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Nodavirus infection induces a great innate cell-mediated cytotoxic activity in resistant, gilthead seabream, and susceptible, European sea bass, teleost fish

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ABSTRACT

Viral nervous necrosis (VNN) virus produces great mortalities in fish having susceptible and reservoir species between the most important marine aquaculture species. Cell-mediated cytotoxicity (CMC) is considered, towards the interferon (IFN), the most important mechanism of the immune response to fight against viral infections but it has been very scarcely evaluated. We aimed to evaluate the effects of VNNV infection in the reservoir gilthead seabream (*Sparus aurata*) and susceptible European sea bass (*Dicentrarchus labrax*). Firstly, after experimental infection we found mortalities in the sea bass (55%) but no in the seabream. Moreover, VNN virus replicates in the brain of both species as it was reflected by the high up-regulation of the Mx gene expression. Interestingly, the head-kidney leucocyte cell-mediated cytotoxic activity was significantly increased in both species reaching highest activity at 7 days: 3.65- and 2.7-fold increase in seabream and sea bass, respectively. This is supported by the significant up-regulation of the non-specific cytotoxic cell receptor (NCCRP-1) in the two fish species. By contrast, phagocytosis was unaffected in both species. The respiratory burst was increased in seabream 7 days post-infection whilst in sea bass this activity was significantly decreased at days 7 and 15. Our results demonstrate the significance of the CMC activity in both gilthead seabream and European sea bass against nodavirus infections but further studies are still needed to understand the role of cytotoxic cells in the antiviral immune response and the mechanisms involved in either reservoir or susceptible fish species.

1. Introduction

Viral diseases are responsible for many of the economical losses suffered in modern aquaculture since they produce high levels of mortality and no effective antiviral treatments are available. Moreover, fish farming management such as growth under very high densities, introduction of new species, continuous transport between hatcheries, nurseries and growing plants are increasing the spread of pathogens and the number of susceptible and reservoir species. Among the fish virus, viral nervous necrosis virus (VNNV) produces the viral encephalopathy and retinopathy (VER) disease altering therefore the brain and retina structure and function. This is considered one of the most serious viral diseases in marine aquaculture being larvae and juveniles the most susceptible stages [1]. Nodavirus belongs to the family Nodaviridae, genus *Betanodavirus* and has a bi-segmented genome made of two single-stranded, positive-sense RNA molecules, RNA1 and RNA2 [2]. The RNA1 (3.1 kb) encodes the RNA-dependent RNA-polymerase (RdRP) and also contains a subgenomic fragment (RNA3 in a different open reading frame) only present in infected cells but not in viral particles [3]. The RNA2 (1.4 kb) encodes the capsid protein (CP) [4]. VNNV has more than 30 susceptible fish species and European sea bass (*Dicentrarchus labrax*) represents the most susceptible [5]. Furthermore, other fish species usually cultured in the same or close farms such as the gilthead seabream (*Sparus aurata*) are reservoir or carries species that support viral replication but they do not suffer from VER disease [6,7]. Therefore, while most available information focuses on the mechanisms involved in pathogen susceptible fish further knowledge is also important in pathogen-reservoir fish systems in order to avoid or control the spread of pathogens.

Among the major immune mechanisms to fight viral infections in fish, the interferon (IFN) pathway and the cell-mediated cytotoxic (CMC) activity are the most important, but most efforts have only focused on the IFN pathway [8,9]. In teleost fish, this CMC activity is carried out by nonspecific cytotoxic cells (NCCs), natural

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killer-like (NK) cells and cytotoxic-T lymphocytes (CTL) [10,11]. Among them, innate NCCs and NK-like cells are characterized by the presence or absence, respectively, of the NCC receptor protein-1 (NCCRP-1) marker [12,13] whilst the specific CTLs are characterized by the presence of T-cell receptor (TCR) and CD8 co-receptor [10,11]. However, few studies of the fish CMC activity have focused on the role of cytotoxic cells against viral infections [14–25]. They have demonstrated that fish cytotoxic activity is increased both *in vitro* and *in vivo* by viral infections. Even though, very little is still known about the mechanisms involved in the fish CMC activity and their involvement in the antiviral response. In the case of VNNV, infected orange-spotted groupers (*Epinephelus coioides*) elicited a specific cytotoxic activity against VNNV-infected cells mediated by $CD8^+$ lymphocytes in a MHC I-restricted manner [15]. However, no other study has focused on the CMC activity against VNNV and only one has evaluated the innate CMC activity of gilthead seabream (*S. aurata*) or European sea bass against viral infections [16]. Apart from the CMC, other cellular innate immune responses carried out by phagocytes (granulocytes and macrophages) may be important in the clearance of viral infections. In this way, few studies have related increments or reductions in the phagocytosis and in the production of reactive oxygen species (ROS) or nitric oxide (NO) after viral infections [16,26–31]. Focussing on nodavirus, a single study has found that VNNV infection of turbot (*Scophthalmus maximus*) (specie showing VER disease symptoms and mortalities) elicited an increase in the NO production by macrophages [32]. Overall, further studies are needed to understand the innate immune responses at cellular level in fish suffering viral infections.

