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Immunological interactions between *Piscine orthoreovirus* and *Salmonid alphavirus* infections in Atlantic salmon

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ABSTRACT

Heart and skeletal muscle inflammation (HSMI) and pancreas disease (PD) cause substantial losses in Atlantic salmon (*Salmo salar*) aquaculture. The respective causative agents, *Piscine orthoreovirus* (PRV) and *Salmonid alphavirus* (SAV), are widespread and often concurrently present in farmed salmon. An experimental infection in Atlantic salmon was conducted to study the interaction between the two viruses, including the immunological mechanisms involved. The co-infected fish were infected with PRV four or ten weeks before they were infected with SAV. The SAV RNA level and the PD specific lesions were significantly lower in co-infected groups compared to the group infected by only SAV. The expression profiles of a panel of innate antiviral response genes and the plasma SAV neutralization titers were examined. The innate antiviral response genes were in general upregulated for at least ten weeks after the primary PRV infection. Plasma from co-infected fish had lower SAV neutralizing titers compared to the controls infected with only SAV. Plasma from some individuals infected with only PRV neutralized SAV, but heat treatment removed this effect. Field studies of co-infected fish populations indicated a negative correlation between the two viruses in randomly sampled apparently healthy fish, in line with the experimental findings, but a positive correlation in moribund or dead fish.

The results indicate that the innate antiviral response induced by PRV may temporary protect against a secondary SAV infection.

1. Introduction

Piscine orthoreovirus (PRV) was first described in association with heart and skeletal muscle inflammation (HSMI) in Atlantic salmon (*Salmo salar*) in 2010 [1]. PRV clusters within the genus *Orthoreovirus* in the family *Reoviridae*, which are non-enveloped viruses with a genome consisting of ten segments of double-stranded RNA [1–3]. Erythrocytes are important target cells for PRV, but the virus also infects cardiomyocytes and red skeletal muscle cells [4,5], and the inflammation in heart and red skeletal muscle named the disease [6]. Although PRV is detected in both clinically healthy and HSMI diseased farmed Atlantic salmon, there is a correlation between HSMI and high load of viral RNA in hearts [7,8]. The prevalence of PRV positive salmon during the production cycle varies, but the vast majority of sea reared farmed Atlantic salmon becomes infected during the production [7].

The high prevalence of PRV infection in farmed Atlantic salmon in seawater makes the likelihood for co-infections with other viruses plausible. Co-infections between PRV and *Salmon pancreas disease virus* (SPDV) and between PRV and *Piscine myocarditis virus* (PMCV) have been described [8–10]. SPDV, more commonly known as salmonid alphavirus (SAV), is the etiological agent of pancreas disease (PD) in Atlantic salmon, and it causes significant economic losses [11–14]. The abbreviation SAV is used in this study. Six subtypes of SAV have been described [15], of which subtypes 2 and 3 are endemic in Norway [16,17].

A primary PRV infection reduced the effect of a subsequent SAV infection in an experimental challenge by reducing the prevalence and severity of the SAV infection at four and ten weeks after the

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initial challenges [18]. This is in contrast with a recent finding where primary PRV infection had no effect on a secondary infection with infectious hematopoietic necrosis virus (IHNV), conducted two weeks after the PRV infection in Sockeye salmon (*Oncorhyncus nerka*) [19]. Various cross protection between viral infections in fish has been demonstrated, although with differences in challenges settings and in duration of the protection [20–29].

PRV infection in Atlantic salmon upregulates genes of the innate antiviral immune response, including the IFN cascade, and down-regulates a large part of non-immune related genes [30–32]. A study on Atlantic salmon post-smolts demonstrated a coinciding peak expression of Mx and PRV RNA in hearts [33] (PRV was called Atlantic salmon reovirus in that study). Together, these studies indicate a strong innate immune response induction by PRV infection in Atlantic salmon.

In this study, using the subtype SAV2, the mechanisms of the PRV-SAV cross-protection were targeted. We investigated the expression of innate antiviral immune genes and the SAV neutralizing titer of plasma in experimentally PRV-infected and PRV-SAV co-infected fish. To investigate if cross protection is present under farming conditions, two different datasets from farmed fish infected by the two viruses was included, and correlation analysis performed.

2. Materials and methods

2.1. Challenge trial

The samples were collected in a challenge trial described in detail earlier [18]. Briefly, seawater adapted Atlantic salmon post-smolts were challenged with PRV by cohabitation in a 1:1, shedder:cohabitant, ratio (Fig. 1). The PRV shedders were removed after four weeks and the cohabitants were distributed in two tanks. PRV cohabitants in one tank were exposed to SAV shedders at 4 weeks after PRV challenge (SAV-early), while the fish in the remaining tank were exposed to SAV shedders 10 weeks after PRV challenge (SAV-late). The PRV-SAV co-infections lasted for 6 weeks and sampling was performed regularly. In the original challenge trial both SAV subtypes 2 and 3 were studied in parallel. For clarity and relevance to field samples, only the SAV2 groups are included in the present work, and hence SAV addresses only SAV2. The challenge trial was approved by the Norwegian Animal Research Authority, and performed in accordance with the recommendations of the current animal welfare regulations: FOR-1996-01-15-23 (Norway).

