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Comparison of transcriptomic responses to pancreas disease (PD) and heart and skeletal muscle inflammation (HSMI) in heart of Atlantic salmon (*Salmo salar* L)

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ABSTRACT

Pancreas disease (PD) and heart and skeletal muscle inflammation (HSMI) are viral diseases associated with SAV (salmonid alphavirus) and PRV (piscine reovirus), which induce systemic infections and pathologies in cardiac and skeletal muscle tissue of farmed Atlantic salmon (*Salmo salar* L), resulting in severe morbidity and mortality. While general features of the clinical symptoms and pathogenesis of salmonid viral diseases are relatively well studied, much less is known about molecular mechanisms associated with immunity and disease-specific changes. In this study, transcriptomic analyses of heart tissue from PD and HSMI challenged Atlantic salmon were done, focusing on the mature phases of both diseases at respectively 28–35 and 42–77 days post infection. A large number of immune genes was activated in both trials with prevalence of genes associated with early innate antiviral responses, their expression levels being slightly higher in PD challenged fish. Activation of the IFN axis was in parallel with inflammatory changes that involved diverse humoral and cellular factors. Adaptive immune response genes were more pronounced in fish with HSMI, as suggested by increased expression of a large number of genes associated with differentiation and maturation of B lymphocytes and cytotoxic T cells. A similar down-regulation of non-immune genes such as myofiber and mitochondrial proteins between diseases was most likely reflecting myocardial pathology. A suite of genes important for cardiac function including B-type natriuretic peptide and four neuropeptides displayed differential expression between PD and HSMI. Comparison of results revealed common and distinct features and added to the understanding of both diseases at their mature phases with typical clinical pictures. A number of genes that showed disease-specific changes can be of interest for diagnostics.

1. Introduction

Pancreas disease (PD) and heart and skeletal muscle inflammation (HSMI) are viral diseases that affect farmed Atlantic salmon (*Salmo salar* L) during the sea water phase. PD was first described in Scotland in 1976 [41] and the first case in Norway was detected in 1989 [47]. PD is caused by an alphavirus named Salmon pancreas disease virus or salmonid alphavirus (SAV) [32] and characterized by damage of pancreas and severe cardiac and skeletal myopathies [12,36,47,54]. The first case of HSMI was reported in Norway in 1999 [23]. HSMI is diagnosed by the detection of inflammatory lesions in cardiac and skeletal muscle [24]. Under experimental conditions, epi- and myo-carditis develop gradually during six to eight weeks [25]. Piscine orthoreovirus (PRV) has been associated with HSMI [34,44]. Moreover, PRV viral loads have been correlated with the severity of disease and an association between PRV particles and cardiac histopathology has been presented [13].

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However, high prevalence of PRV in healthy salmon [33] suggests that development of disease may require an additional trigger. Thus, the causative relationship between PRV and HSMI needs further confirmation. At present, both PD and HSMI are major concerns to commercial Atlantic salmon aquaculture. Although mortalities are relatively low in comparison with acute viral diseases such as infectious salmon anaemia (ISA), PD can result in reduction of growth and affect feed conversion and product quality thereby giving heavy losses. Moreover, detection of SAV at a facility can result in a decision of stamping out to prevent further spread of the disease [17]. Survivors may become asymptomatic carriers of infection [2]. Vaccination against PD in marine farms of Norway has not provided complete protection but has reduced the number of outbreaks and mortality [5]. Resistance to PD can also be improved by means of selective breeding [43]. At present, feed additives and functional feeds are the only available measures to alleviate HSMI pathology [1,35].

Viral diseases induce large-scale gene expression changes with both common and pathogen-specific features. RNA viruses infecting Atlantic salmon can stimulate a rapid and strong innate immune response activated by pathogen recognition receptor (PRR) signalling and amplified by the type I IFN pathway. A suite of virus responsive genes (VRG) was identified in Atlantic salmon by targeted cloning (reviewed in Refs. [48,49]. Use of transcriptomic tools further revealed a large group of VRG induced by all viral pathogens and synthetic double stranded RNA [26,58]. The innate antiviral responses are highly similar and differ only by the magnitude, which is determined by the viral loads. Much less is known about disease-specific gene expression changes. Adaptive immunity may show greater diversity than the type I IFN responses. For example, Atlantic salmon challenged with ISA developed similar early innate responses, while expression of B cell-specific genes was markedly different in fish with early and late mortality [20]. Furthermore, Atlantic salmon with high and low resistance to cardiac myopathy syndrome (CMS) showed discernible T cell responses that closely correlated with cardiac pathology [57]. One may expect even greater differences in the acquired immune responses to diverse pathogens. Given that each pathogen produces characteristic lesions, differences can also be predicted in the inflammatory pathways and non-immune genes. Better understanding of disease-specific features and molecular mechanisms of pathology is of high importance for precise diagnostics and development of appropriate strategies for prevention and mitigation. Until present, most studies have addressed one viral disease affecting Atlantic salmon at a time and examined different immune parameters and genes, making the results difficult or impossible to compare. Herein, a common oligonucleotide microarray platform was used for gene expression profiling in samples from two controlled trials and analyses were designed with focus on disease comparison. Challenge models to study PD and HSMI are different with respect to the routes of infection and time-course. Therefore it was prioritized to compare these diseases at their mature stages with fully manifested lesions. Analyses that included individuals with high viral loads and characteristic cardiac pathology provided a comprehensive survey of host–pathogen interactions and revealed both common and specific features.

