Nodavirus increases the expression of Mx and inflammatory cytokines in fish brain.

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Abstract

Nodavirus has become a serious pathogen for a wide range of cultured marine fish species. In the present work, the expression of genes related to immune and inflammatory responses of sea bream (*Sparus aurata* L.), considered as non susceptible species, was studied both *in vitro* and *in vivo*. No replication of the virus was observed in head kidney macrophages and blood leukocytes. Moreover, the enhancement of expression of several immune genes (tumor necrosis factor alpha (TNF-α), interleukin-1-beta (IL-1β), interferon-induced Mx protein) was not detected in both head kidney macrophages and blood leucocytes in response to an *in vitro* infection with nodavirus. However, *in vivo*, nodavirus was detected 1 day post-infection (p.i.) by a reverse transcription-polymerase chain reaction (RT-PCR) in blood, liver, head kidney and brain of experimentally infected sea bream, while its presence clearly decreased in blood after 3 days p.i. Also, a transitory increment of the expression of TNFα and IL-1β was detected in the brain of intramuscular (i.m.) infected sea bream 3 days p.i. In head kidney, the over expression of TNFα was only observed 1 day p.i. The expression of Mx, an interferon induced gene, was increased in brain and head kidney of infected sea bream, reaching values of 1300 fold compared to controls in brain three days post infection.

For comparative purposes, we analyzed the expression of the same genes on a susceptible species, such as sea bass (*Dicentrarchus labrax*) and, although the same pattern of expression was observed both in brain and kidney, the magnitude was different mainly in the case of brain, the key organ of the infection, where higher expression of TNFα and lower expression of Mx compared with control was observed.

Keywords: Nodavirus, sea bream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*), immune system, cytokines, TNFα, IL-1β and Mx
1. Introduction

Viral encephalopathy and retinopathy (VER), also referred to as viral nervous necrosis (VNN) is an emerging disease caused by several Betanodaviruses, members of the family Nodaviridae inducing high mortalities in infected marine fish. The disease caused by these viruses is identified by abnormal swimming behaviour and neurological lesions, which are characterized by cellular vacuolization and neuronal degeneration mostly found in the brain, retina, spinal cord and ganglia of the affected fish. Since its first description in larvae and juvenile sea bass (Dicentrarchus labrax) reared in Martinique (Bellance and Gallet de Saint-Aurin, 1988), the disease has spread to many other marine species worldwide (Nakai et al., 1994; Munday and Nakai, 1997; Curtis et al., 2001; Barke et al., 2002) and recently in freshwater species (Hegde et al., 2003; Athanassopoulou et al., 2004).

Sea bream and sea bass are species of a high economic value cultured in the Mediterranean Sea. Sea bream has been initially reported as an asymptomatic carrier of the disease (Castric et al., 2001). However, we have previously shown that sea bream can be experimentally susceptible to nodavirus, depending upon the temperature and route of infection (Aranguren et al., 2002). Also, sea bream is often cultured in the Mediterranean in the vicinity of sea bass and other susceptible species, raising the possibility of cross infection.

So far, little is known about the interactions between nodavirus and the fish immune system. Antibodies to nodavirus were detected by ELISA (Enzyme-Linked Immuno Sorbent Assay) in the serum of adults of striped jack (Mushiake et al., 1992), sea bass (Breuil et al., 2000), barfin flounder (Watanabe et al., 2000) and barramundi (Huang et al., 2001), regardless of the sex or origin (wild or cultivated) of the fish examined. Vaccines have been experimentally tested in fish with preliminary positive results (Husgaro et al., 2001; Sommerset et al., 2003; 2005) and the effect of nodavirus-neutralizing antibodies on virus clearance or survival has been reported (Tanaka et al., 2001).
The aim of this work was to study if the experimental infection of sea bream and sea bass with nodavirus could affect the expression of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and an interferon-induced Mx protein, both in vitro and in vivo. Moreover, we have compared the viral replication and the gene expression between the two fish species with the aim to find possible explanations of the differential susceptibility to the disease.

