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Naïve Atlantic salmon (*Salmo Salar* L.) surviving a lethal challenge with infectious pancreatic necrosis virus (IPNV) shows upregulation of antiviral genes in head-kidney, including Vig-2

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ABSTRACT

The differential gene expression profile of survivors of unvaccinated Atlantic salmon challenged by cohabitation (81% mortality) with a highly virulent strain of IPNV was studied using suppressive subtractive hybridisation (SSH). RNA was extracted from head-kidney from fish prior to IPNV challenge and 4 weeks post challenge (survivors). Five hundred and seventy six cDNA clones were randomly selected for sequencing, BLAST N and BLAST X analyses were performed. 180 unique genes were found, out of these 167 were singletons and 13 were contigs. These genes were grouped according to their putative functions using Blast2Go and TrEMBL. Ten out of 14 genes identified by SSH were confirmed by quantitative real time PCR. Representatives of almost all the biological functional groups were found, including immune and stress response, transcription, translation, metabolism and protein transport and all were upregulated in the survivor fish. Viral Haemorrhagic Septicaemia Virus induced gene 2 (Vig-2) was among the genes found to be upregulated in the survivors and this prompted us to assess the expression level of IFN-α and Mx, which were both found to be significantly upregulated, alongside inflammatory markers (P-selectin) and proteolysis genes (proteasome subunit alpha type 2). In summary the results obtained point towards innate responses being induced in surviving fish combined with factors involved in protein degradation and yet un-classified genes with regard to antiviral functions (Vig-2).

1. Introduction

Infectious pancreatic necrosis (IPN), a highly contagious disease of salmonid fish is caused by the IPN virus (IPNV), and is one of the most serious economic diseases in the aquaculture industry. IPN is a disease of salmonid species isolated from freshwater and marine sources. Closely related marine *Aquabirnaviruses* have been isolated from non-salmonid species of fish, molluscs and crustaceans throughout the world (Hill, 1982; Hill and Way, 1995). Outbreaks of IPN in Atlantic salmon (*Salmo salar* L.) are often seen in first feeding fry and soon after the smolts have been transferred to the sea. The most common clinical signs of disease are abnormal swimming, distended abdomen and darkened pigmentation. In the inner organs necrosis of the exocrine pancreas and also, to some degree in the liver are observed (Hill, 1982; Santi et al., 2004; Taksdal et al., 2009). It is believed that fish surviving an IPNV challenge become carriers of the virus (persistently infected), but without clinical signs of IPN, unless reactivation of the virus occurs, which could lead to clinical disease.

IPNV is the prototype of the *Birnaviridae* family and belongs to the genus *Aquabirnavirus*. The genome consists of two double-stranded RNA segments, segment A and segment B, which is packed into a non-enveloped icosohedral capsid of approximately, 60 nm. Segment B is monocistronic and encodes an internal polypeptide VP1, the RNA dependent RNA polymerase (RdRp), while segment A contains two open reading frames (ORFs). The single large ORF encodes three gene products, the VP2, VP3 and VP4, and VP5 is a smaller conserved ORF, overlapping the 5′ end of the VP2 gene.

Aquabirnaviruses consist of two distinct serogroups, A and B. Serogroup A consists of 9 serotypes and are all pathogenic to fish, while serogroup B consists of 1 serotype, which is avirulent. There is a high degree of genomic variation within the serogroups (Heppell et al., 1995), but also some variation in the genome of the IPNV within a serotype exists (Santi et al., 2004). These variations are also reflected in variation in pathogenicity of the different IPNV isolates (Song et al., 2005). All of the Norwegian IPNV-isolates belong to the Sp serotype and sequencing of many of these isolates has led to the identification of a highly polymorphic area in the gene coding for the VP2. At least four positions in the VP2 protein; 217, 221, 247 and 500 are polymorphic and

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the amino acids at positions 217 and 221 determine the virulence characteristics of the virus (Song et al., 2005). Based on field studies the different isolates were regarded as causing high, moderate and low mortality, and the high mortality group often had the amino acid motif TATY (NVI 015, AY379740) while the low mortality group had PTAY (NVI 016, AY379742) at these four positions (Santi et al., 2004). Typically the highly virulent strains cause mortality in the range of 80–90% while the low virulent strains cause mortality of 10% and below (Santi et al., 2004; Santi et al., 2005).

The knowledge of transcriptional response in Atlantic salmon and fish cell lines to IPNV is limited. In a previous study we used suppressive subtractive hybridisation (SSH) on CHSE cells persistently infected with IPNV and could confirm that some of the genes found upregulated in CHSE cells were indeed also upregulated in IPNV persistently infected Atlantic salmon (Marjara et al., 2010). IFN-α and Mx are also known to be upregulated in IPNV infected Atlantic salmon (Ingerslev et al., 2009; Jensen and Robertsen, 2002; Lockhart et al., 2007; McBeath et al., 2007; Robertsen, 2008; Saint-Jean and Perez-Prieto, 2007). Studies of transcriptional host response in IPNV infected Atlantic halibut and Atlantic cod (Jensen et al., 2009; Patel et al., 2009) have recently been published.