2. Materials and methods

2.1. Challenge trials

The experimental HSMI and PD challenges were performed at Tromsø Aquaculture Research Station (Troms, Norway) and unvaccinated Atlantic salmon smolt (Aquagen standard, Kyrksæterøra, Norway) was used. The fish were treated according to Norwegian legislation and anesthetized prior to handling with Benzoak (benzocaine; 0.1 mg/ml) or euthanized with an overdose of Benzoak prior to all samplings.

2.1.1. HSMI challenge trial

2.1.1.1. Fish and inoculum intraperitoneal injection challenge. The fish, with a mean weight of approximately 50 g at time of challenge were kept in tanks supplied with running sea water at 10 °C and 24 h light/0 h dark regime and fed based on appetite with commercial dry feed (Skretting, Norway). Before challenge, 10 fish was tested and found negative for infectious pancreatic necrosis virus (IPNV) in a carrier test, performed as described earlier [19]. The infection material originated from a field outbreak of HSMI as determined by histopathological examination and quantitative Real Time RT-qPCR (RT-qPCR) showing high loads of PRV. The material was confirmed free of infectious salmon anaemia virus (ISAV), salmonid alphavirus (SAV), piscine myocarditis virus (PMCV) and infectious pancreatic necrosis virus (IPNV) by RT-qPCR. The inoculum was prepared by homogenizing half hearts from 14 fish and mean Quantification cycle (Cq)-value was 25.7 for PRV. A total of 47 fish were injected intraperitoneal (i.p.) with 0.1 ml inoculum. Tissue samples from 6 individuals per time point were harvested at 35, 42, 49, 56, 63 and 77 days post infection (dpi) in RNAlater (Ambion, Applied Biosystems, USA) and stored according to the manufacturers guidelines. Sampling points were chosen based on the expected disease development under experimental conditions [39]. Parallel samples were harvested in 10% phosphate-buffered formalin (pH 7.0), embedded in paraffin and prepared for histological analyses. Control samples from 8 uninfected fish were sampled at time of infection (0 dpi).

2.1.1.2. RNA isolation from heart tissue and quantification of PRV by RT-qPCR. Total RNA was extracted by first homogenizing equal sized tissue samples using MagNA Lyser Green Beads and the MagNa Lyser Instrument (Roche Diagnostics GmbH, Mannheim, Germany) and then purifying the RNA using the ABI Prism 6100 Nucleic Acid Prep Station (Applied Biosystems) with the recommended on-column DNAse treatment (NanoDrop Technologies, Rockland, DE, USA). RNA integrity was verified by the 2100 Bioanalyzer in combination with an RNA Nano Chip (Agilent Technologies), and RNA purity and concentrations were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). Total RNA was stored at −80 °C until use. Super Script VILO cDNA synthesis kit (Invitrogen) was used for cDNA synthesis following the manufacturer's instruction. RNA input was 400 ng per reaction. Obtained cDNA was diluted 1:10 in nuclease free water (Ambion) and stored at −20 °C before use. The cDNA was further diluted for use in Real Time-qPCR. Absence of genomic DNA in the RNA (Non-template control; NTC) was verified by a selection of samples being subjected to RT-qPCR without prior cDNA synthesis. To determine the relative amount of PRV virus in heart samples, RT-qPCR was performed by running 20 μl reactions in duplicates in 384 well plates using the 7900HT Fast real-time PCR system and Power SYBR green PCR master mix (Applied Biosystems). Primers used were for the L1 segment of PRV, a kind gift from Torstein Tengs, Norwegian Veterinary Institute, Oslo, Norway. Elongation factor 1α (EF1α) (Table 1) was analysed as a reference [21]. Gene expression data were analysed with the SDS 2.3 software.

2.1.1.3. HSMI histopathology. HSMI induced heart lesions were evaluated from heart tissue harvested at 42, 49, 56, 63 and 77 dpi. Each of the heart ventricle layers: epicardium, compactum and spongiosum were scored separately on a scale from 0 (no changes) to 3 (severe changes). HSMI was diagnosed when the combined score per fish was >4. Samples were blinded prior to analysis.