Fish and Shellfish Immunology 75 (2018) 139–148

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Immunological response of the Sub-Antarctic Notothenioid fish *Eleginops maclovinus* injected with two strains of *Piscirickettsia salmonis*

D. Martínez[a,b,c,**], D. Díaz-Ibarrola[a], C. Vargas-Lagos[a,d,e], R. Oyarzún[a,b,c], J.P. Pontigo[a], J.L.P. Muñoz[f], A.J. Yáñez[e,g], L. Vargas-Chacoff[a,c,*]

[a] Instituto de Ciencias Marinas y Limnológicas, Laboratorio de Fisiología de Peces, Universidad Austral de Chile, Valdivia, Chile
[b] Escuela de Graduados, Programa de Doctorado en Ciencias de l6a Acuicultura, Universidad Austral de Chile, Av. Los Pinos s/n Balneario Pelluco, Puerto Montt, Chile
[c] Centro Fondap de Investigación de Altas Latitudes (IDEAL), Universidad Austral de Chile, Casilla 567, Valdivia, Chile
[d] Escuela de Graduados, Programa de Magister en Microbiología, Universidad Austral de Chile, Valdivia, Chile
[e] Centro Fondap Interdisciplinary Center for Aquaculture Research (INCAR), Universidad Austral de Chile, Valdivia, Chile
[f] Centro de Investigación y Desarrollo i ~ mar, Universidad de los Lagos, Casilla 557, Puerto Montt, Chile
[g] Instituto de Bioquímica y Microbiología, Universidad Austral de Chile, Valdivia, Chile

ARTICLE INFO

Keywords:
Eleginops maclovinus
Immune system
Sub-Antarctic Notothenioid
Gene expression
P. salmonis

ABSTRACT

Eleginops maclovinus is an endemic fish to Chile that lives in proximity to salmonid culture centers, feeding off of uneaten pellet and salmonid feces. Occurring in the natural environment, this interaction between native and farmed fish could result in the horizontal transmission of pathogens affecting the aquaculture industry. The aim of this study was to evaluate the innate and adaptive immune responses of *E. maclovinus* challenged with *P. salmonis*. Treatment injections (in duplicate) were as follows: control (100 μL of culture medium), wild type LF-89 strain (100 μL, 1×10^8 live bacteria), and antibiotic resistant strain Austral-005 (100 μL, 1×10^8 live bacteria). The fish were sampled at various time-points during the 35-day experimental period. The gene expression of TLRs (1, 5, and 8), NLRCs (3 and 5), C3, IL-1β, MHCII, and IgMs were significantly modulated during the experimental period in both the spleen and gut (excepting TLR1 and TLR8 spleen expressions), with tissue-specific expression profiles and punctual differences between the injected strains. Anti-*P. salmonis* antibodies increased in *E. maclovinus* serum from day 14–28 for the LF-89 strain and from day 14–35 for the Austral-005 strain. These results suggest temporal activation of the innate and adaptive immune responses in *E. maclovinus* tissues when injected by distinct *P. salmonis* strains. The Austral-005 strain did not always cause the greatest increases/decreases in the number of transcripts, so the magnitude of the observed immune response (mRNA) may not be related to antibiotic resistance. This is the first immunological study to relate a pathogen widely studied in salmonids with a native fish.

1. Introduction

Fish are the first vertebrates to present an immune system similar to mammals, with lymphoid organs that include the thymus, spleen, and kidney, but lacking bone marrow and lymph nodes [1]. As in mammals, the immune system in fish is divided into innate (nonspecific) and acquired (specific) responses, both of which are composed by humoral and cellular components [2]. The cells and molecules of the innate immune system use non-clonal pattern recognition receptors, including C-type lectin receptors, Toll-like receptors (TLRs), and NOD-like receptors (NLRs) [3]. This is in contrast to cells of the adaptive immune system, which express clonal receptors able to specifically recognize antigens and their derived peptides [3]. Both systems communicate through soluble mediators known as cytokines, which mediate the immune response and alert the recognition of a pathogenic stressor, rapidly controlling pathogen growth, promoting inflammation, and triggering an adaptive immune response [3].