2.2. Sampling

Samples from heart and spleen were collected in 0.6 ml RNA*later*™ (Ambion Inc., USA) in prefilled 1.0 ml tubes (FluidX® Ltd, UK). Blood was collected from the caudal vein on heparinized vacutainer tubes (Fisher Scientific) and centrifuged (850 × g, 10 min, 4 °C). Plasma was separated from the blood cell pellet and both were stored at −80 °C.

2.3. Gene expression analysis

RNA extraction from heart, spleen and blood cell pellet (N = 8 per time point) and subsequent RTqPCR were performed for PRV, SAV and elongation factor 1α (EF1α), using assays described earlier [34–36]. RNA quantity was determined by using a NanoDrop 2000 UV–Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNase Out (Life technologies) was added to a final concentration of 0.4 U/μl and the RNA was stored at −80 °C awaiting gene expression analysis. cDNA was synthetized from 600 ng total RNA using QuantiTect Reverse Transcription Kit (Qiagen) containing gDNA wipeout buffer according to the manufacturer's instructions. A representative sample mix was prepared for cDNA synthesis with and without addition of RT-enzyme, and used for assessing efficiency and control background contamination of genomic DNA for all assays prior to the analysis of individual samples. Quantitative PCR was performed using 15 ng (5 μl of 3 ng/μl) cDNA input per reaction. The genes targeted for expression analysis, primer and probe sequences and their origins, are shown in Table 1. For new primers targeting genes containing introns, one of the primers was designed to cross exon-exon junctions. Both Maxima SYBR Green (Thermo Scientific) with 10 μM of both primers and QuantiFast Probe (10 μM) PCR + ROX Vial kit (Qiagen) were used. The cyclic conditions were 95 °C for 10 min, then 40 cycles of 95°C/15 s, 60°C/30 s and 72°C/30 s in a Mx3005P (Stratagene, La Jolla, CA, USA) for all analyses. Melting curve analyses were performed for each SYBR-Green assay. All samples were run in duplicates on the same plate for each qPCR assay. A seven-point concentration grade standard curve (40–0.675 ng) was run for validation of the primer pairs.

2.4. SAV neutralizing assay

A SAV neutralization assay was performed for plasma samples. After thawing, the plasma samples were split into two equally large aliquots. Complement was inactivated in one aliquot by heating (48 °C for 20 min) as described by Lamas *et al* [40]. The assay was performed with chinook salmon embryo (CHSE-214) cells as earlier

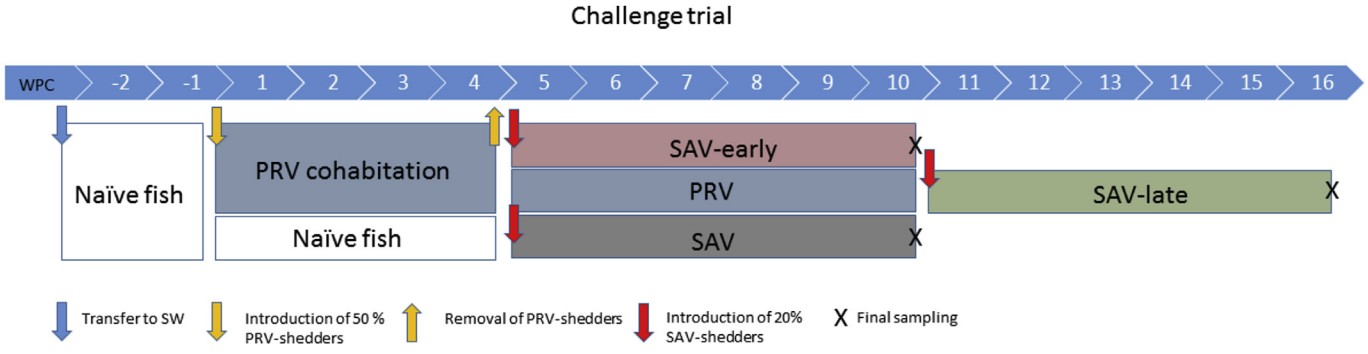

Fig. 1. The challenge trial. Timeline indicates weeks post introduction of PRV shedders (WPC-PRV). Naïve fish were transferred to seawater and acclimatized for two weeks before 50% were injected to become PRV-shedders. The PRV shedders were removed after four weeks and 20% SAV shedders were added to one tank (i.e. the SAV-early group). To the remaining PRV cohabitant group, 20% SAV-shedders were added at 10 WPC-PRV (i.e. the SAV-late group). In addition, one group was infected with only SAV, this group was called the SAV group.