2. Materials and methods

2.1. Fish

Adult sea bream and sea bass of approximately 200 g were obtained from a commercial fish farm. Fish were then acclimatized to laboratory conditions for 2 weeks, maintained at 20 ºC and fed daily with a commercial diet (Trouw, Spain).

2.2. Virus

The nodavirus strain, 475-9/99, was provided by The Institute Zooprofilattico delle Venize (Italy) after isolation from diseased sea bass. The virus was propagated in the SSN-1 cell line (Frerichs et al., 1996) and then titrated in 96-well plates (Falcon). TCID$_{50}$ ml$^{-1}$ (tissue culture infectious dose infecting 50 % of inoculated cultures) was calculated according to Reed and Muench (1938).

2.3. Isolation of head kidney macrophages and blood leukocytes
Head kidney macrophages and blood leukocytes were isolated following the method previously described by Chung and Secombes (1988). The viable cell concentration was determined by Trypan blue exclusion.

2.4. Replication of nodavirus in sea bream and sea bass leukocytes and kidney macrophages

Primary cultures of total blood leukocytes and kidney macrophages from sea bass and sea bream were infected with nodavirus (1 x 10⁴ TCID₅₀ ml⁻¹). After 1 h of incubation with the virus at 25 ºC, cells were washed twice with L-15 medium and incubated at 25 ºC with L-15 + 5 % fetal calf serum (FCS). After 1, 3, 5 and 7 days, supernatants and cells were collected by scraping the bottom of the wells and separated by centrifuging at 12000 x g for 10 minutes at 4 ºC. Cells were then suspended in the same medium previously used for the culture. Supernatants and cells were frozen until use and, in the case of cells, another freezing cycle was conducted in order to lyse them. Titration of supernatants and cells was made in SSN-1 96-well plates and the TCID₅₀ calculated.

2.5. Cytokines induction after a nodavirus infection

The level of expression of TNFα, IL-1β and Mx was tested after infection both in vitro and in vivo using quantitative Real Time PCR (qPCR).

The in vitro induction of these genes was tested after infecting head kidney macrophages and blood leukocytes (5 x 10⁶ cells ml⁻¹) with nodavirus at a final concentration of 7.8 x 10⁵ TCID₅₀ ml⁻¹. After 6 hours of incubation at 25 ºC, supernatants were removed by centrifuging 5 min at 12000xg and RNA was extracted from the cells using Trizol (Gibco). RNA was then used to obtain cDNA by Superscript Preamplification System (Gibco), which was stored at –20 ºC.
The *in vivo* induction was tested by intramuscular injection of sea bream and sea bass. Eighteen fish from each species were challenged with 50 µl of nodavirus (3 x 10^5 TCID\(_{50}\) ml\(^{-1}\)/fish) and eighteen fish were injected with 50 µl of cell culture medium as control. Fish were sacrificed by MS-222 overdose 1, 3 and 7 days post challenge (three pools of two fish each one) and brain and head kidney were removed aseptically and frozen for RNA isolation and cDNA transcription, as previously described.

Quantitative PCR assays were performed using the 7300 Real Time PCR System (Applied Biosystems). cDNA amplification was performed using specific primers designed by Primer 3 software (Rozen and Skaletsky, 2000). 0.5 µl of each primer (10 µM) was mixed with 12.5 µl of SYBR green PCR master mix (Applied Biosystems) in a final volume of 25 µl. The standard cycling conditions were 95 ° for 10 min, followed by 40 cycles of 95 ° 15 s and 60 ° for 1 min. The comparative CT method (2-∆∆CT method) was used to determine the expression level of analyzed genes (Livak and Schmittgen, 2001). The expression of the candidate genes was normalized using β–actin as a housekeeping gene. Fold units were calculated dividing the normalized expression values of infected tissues by the normalized expression values of the controls. Primer sequences are shown in Table 1.