Although the CMC activity is a major component of the antiviral immune response its characterization has almost been ignored in fish and never evaluated in the case of gilthead seabream and European sea bass infected with nodavirus, for which they serve as reservoir and susceptible species, respectively. Therefore, we aimed in this study to evaluate the effects of the nodavirus infections in the cellular innate immune response focussing on the cytotoxic activity but also evaluating others such as phagocytosis or ROS production. We will also try to find differences in the immune responses between the two species in an attempt to understand the reasons behind the resistance to the nodavirus. Finally, potential correlations between the CMC activity and IFN/Mx pathway will be pointed for the first time in fish.

2. Material and methods

2.1. Animals

Adult specimens of the marine teleost gilthead seabream (*S. aurata*) and European sea bass (*D. labrax*) (125 ± 25 and 305 ± 77 g body weight, respectively) were purchased from the IEO installations and translated to the University of Murcia aquaria. Fish were kept in 450–500 L running seawater (28‰ salinity) aquaria at 24 ± 2 °C and with a 12 h light:12 h dark photoperiod and fed daily with 1 g per fish of a commercial pellet diet (Skretting). Animals were acclimatised for 15 days prior to the experiments. All animal studies were carried out in accordance with the European Union regulations for animal experimentation and the Bioethical Committee of the University of Murcia.

2.2. Nodavirus stocks

Nodavirus (strain 411/96, genotype RGNNV) were propagated in the SSN-1 cell line which is persistently infected with a snakehead retrovirus (SnRV) [33]. The SSN-1 cells were grown at 25 °C in Leibovitz's L15-medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 2 mM L-glutamine (Gibco), 100 i.u. ml^{-1} penicillin (Gibco), 100 μg ml^{-1} streptomycin (Gibco) and 2.5 μg ml^{-1} fungizone (Gibco) using Falcon Primaria cell culture flasks (Becton Dickinson). Cells were inoculated with nodavirus and incubated at 25 °C until the cytopathic effect was extensive. Supernatants were harvested and centrifuged to eliminate cell debris. Virus stocks were titrated in 96-well plates according to Reed and Müench [34] before used in the experiments.

2.3. Nodavirus infections

Thirty specimens of gilthead seabream or European sea bass were randomly divided into two tanks. Each group received a single intramuscular injection of 100 μl of SSN-1 culture medium (mock-infected) or culture medium containing 10^6 VNNV $TCID_{50}$ $fish^{-1}$ since this route of infection has been proven as the most effective [35]. Fish were sampled 1, 7, 15 or 30 days after the viral injection. Mortality was also recorded through the experiment.

2.4. Fish sampling

Fragments of brain and head-kidney tissues were removed for RNA isolation. Gilthead seabream and European sea bass head-kidney leucocytes (HKL) were isolated to evaluate the innate immune parameters as described elsewhere [36]. Briefly, after bleeding, head-kidney was cut into small fragments and transferred to 8 ml of sRPMI [RPMI-1640 culture medium (Gibco) supplemented with 0.35% sodium chloride (Sigma), 5% FBS, 100 i.u. ml^{-1} penicillin and 100 μg ml^{-1} streptomycin]. Cell suspensions were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100 μm). Cells were washed twice in sRPMI, counted in a Z2 Coulter Particle Counter (Beckman Coulter) and adjusted to 10^7 cells ml^{-1}. Cell viability was determined by the trypan blue exclusion test.

2.5. Gene expression by real-time PCR

VNN virus replication was determined by the detection of the viral RNA-dependent RNA polymerase (RdRP) and coat protein (CP) genes in brain and head-kidney tissues. To evaluate the immune response in both tissues we determined the transcript levels of myxovirus (influenza) resistance protein (Mx) and NCCRP-1 genes. For this, total RNA was isolated from TRIzol Reagent frozen samples following the manufacturer's instructions. One μg of total RNA was treated with DNAse I to remove genomic DNA and the first strand of cDNA synthesized by reverse transcription using the Thermo-ScriptTM RNAse H- Reverse Transcriptase (Invitrogen) with an oligo-dT12-18 primer (Invitrogen) followed by RNAse H (Invitrogen) treatment.

Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min 60 °C and 15 s at 95 °C. For each mRNA, gene expression was corrected by the elongation factor 1-alpha (EF1α) content in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the EF1α Ct value from the target Ct. The primers used are shown in Table 1. Before the experiments, the specificity of each primer pair was studied using positive and negative samples. Amplified products from positive samples were run in 2% agarose gels and sequenced. After these verifications, all amplifications were performed in duplicate cDNAs and repeated once to confirm the results. Negative controls with no template were always included in the reactions.