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Short communication

Expression of interferon and interferon – Induced genes in Atlantic salmon *Salmo salar* cell lines SHK-1 and TO following infection with Salmon AlphaVirus SAV

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ABSTRACT

Salmon AlphaVirus (SAV) is the aetiological agent of Salmon Pancreas Disease (SPD), a serious disease in farmed Atlantic salmon. Currently there is no available information on the ability of this virus to stimulate or suppress aspects of innate immunity in host cells. Two different Atlantic salmon cell lines (SHK-1 and TO), both derived from head kidney leucocytes, were infected with SAV and the kinetics and magnitude of gene expression were studied by real-time quantitative PCR. SAV *nsP1* gene transcripts for strain P42P increased rapidly in TO cells with subsequent development of a cytopathic effect (CPE) while this virus strain hardly replicated at all SHK-1 cells causing no CPE. SAV P42P induced strong expression of type I *IFN* (*IFN*) and the antiviral *IFN*-induced gene *MX* transcripts in SHK-1 cells. Although the *IFN* response in infected TO cells was higher than in SHK-1 cells, the level of *MX* transcripts was lower. This may be because the virus was able to interfere with *IFN*-signaling and suppress *MX* transcription or that the TO cells are less able to transcribe the *MX* gene. Either way, it may account for why the SHK-1 cells suppress SAV replication while the TO cells are highly susceptible and succumb to the virus.
The present results provide the first evidence for differential induction of expression of the interferon-induced antiviral gene, *MX*, correlating with resistant (SHK-1) and susceptible (TO) Atlantic salmon cell lines in response to infection by SAV.

1. Introduction

Pancreas disease (PD) in farmed Atlantic salmon *Salmo salar* L. was first recognized in Scotland in 1976 [1–6], and has continued to cause serious economic losses in Europe. This disease is caused by a Salmon AlphaVirus (SAV) subtype 1 [7,8]. The pathology is characterized by a complete necrosis of the pancreatic acinar tissue, but heart and muscle lesions have also been noted [1,9]. Recovered fish develop a strong and long lasting immunity to the disease [10] and neutralizing antibodies have been detected [11] but currently there is no information available about the innate immune responses to this virus.

Teleost fish are armed with an innate antiviral defense system based on interferon (*IFN*) production [12], which represents the first line of defense against viral infective agents such as Infectious Pancreatic Necrosis Virus (IPNV) and Infectious Hematopoietic Necrosis Virus (IHNV) [13]. *IFN* molecules are typically secreted by nucleated cells in response to a viral infection. A rapid signaling pathway induces the expression of a number of proteins, including *MX*, with direct and indirect antiviral properties [14]. There are two classes of *IFN*s; type I (α/β *IFN*) produced by all cell types, and type II (γ*IFN*) which is produced by more specialized immune cells. In higher vertebrates, type II *IFN* is generally accepted to be part of adaptive immunity, whereas type I *IFN* is an important mediator of the innate response. Most viruses are equipped with a diverse array of strategies to evade this powerful early cellular defense mechanism [15], often involving specially encoded proteins such as the non-structural (NS) protein of influenza A virus. In fish, it has been well demonstrated that type I *IFN* can rapidly induce expression of *MX* which has been shown to have antiviral activity against IPNV [16]. Presently there is no information available on the interaction between SAV and the induction of the *IFN* system which may play a role in determining states of resistance/susceptibility.

In the present study, a time-course sampling and real-time PCR was done to measure the induction of several Atlantic salmon innate immune genes following *in vitro* infection of two Atlantic salmon cell lines with SAV. The cell lines used, SHK-1 and TO cells

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were both developed from adherent Atlantic salmon head kidney (HK) leucocytes in attempts to produce cell lines to use in the propagation of Infectious Salmon Anemia (ISA) virus. The SHK-1 cells had some characteristics of macrophages, e.g. they were positive for acid phosphatase, non-specific esterase and had the ability to phagocytose the bacterium *Aeromonas salmonicida*, but lacked the ability to kill the bacteria [17]. The TO cells have not been well characterized as to their nature or lineage but are claimed to be a better host than SHK-1 cells for replication of ISAV [18] and SAV [8]. However, SHK-1 is sensitive to ISAV and is also used in ISAV viral diagnostics [19,20]. They have recently been shown to have a high phagocytic capacity and bactericidal function [21]. Preliminary work on culture of SAV (unpublished) showed that SHK cells were resistant (showed no CytoPathic Effect, CPE), while TO cells developed a CPE at day 7 progressing to complete CPE by day 14, in agreement with results from Graham et al. [8]. In an attempt to elucidate the differences in susceptibility of these cell lines to SAV in terms of *IFN* and *IFN*-induced antiviral responses and to further elucidate the nature of the cell types, the expression of a number of available salmon immune genes was studied post-infection. Host factors measure included Translation Elongation Factor 1 α (*ELF*) as the reference house-keeping gene, type I interferon (α/β *IFN*, will be referred as *IFN*), the antiviral *IFN*-induced gene *MX*, and a marker of type II *IFN*, Gamma Interferon-Induced 10 kDa Protein (γIP, will be referred as gIP), while the gene encoding nonstructural Protein 1 (*nsP1*) was measured to assess viral replication.

