stimulate cells to produce a capsule (Acosta et al., 2004) [18]. The following inactivated vaccines were prepared in our laboratory using the 94/99 strain: a formalin-killed vaccine: bacteria was incubated with 5‰ concentration of formalin overnight with continuous shaking [18] a heat-shock vaccine: inactivation was reached by exposing bacteria to a temperature of 80 °C for 10 min, as described by López-Dóriga et al. (2000) [19] and a UV-light vaccine: bacteria was grown in TSA plates and exposed to UV rays for two hours. Final concentration of each of the three prepared bacterins was adjusted to 10^8 cfu/ml using a spectrophotometer (optical density = 600 nm). As positive control we used the commercial vaccine Ictiovac PD® (Hipra, Spain).

2.3. Immunization of fish

1500 sea bream of 5 g average body weight were immunized by direct immersion in a 10 fold dilution of each bacterin (9 L seawater: 1 L vaccine) during 60 s (short bath). After receiving a bath with clean water they were replaced into their respective tank. As a negative control a group of fish had the same treatment but received a 60 s bath in Phosphate Buffered Saline (9 L seawater: 1 L PBS). As a positive control a group received a bath with the commercial vaccine Ictiovac PD®.

2.4. Collection of samples

Collection of sample was performed as follow: 3 fish per tank were anesthetized with 2-phenoxy ethanol and then slaughtered on ice, sample of liver were aseptically taken at day 1, 2, 3 and 4 post immunization and stored in 500 μl of RNA later at −80 °C until analysis.

2.5. Analysis of gene expression

Total RNA was extracted from liver of sea bream with a chloroform protocol [20] Briefly, frozen sample of liver were defrosted using an ultra sonicator, then 500 μl of chloroform (PANREAC) was added to the sample and mixed vigorously. After centrifugation at 4000 g for 10 min at 4 °C equal volume of the aqueous phase and isopropyl alcohol (PANREAC) were mixed and incubated 5 min at room temperature. After centrifugation at 4000 g for 10 min at 4 °C the pellet was washed twice with cold 75% ethanol, dried with an ultra-vacuum centrifugator (Beckman Coulter) dissolved in 100 μl of RNase-free dH_2O and finally quantified with a NanoDrop-1000 spectrophotometer. Sample were adjusted in RNase-free dH_2O to the same concentration of 2 ng/ml and RNA was reverse transcribed to cDNA using the iScript Reverse Transcription Reagent kit (Bio-Rad) as follows: 2 μl of total RNA 5 μl of buffer reaction 5×, and 12 μl of RNase-free dH_2O, heated the mixture to 70 °C for 10 min and chilled on ice. Then, were added 1 μl of enzyme reverse transcriptase (200 U ml^{-1}) and 5 μl of RNase-free dH_2O. This was subsequently incubated at 25 ° for 10 min, 50 min at 42 °C and 15 min at 70 °C the cycle ending at 16 °C or ice.

The expression of five selected immune-relevant genes (Mx, IL-1β, IL-Ir2, COX-2, TNF-α) was analyzed by real-time PCR using a SYBR Green Supermix (Biorad). Specific PCR primers for Mx, were designed using the GeneRunner® software. For the amplification of the genes IL-1β, TNF-α and Cox-2 we used the sequence of primers described by Sepulcre et al. (2007) [21]; the sequence described by Roca et al. (2008) [22] for IL-Ir2 gene, and the sequence described by Bravo et al. (2011) [9] for β-actin. β-actin was use as housekeeping gene. Primers, concentration and reference are given in Table 1.

The real-time analysis consisted of 1 cycle of 95 °C for 5 min, 40 cycles of 95 °C for 15 s and annealing temperature for 30 s, 1 cycle of 95 °C for 1 min, 1 cycle of 70 °C for 1 min, and a melting curve of 81 cycles (from 55 °C to 95 °C) for 30 s. Reactions were performed in triplicate for each template cDNA that was replaced with water in all blank control reactions. The annealing temperatures were 58,3 °C for β actin, 60 °C for Mx, Cox-2 and TNF and 59,7 °C for IL-1β and IL-Ir2.

The relative gene expressions were determined according to the ΔΔCt method using IQ5 software (Bio-Rad). The ΔΔCt method is also known as the comparative Ct method where:

$$\Delta\Delta Ct = [\Delta]Ct_{sample} - [\Delta]Ct_{control};$$

here ΔCtsample is the Ct value for any sample normalized to the endogenous housekeeping gene and ΔCtcontrol is the Ct value for the control sample also normalized to the endogenous housekeeping gene. The IQ5 software allows automatic normalization of