Various infectious agents can stimulate the immune system, including Gram negative bacteria such as *Piscirickettsia salmonis*. This facultative intracellular bacterium [4–8] is the etiological agent of Piscirickettsiosis, a systemic disease that colonizes several salmonid organs, including the kidney; liver; spleen; heart; skeletal muscle; brain; intestine; ovary, and gills [9–11]. The most prominent microscopic lesions occur in the liver, kidney, spleen, and gut [12,13].

* Corresponding author. Instituto de Ciencias Marinas y Limnológicas, Laboratorio de Fisiología de Peces, Universidad Austral de Chile, Valdivia, Chile.
** Corresponding author. Instituto de Ciencias Marinas y Limnológicas, Laboratorio de Fisiología de Peces, Universidad Austral de Chile, Valdivia, Chile.
E-mail addresses: danixa.martinez@alumnos.uach.cl (D. Martínez), luis.vargas@uach.cl (L. Vargas-Chacoff).

https://doi.org/10.1016/j.fsi.2018.01.012
Received 8 November 2017; Received in revised form 3 January 2018; Accepted 11 January 2018
Available online 06 February 2018

Piscirickettsiosis was initially reported as a salmonid disease, although there is evidence that this disease can occur in non-salmonid species, such as *Dicentrarchus labrax* [14], *Atractoscion nobilis* [15], *Oreochromis mossambicus*, and *Sarotherodon melanotheron* [16]. Furthermore, genetic material of this bacterium has been detected in fish native to Chile, including *Eleginops maclovinus*, *Odontesthes regia*, *Sebastes capensis*, and *Salilota australis* [17].

P. salmonis, a bacterium responsible for significant economic losses, can infect, survive, replicate, and disseminate within host monocytes/macrophages without inducing the cytopathic effect [18]. This bacterium can induce the apoptosis of infected salmonid macrophages *in vitro* during the early, intermediate, and late phases of infection [19]. In particular, *P. salmonis* can modulate the expression of genes involved in the innate and adaptive immune responses in the *Salmo salar* head kidney [20,21]. Between 2 and 14 days post-infection with *P. salmonis*, *S. salar* overexpresses genes involved in the inflammatory and oxidative responses and under-expresses genes involved in the adaptive immune response, the G protein signaling pathway, and apoptotic processes [22,23].

Immunological studies evaluating how the tissues of native fish respond to the presence of *P. salmonis* are scarce, mainly in native fish that normally lives in proximity to salmonid culture centers. *E. maclovinus*, known as the Patagonian blenny, is a Sub-Antarctic Notothenioid of the Eleginopidae (Osteichthyes) family, Notothenioidei suborder. The Patagonian blenny is endemic to Patagonia, in South America, and is one of the most eurythermal, euryhaline, and stenobathic representatives of the suborder [24,25]. *E. maclovinus* subsists off of unconsumed pellet feed and salmonid excrements [26]. These feeding behaviors are indicative of a native-farmed fish interaction in the natural environment. One consequence of this interaction would be a transference of microorganisms (e.g., *P. salmonis*) with different degrees of pathogenicity and antibiotic resistance. In Chile, Piscirickettsiosis is controlled primarily through antimicrobial agents and vaccines [13]. Unfortunately, existing treatments are inefficient and frequently lead to the emergence of antibiotic-resistant isolates [27].

The role that native fish play in transmitting Piscirickettsiosis remains unknown, although *E. maclovinus* increases serum immunoglobulin levels in response to an injected extract of total *P. salmonis* proteins [28]. Additionally, Martínez et al. [29] reported that *E. maclovinus* injected with live *P. salmonis* modulate the genic expression of ferritin-H, suggesting the possible activation of nutritional immunity in the presence of this bacterium [30].

The objective of this study was to evaluate the temporal activation of the innate and adaptive immune responses in two poorly investigated tissues of *E. maclovinus*. Spleen and gut tissue, as well as serum, were obtained after *E. maclovinus* was challenged with two *P. salmonis* strains: the LF-89 reference strain (ATCC® VR-1361™) and the antibiotic-resistant Austral-005 strain. Both strains exhibit genomic differences that would seem to determine different degrees of virulence or pathogenesis [27,31].