2. Materials and methods

Atlantic salmon head kidney cell line SHK-1 (passage no. P64, European Collection of Cell Cultures, ECACC 97111106) derived from a macrophage-enriched culture [17] and the TO cell line (passage no. P95) from head kidney leucocytes [18] were propagated in Leibovitz L-15 supplemented with 5% foetal bovine Serum (FBS), 0.08% 2-mercaptoethanol and HMEM supplemented with 5% FBS, 1% 200 mM L-glutamine, and 1% non-essential amino acids, respectively. Stock cultures were incubated at 20 °C for 7–21 days. Cell mono-layers in 25 cm^2 flasks and in the above media, that were between 50 and 60% confluent and 24–48 h old, were used for this study.

SAV subtype 2 P42P [22] were propagated in CHSE-214 cells for 7 days as previously described in Ref. [23]. The supernatant was harvested and stored at −80 °C until use. The virus titre was 1.11×10^8 $TCID_{50}$ ml^{-1}. SHK-1 and TO cell mono-layers were challenged with $1.11 \times 10^{6.5}$ $TCID_{50}$ SAV ml^{-1} and incubated at 15 °C. On days 0, 2, 3, 4, 7, 10 and 14 the cells in 3 flasks of infected or un-infected cells (control) were washed with phosphate buffered saline (PBS), lysed with trypsin EDTA, centrifuged, the pellets drained and stored at −80 °C prior to RNA extraction.

RNA was extracted from the infected cell cultures using the RNeasy® Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA was eluted in 50 μl RNase-free dH_2O. RNA was reverse transcribed to cDNA using the TaqMan® Reverse Transcription Reagent kit (ABI) with oligo-$d(T)_{16}$ (for host genes) or random hexamers (for SAV *nsP1*) as follows: 9.6 μl of total RNA (approx. 0.5 μg) and 1.25 μl 50 μM oligo-$d(T)_{16}$ or random hexamers were mixed and heated to 70 °C for 10 min and chilled on ice. The final volume was adjusted to 25 μl by adding Master mix comprised of the following: 1x RT buffer (25 mM Tris–HCl pH 8.3, 37.5 mM KCl, 5.5 mM $MgCl_2$), 0.5 mM each dNTP, 0.4 U RNase inhibitor and 1.25 U Multiscribe Reverse Transcriptase. Both Random hexamers and oligo-$d(T)_{16}$ reactions were incubated at 48 °C for 90 min, heat inactivated at 95 °C for 5 min and stored at −80 °C until use.

Real-time PCR assays were performed on an ABI 7000 Sequence Detection System (Applied Biosystems). TaqMan® probes to amplify the Elongation factor 1 α gene (*ELF*), *MX*, type I *IFN* (*IFN*), γ*IFN*-induced Protein (γIP) and SAV nonstructural protein P1 (*nsP1*) genes are presented in Table 1. One microlitre cDNA was added to the following mix contained in individual wells of a 96-well optical plate (Applied Biosystems): 10 μl of TaqMan® 2x PCR mix with UNG (Applied Biosystems), 8 μl of dH_2O and 1 μl of a 20x mix containing forward primer (18 μM), reverse primer (18 μM) and probe (5 μM). The standard cycling conditions were 50 °C for 2 min, 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. The fluorescence output for each cycle was measured and recorded upon the completion of the entire run. Absolute quantification of transcripts was carried out. The efficiencies of the primers and probes were tested using a 10-fold serial dilution of known template (either cDNA or plasmid) to produce a standard curve. The cycle threshold (C_t) values were converted into expression values normalised against the reference gene, *ELF*, using the statistical standard curve method as described in the Applied Biosystems User Manual.

Data were analysed using Minitab software package for Windows (Minitab, Inc.). Effect of time was tested by one-way analysis of variance and level of expression at a given time point was compared to the initial time point by Tukey's multiple comparisons.

Table 1
Sequences of primers and probes used in real-time PCR analysis. The probes are labelled with 6-FAM in 5′ and MGB in 3′ (AMI). The column "Binds" indicates which strand (+ = coding; − = non-coding) the primer or probe anneals to. F, forward primer; R, reverse primer; P, probe.

Gene	Genbank AN	Name		Sequence 5'–3'	Binds	Size (bp)
Translation elongation factor 1 α	AF321836	*ELF*	F	CCCCTCCAGGACGTTTACAAA	−	57
			P	6-FAM-ATCGGTGGTATTGGAAC-MGB	−	
			R	CACACGGCCCACAGGTACA	+	
Mx1 and *Mx2*	U66475	*MX*	F	GATGCTGCACCTCAAGTCCTATTA	−	73
	U66476		P	6-FAM-CAGGATATCCAGTCAACGTT-MGB	−	
			R	CGGATCACCATGGGAATCTGA	+	
Type I interferon 1 and 2	AY216595	*IFN*	F	ACTGAAACGCTACTTCAAGAAGTTGA	−	104
	AY216594		P	6-FAM-CTGTGCACTGTAGTTCATTT-MGB	+	
			R	AGGAAAGAGACAAAACGTCATCTGC	+	
Gamma interferon-induced 10 kDa protein (IP10, gIP)	AY795563	IP10	F	CCAAACACTCCTGCCAGAACAT	−	65
	AJ841811		P	6-FAM-CAGAGTGACAATGATCTCC-MGB	+	
			R	ACACTTCATCCCTTTTCCGTTCTT	+	
Salmon AlphaVirus (SAV) nonstructural Protein 1	AY604235	*nsP1*	F	CCGGCCCTGAACCAGTT	−	107
			P	6-FAM-CTGGCCACCACTTCGA-MGB	+	
			R	GTAGCCAAGTGGGAGAAAGCT	+	