### 2.6. Nodavirus detection by RT-PCR

In order to determine whether nodavirus was present in the different organs of sea bream in which the expression of cytokines was studied in a similar way than it happens in sea bass, viral detection was performed using an RT-PCR based on the amplification of a highly conserved region of the coat protein gene as previously described by Dalla Valle et al. (2000). Products of the amplification reaction were visualized on a 2 % agarose gel.

### 2.7. Statistics
Data were compared using Student’s *t* test. Results are expressed as mean ± standard deviation and differences were considered statistically significant at *p* < 0.05.

3. Results

3.1. Replication of nodavirus in head kidney macrophages and blood leukocytes

The viral titer did not increase with time in sea bream and sea bass kidney macrophages or in blood leukocytes (Figure 1), neither in the cells nor in the supernatants, indicating that these cell populations do not support viral replication in any of the two studied species. No cytopathic effect was ever observed in head kidney macrophage or blood leukocyte cultures during the nodavirus infection.

3.2. Nodavirus detection by RT-PCR

In order to confirm that the lack of susceptibility of sea bream to nodavirus infection was due to a problem in the accessibility to the key organ, the presence of nodavirus was assessed in infected sea bream at days 1 and 3 post-infection in blood, liver, kidney and brain. Nodavirus, as in the case of sea bass (data not shown), was strongly detected in blood 1 day post-infection but the amount of virus detected highly decreased 3 days after infection (Figure 2a). However, nodavirus presence was confirmed 1 and 3 days p.i. in the remaining tissues, especially in brain as the target organ of the disease (Figure 2b, 2c and 2d). Nodavirus was never detected in control sea bream tissues (Figure 2a, 2b, 2c and 2d).

3.3. Cytokines expression analysis in sea bream and sea bass
The expression of TNF-α, IL-1β and Mx both in sea bream and sea bass macrophages and blood leukocytes was not enhanced after exposure to nodavirus in vitro in this study (data not shown).

However, with regard to the in vivo infection of sea bream, a significant but transitory up-regulation of the expression of TNFα and IL-1β was detected in the brain of infected sea bream 3 days p.i. (Figure 3a and 3c, respectively). In head kidney, the over expression of TNF-α was only observed 1 day p.i. (Figure 4a), and a down-regulation was detected 3 days p.i. in the case of IL-1β (Figure 4c). The expression of Mx protein was increased both in brain and head kidney (Figures 3e and 4e, respectively), reaching values of 1300 fold compared to controls in brain three days post infection (Figure 3e).

The pattern of expression described above for sea bream was similar to the one observed both in brain and kidney of infected sea bass. Nevertheless, the magnitude was different mainly in the case of brain, the target organ of the infection, where higher expression of TNF-α and lower expression of Mx compared with control was observed (Figure 3b and 3f).

4. Discussion

Nodavirus is an increasingly important pathogen for several marine fish species, causing mortalities mainly in larvae and juveniles due to a degenerative process in brain, retina and spinal cord. Despite of many species are affected by this disease including sea bass and sea bream, the pathogenesis and immune response of nodaviriosis is not well known so far. Innate immunity is the first line of defense in fish and other invertebrates and therefore has a relevant role after body injury or infection. Pro-inflammatory cytokines such as interleukins and tumor necrosis factors that participate in the Acute Phase Response (APR) or the effectors molecules involved in the antiviral interferon (IFN) pathway such as Mx proteins are one of the most studied.
In the present study, in contrast to what occurs with other fish viruses (Chilmonczyk et al., 1995; Tafalla et al., 1998), the *in vitro* experiments suggested that nodavirus replication in sea bream and sea bass immune system cells (head kidney macrophages and blood leukocytes) was limited or non-existent. Even when viral replication is not supported by cells of the immune system, viruses often cause an alteration of their immune functions (Stolhman et al., 1982). However, this seems not to be the case in sea bream and sea bass macrophages and leukocytes infected *in vitro*, as at least in the genes analyzed (TNF-α, IL-1β and Mx), no modulation of expression was detected (data not shown). *In vivo* studies seem to support the *in vitro* results since, although nodavirus was present in the blood and several organs 1 day post-infection, after three days, the analysis of RT-PCR products indicated low nodavirus concentration in the blood of infected sea bream but a high concentration in liver, head kidney and above all in brain. This confirmed the spread of nodaviruses through the circulating system towards the target organ for replication and development of the disease. This result indicates that the virus behaves in a similar way in both fish species reaching the brain where the viral pathogenesis is evident. The lack of susceptibility of sea bream cannot then be explained by a different ability to reach and replicate in the brain, indicating that a stronger response should be present in sea bream which confers resistance against the disease.