The purpose of this study was to investigate the host immune response, with emphasis on protective transcript profiles, to IPNV infection in naïve Atlantic salmon postsmolts. As little is known about the genes involved in protection against IPN in Atlantic salmon (*Salmo salar* L.), suppressive subtractive hybridisation (SSH) was used rather than targeted gene analysis to investigate and obtain a broader profile of the response. We found upregulation of genes involved in the immune and stress response/apoptosis, protein interactions and transport, metabolic processes and solute transport, transcription, transposition and replication, and the proteolysis/hydrolysis group. Fourteen genes were selected for quantitative real-time analysis relating to the characterisation of genes identified by SSH as being upregulated in response to IPNV infection. We also analysed the level of interferon-γ (IFN-γ), interferon-α (IFN-α) and Myxovirus resistance (Mx) in these fish which were all upregulated and interestingly, we found the Vig-2 gene being highly upregulated in the survivor fish. To our knowledge this is the first time Vig-2 has been found to be induced by IPNV infection. Genes functionally belonging to the proteolysis, immune response, transcription, translation, protein interaction, protein transport and the metabolic groups were also found to be significantly upregulated. Our data may help in understanding the molecular mechanisms on how the fish are protected against IPN.

2. Materials and methods

2.1. Cell work and propagation of viruses

Viruses used for challenge studies were propagated in Rainbow trout gonad cells (RTG-2, ATCC CCL-55) grown at 15 °C in Leibovitz's L-15 media supplemented with 10% foetal bovine serum (FBS), 2% L-glutamine and gentamycin 25 μg ml^{-1}. All of the viruses used in this study were recovered by reverse genetics (rNVI15r) as derivatives of a highly virulent Norwegian IPNV sp strain NVI-015 (GenBank accession nos. AY379740 and AY379741) (Santi et al., 2004; Santi et al., 2005).

2.2. Experimental fish, IPNV challenge and sampling

Atlantic salmon (*Salmo salar* L.) parr, AquaGen AS breed, of IPN susceptible families were hatched and reared at VESO's hatchery (Namsos, Norway). All the fish experiments were conducted following the code of ethics of the world medical association (Declaration of Helsinki) for animal experiments. Fish used in the current study were part of a larger experiment including a total of approx. 2400 fish. In total we had 12 different groups with 180 fish in each group; 11 different vaccine groups and one non-vaccinated control. In the present study only fish from the control group (non-vaccinated fish) were analysed and therefore the details for the other groups are not given. Fish were marked by ink jet and fin clipping to represent the control group and the virus shedders (21 fish, see details below). The water temperature throughout the experiment was 10 °C ± 1 °C. The water flow was kept to maintain a minimum of 8 mg/O_2 per ml of outlet water.

One hundred and forty one fish were kept in tanks with UV-treated fresh water and fed commercial diets (EWOS Micro, EWOS, Norway) twice a day at 1.0% of the body weight. The fish were prepared for seawater transfer using a standard light regime. In brief, the fish were kept in fresh water and exposed to 12 h darkness and 12 h daylight followed by a period with full (24 h) daylight. This induced smoltification and the fish were transferred to seawater when they carried the external signs of being prepared for seawater transfer, i.e. they were smoltified. The average weight of the fish was 121.6 g when transferred to seawater.

After sea transfer, samples were taken from 12 fish to serve as non-vaccinated and non-challenged controls while the remaining fish were divided into 3 tanks each containing 40 fish. T_0 corresponds with the time point when the fish were challenged.

Challenge was carried out by cohabitation. This was performed by injecting 21 fish from the same fish group intraperitoneally with 0.1 ml of plaque purified IPNV strain rNVI15r at a concentration of $1.0 \times 10^{7.0}$ $TCID_{50}$/ml. The injected fish were added to the 3 tanks (of 40 fish each), 7 injected fish in each tank. The injected fish served as virus shedders and were not analysed further, apart from documenting that the fish died from IPN.

Recording of mortality was done daily. At all sampling stages, the fish were anaesthetised with methiomidate before being killed with a blow to the head. The second sample collection was performed 4 weeks post challenge (4WPC) from 12 of the survivors (of 26 surviving fish in total). At this point the average mortality (of the three parallel tanks) was 81%. Head kidney tissues were preserved in RNAlater (Ambion) and stored at −20 °C, part of the head kidney from each fish was also kept in Leibovitz's L-15 media containing gentamycin 50 μg ml^{-1} and 30% glycerol. All samples were tested for IPNV both by RT-PCR (RNAlater samples) and cell-culture (L15-media samples).

2.3. Virus re-isolation and sequencing

Head kidney tissues were homogenised in a stomacher, 1:5 (w/v) in L-15 medium supplemented with gentamycin 50 μg ml^{-1} followed by centrifugation at 1200 *g* for 10 min. One hundred microliters were used for inoculation of RTG-2 cells grown on 24 well plates to final dilutions of 1 and 1:10. The plates were incubated at 15 °C for 7 days, 0.1 ml of cell culture media was transferred to new monolayers for a second passage. The second passage plates were scored for cytopathic effects (CPE) (positive if CPE) after 7 days. All head-kidney samples from RNAlater were tested for the presence of IPNV using RT-PCR. RNA extraction was carried out using the QIAamp Viral RNA Mini Kit according to the manufacturer's recommendations (Qiagen). Reverse transcription-PCR was performed using virus specific primers (see Table 1) for sequencing and for quantitative real time PCR for determining the virus load, where a known titre (10^6 $TCID_{50}$) of virus supernatant was used as a control.

2.4. Total RNA extraction

Total RNA was isolated from head kidney using the RNeasy Mini Kit (Qiagen) according to the manufacturer's animal tissue protocol, with the DNAse treatment included. RNA was quantified on the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc.). All samples were then run on the Bioanalyzer Agilent 2100 (Agilent Technologies) to assess the quality of the RNA. Equal amounts of total RNA from 7 samples from each group (Non-challenged control and survivors) were