2. Materials and methods

2.1. Samples

The present study used the same specimens and experimental procedures as in Martínez et al. [29,30]. Briefly, immature *E. maclovinus* (20 ± 5 g body weight) were captured and transferred to the Calfuco Coastal Laboratory (Faculty of Sciences, Universidad Austral de Chile, Valdivia, Chile). Fish were acclimated (four weeks) in 500 L tanks with seawater (32 psμ, 1085 mOsm kg^{-1}), at a density of 3.1 kg m^{-3}, using a flow-through system, natural photoperiod, and a temperature of 12.0 ± 0.5 °C, following indications given in Vargas-Chacoff et al. [32]. Fish were fed once daily with a 1% body weight ratio of commercial dry pellets (Skretting Nutrece 100 ML; containing 48% protein, 22% fat, 13.5% carbohydrates, 8% moisture, and 8.5% ash). All experiments were performed in compliance with guidelines established by the Comisión Nacional de Ciencia y Tecnología de Chile (CONICYT) and the Universidad Austral de Chile for the use of laboratory animals.

2.2. Primers design

All steps involving massive cDNA sequencing were performed at the Austral-omics Laboratory on a GS Junior Titanium Series (Roche) following manufacturer protocols. In detail, total RNA was extracted from one tissue (head kidney of a healthy fish) with the commercial RNA NucleoSpin® RNA Kit (Macherey-Nagel). RNA was selected with optimal integrity (RIN > 7). Subsequently, mRNA isolation was performed with the PolyATtract III Kit® in an mRNA Isolation System (Promega). The quantity and quality of total mRNA was evaluated using an A260 ND-1000 spectrophotometer (NanoDropH Technologies) and Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. The cDNA library was constructed following the "Rapid cDNA library preparation" protocol recommended by Roche. Bioinformatics analysis of the obtained data included the formation of transcripts (contigs); specifically by using a combination of specialized transcriptomic analysis software followed by transcript annotation against the non-redundant NCBI database. After this process, mRNA sequences of TLR1, TLR8, NLRC3, NLRC5, major histocompatibility complex class II (MHCII), and immunoglobulin M (IgM) were obtained and analyzed using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/). RNA-seq revealed some genes for the immune response, and the other genes selected for study were amplified from heterologous primers (C3, IL-1β, and TLR5). Genes related to the innate immune response included C3, TLR (1, 5, and 8), NLRC (3 and 5), and IL-1β. Genes related to the adaptive humoral immune response included MHCII and IgMs. Partial cDNA coding sequences were obtained and deposited in GenBank, the accession numbers of which are indicated in Table 1.

2.3. P. salmonis LF-89 and Austral-005

Inoculates of *P. salmonis* were kindly donated by the Laboratory of Metabolism and Biotechnology, Institute of Biochemistry and Microbiology, Faculty of Sciences, Universidad Austral de Chile (Valdivía, Chile). The LF-89 strain was used as a reference [33], and Austral-005 was used as an antibiotic-resistant strain [34]. Both strains of *P. salmonis* were grown in AUSTRAL-TSFe agar plates and incubated at 18 °C for ten days, according to standard conditions [8]. Subsequently, *P. salmonis* colonies were grown in 4.5 mL AUSTRAL-SRS broth at 18 °C for 72 h with moderate agitation (75 rpm) [7].

2.4. Infection assays with P. salmonis LF-89 and Austral-005

After acclimation, *E. maclovinus* specimens were randomly distributed among rectangular tanks (100 L), each of which corresponded to a control/treatment group, in duplicate (n = 126 total). The groups were as follows: (i) Control group, injected only with the culture medium (100 μL, n = 42); (ii) LF-89 Experimental group, injected with the *P. salmonis* LF-89 type strain (100 μL; 1×10^8 concentration of live bacteria, n = 42); and (iii) Austral-005 Experimental group, injected with the *P. salmonis* Austral-005 antibiotic-resistant strain (100 μL; 1×10^8 concentration of live bacteria, n = 42). Six fish per group were collected at 1; 3; 7; 14; 21; 28 and 35 days post-injection (dpi) (i.e., 18 fish total sampled per time-point). Specimens were sampled to evaluate the temporal activation of genes associated with the innate immune system (initial dpi) and adaptive immune system (latter dpi). During the experiment, the fish were maintained at a density of 3.1 kg m^{-3}, temperature of 12.0 ± 0.5 °C, with a flow-through system, and under a natural photoperiod, as per Vargas-Chacoff et al. [32]. Fish were fed once daily at a ratio of 1% body weight using commercial dry pellets (Skretting Nutrece Defense 100). The bacterial dose was the same used in previous studies [29,30].