TNF-α is an important mediator in resistance against parasitic, bacterial and viral infections among other therapeutic roles (Aggarwal and Vilcek, 1991; Vilcek and Lee, 1991; Czarniecki, 1993; Wride and Sanders, 1995; Goldfeld and Tsai, 1996; Steinshamn et al., 1996; Krueger et al., 1998; Secombes et al., 2001). IL-1β on the other hand plays a pivotal role in the inflammatory response as initiates and/or increases a wide variety of non-structural function associated genes that are characteristically expressed during inflammation, particularly other cytokines (Dinarello, 1994; Bird et al., 2002). In this study, we observed a strong up-regulation of TNF-α expression in head kidney 1 day post-infection both in sea bream and sea bass infected with nodavirus (Figure 4a and 4b,
respectively), this up-regulation was no longer obvious 3 and 7 days after infection. This result could be explained as head kidney is the main immune organ in fish and therefore responds in the APR to fight against the infection (Dinarello, 1996; Bayne et al., 2001). In the case of IL-1β, a down-regulation was detected 3 days p.i. in both species (Figure 4c and 4d) which was not longer observed 7 days post infection. The regulation of these two cytokines in kidney in the first stages of the disease in both species could be then considered as a generalized response against nodavirus.

In the case of brain, the key organ of the disease, TNF-α and IL β over-expression in sea bream was mainly observed 3 days post-infection (Figure 3a and 3c). The fact that TNF-α was modulated in brain 3 days after infection unlike to what happened in kidney (1 day p.i.), may suggest that immune system seems to be activated in brain when nodaviruses reach their target organ and start replication, as we previously reported (Dios et al., 2007). This pattern of expression was similar to the one observed in the brain of infected sea bass. Nevertheless, the expression values for TNF-α were much higher in sea bass (more than 30 times) than in sea bream (Figure 3b). We suggest that the strong up-regulation of this pro-inflammatory cytokine in the brain of a susceptible species like sea bass, may be responsible of the vacuolization and the neuroinflammatory process associated to this disease in brain, retina and spinal cord. In fact, inflammation has been described as an important factor causing irreparable brain damage in the pathogenesis of neurodegenerative diseases and microbial infections of the nervous system (Brabers and Nottet, 2006; Kim and Joh, 2006; Lafon et al., 2006; Sutton et al., 2006; Wei et al., 2006; Ghoshal et al., 2007; Konsman et al., 2007).

The interferon system is one of the most important mechanisms for antiviral defense and the Mx proteins one of its effectors molecules best known (Meier et al., 1990; Staeheli et al., 1993; Arnheiter et al., 1996; Robertsen et al., 1997; Trobridge et al., 1997; Haller et al., 1998; Jensen and Robertsen, 2000; Haller and Kochs, 2002; Ko et al., 2002; Caipang et al., 2003; Plant and Thune, 2003; Larsen et al., 2004; Chen et al., 2006; Wu and Chi, 2006).
In the present work, an over-expression of Mx was not observed *in vitro* however, a significant up-regulation was detected in general both in brain and head kidney of sea bream and sea bass infected with nodavirus in all sampling points (Figures 3e, 3f, 4e, and 4f). These results corroborated the unequivocal participation of Mx proteins in the antiviral responses in sea bream and sea bass. Noteworthy, just like we previously described for TNF-α in brain, even when the Mx expression pattern is similar in both species, the magnitude of expression in terms of fold change values is higher in sea bream in this case (more than 1300 times) (Figure 3e). This strong up-regulation of Mx protein in the brain of sea bream with respect to the one observed in sea bass could be related to the effectiveness in solving the infection and could explain why sea bream is an asymptomatic carrier of the disease. Also, all the results taking together seem to support recent findings, in which was suggested that human neurons, although are not located in an immune organ, have the intrinsic machinery to mount robust inflammatory, chemoattractive, and antiviral responses (Lafon et al., 2006). To our knowledge, this is the first time this response in fish brain against a viral infection is described.

In summary, the results presented here for sea bream and sea bass pointed out the early activation of TNF-α and IL-1β in head kidney as a generalized response against nodavirus infection. Their expression increased 3 days after infection in brain, where the immune responses seem to be activated when nodaviruses reach the target organ and start replication. Also, TNF-α was highly over-expressed in the brain of infected sea bass, which seems to be related to the vacuolization and neurodegenerative symptoms of the disease. Mx protein was also up-regulated as an antiviral mechanism in both species but the expression level (in fold change units) in brain was higher in sea bream than in sea bass, suggesting an explanation why sea bass is a susceptible species and sea bream is an asymptomatic carrier. Moreover, these results support the fact that fish brain, in the same way that human neurons, is able of triggering a strong inflammatory response characterized by the expression of inflammatory cytokines, chemokines, and antiviral molecules.
Further studies will be conducted to elucidate another genes involved in the immune response of sea bream and sea bass against a nodavirus infection.

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References


Figure legends

Figure 1. Nodavirus titers in supernatants and cells from sea bream and sea bass at different times after in vitro infection. (A) Sea bream kidney macrophages; (B) sea bream blood leukocytes; (C) sea bass kidney macrophages and (D) sea bass blood leukocytes. Data are expressed as mean Log TCID₅₀ mL⁻¹ ± SD for 3 replicates.

Figure 2. RT-PCR nodavirus detection in control and experimentally infected sea bream tissues at different times post-infection. (A) Blood; (B) liver; (C) kidney and (D) brain. Lanes 1 and 2: control samples; lanes 3-6: infected.

Figure 3. Real time PCR results for TNFα, IL-1β and Mx expression level in the brain of intramuscular infected sea bream and sea bass. Fold units were calculated dividing the expression values of infected tissues by the expression values of the controls once normalized regarding to the β–actin expression. (A, C and E) sea bream; (B, D and F) sea bass.

Figure 4. Real time PCR results for TNFα, IL-1β and Mx expression level in the head kidney of intramuscular infected sea bream and sea bass. Fold units were calculated dividing the expression values of infected tissues by the expression values of the controls once normalized regarding to the β–actin expression. (A, C and E) sea bream; (B, D and F) sea bass.
Figure 1

(a) and (c) show the log TCID50 mL$^{-1}$ of cells and supernatants over 7 days post-infection. (b) and (d) illustrate similar data but with different scaling.

Days post-infection

log TCID50 mL$^{-1}$

- cells
- supernatants
Figure 2

a  BLOOD

b  LIVER

c  KIDNEY

d  BRAIN
Figure 3

(a) TNF-α gene expression level across 1 day, 3 days, and 7 days, showing a significant increase at 3 days.

(b) Mx gene expression level across 1 day, 3 days, and 7 days, with a significant increase at 3 days.

(c) IL-1β gene expression level across 1 day, 3 days, and 7 days, with a significant increase at 3 days.

(d) Mx gene expression level across 1 day, 3 days, and 7 days, showing a significant increase at 7 days.

(e) TNF-α gene expression level across 1 day, 3 days, and 7 days, with a significant increase at 7 days.

(f) Mx gene expression level across 1 day, 3 days, and 7 days, showing a significant increase at 7 days.
Figure 4

(a) TNF-α gene expression level over time.

(b) TNF-α gene expression level over time with error bars.

(c) IL-1β gene expression level over time.

(d) IL-1β gene expression level over time with error bars.

(e) Mx gene expression level over time.

(f) Mx gene expression level over time with error bars.
Table 1. Primer sequences of the genes analyzed.

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