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Microarray Analysis in the Liver and Kidney of Atlantic halibut (*Hippoglossus hippoglossus*) following Immunostimulation with a Commercial Vaccine Against *Vibrio (Listonella) anguillarum* and *Aeromonas salmonicida*

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Abstract

The response of Atlantic halibut (*Hippoglossus hippoglossus*) to immunostimulation with a commercial vaccine was investigated using an Atlantic halibut cDNA microarray. In this study fish were immunostimulated with the commercial injectable vaccine and held at 10-12°C. At 1, 2, 7, 14 days post-immunostimulation liver and kidney tissues were collected and mRNA was purified and used for microarray, quantitative real time-PCR (qRT-PCR) and reverse transcription-PCR (RT-PCR) analysis. Of the 381 genes on the microarray, we identified 83 different genes that were differentially expressed (≥2-fold change, $p<0.05$) in at least one of the samples: 44 in liver, 48 in kidney and 8 in both. Of those in the liver, 28 were significantly up-regulated, of which the anti-microbial peptide hepcidin type I was the most prominent. Alternately, 16 genes were significantly down-regulated, including anti-trypsin alpha 1 and alpha-2-HS-glycoprotein (fetuin A). In the kidney, 27 genes were significantly up-regulated, including MHC class I A, hepcidin type I, cadherin 1-D (CDH1-D), cathepsin L, and leukocyte elastase inhibitor. Some of important genes directly related to the adaptive immune system were up-regulated ranging between 1.5-2.0 fold. This includes immunoglobulin light chain (Ig-Lc), MHC class II, MHC, class II-associated invariant chain and recombination activating gene-1 (RAG-1). Another 21 genes were significantly down-regulated, including thyroid receptor interacting protein and cathepsin D. We also found, using qRT-PCR, that pro-inflammatory cytokine TNFα-1 was also significantly up regulated in the kidney.

Our data revealed that immunostimulation with a vaccine against Gram-negative bacteria alters the expression of a wide range of other functional genes as well as cell/organism defence-related genes in the early stages of stimulation. Genes related to the innate immune system, including acute phase responsive genes, were significantly differentially expressed in the liver. Genes directly related to the adaptive immune system were, however, slowly differentially expressed in the kidney.

Keywords: Atlantic halibut; Immunostimulation; Vaccine; Microarray; *Vibrio (Listonella) anguillarum; Aeromonas salmonicida*

Introduction

The Atlantic halibut (*Hippoglossus hippoglossus*) has great potential as an aquaculture species due to its high market value and demand [1]. To ensure the success of commercial scale halibut culture we must improve our understanding of the immune response of Atlantic halibut in order to develop effective disease management strategies and tools. There is some information available on humoral immune parameters of Atlantic halibut including immunoglobulin concentration, non-specific antibody activity, haemolytic activity, lysozyme activity, antiprotease activity, iron binding capacity and bactericidal activity of serum [2-4]. However, little is known about a large-scale gene expression of the immune system including how they respond to vaccination and/or to pathogen exposure. The bacterial pathogens atypical *Aeromonas salmonicida* and *Vibrio anguillarum* are often responsible for serious disease outbreaks in farmed Atlantic halibut [1]. As part of our studies on the immune system of Atlantic halibut we generated an EST library from liver, kidney, and spleen of Atlantic halibut that were vaccinated with a commercial vaccine against *Aeromonas salmonicida* and *Vibrio* anguillarium [5]. From this library we identified 182 clones that contained cell/organism defence genes including immunoglobulin light chain, MHC class I and II, interferon consensus sequence binding protein, B-cell receptor –associated protein, early B-cell factor, CC chemokine (similar to MIP-1ß), 10 complement components, heat shock protein 70 and 90, and antimicrobial peptides hepcidin type 1 and 2 [5]. Using these clones and five other clones from our genomic and single gene research, we produced an Atlantic halibut cDNA microarray that contains 381 different cDNA clones including cell/organism defence genes as well as clones of gene involved in cell signalling/cell communication, cell structure/motility, gene/protein expression, metabolism and unknown functions. cDNA microarrays are a recently developed tool that allows for the simultaneous quantification of expression of large numbers of genes from specific tissues or under specific host conditions including infection [6,7]. Recent studies that have used microarrays to examine the fish immune responses against viral hemorrhagic septicemia (VHS) [8], mitogen stimulation and hirame rhabdovirus (HRV) [9], infection with *Piscirickettsia salmonis* [10], challenge with *Aeromonas salmonicida* [11], polyribocytidylic acid (pIC) stimulation [12] and cytokine stimulation [13] have shown the utility of this method to study fish immune responses.

We were interested in innate and adaptive immune responses of cold-water flatfish such as the Atlantic halibut. In this study, we examined the large-scale transcriptional response of Atlantic halibut following immunostimulation with a commercial vaccine against *Vibrio anguillarum* and *Aeromonas salmonicida*. Expression of a portion of these genes was confirmed by qRT-PCR and RT-PCR. A commercial vaccine was used to ensure consistency of antigens between this and other studies [5]. In conclusion, Atlantic halibut showed both innate and adaptive immune responses after immunostimulation against a commercial vaccine; however the up-regulated levels of adaptive immune related genes, such as RAG-1 and Ig-Lc, were comparatively low.

Materials and Methods

Tissue preparation and RNA isolation

Atlantic halibut obtained from Scotian Halibut Ltd. were maintained at 10°C at the Institute for Marine Biosciences, National Research Council of Canada. Fifteen juveniles (average 225 grams) were intraperitoneally injected with a commercial, oil in water adjuvanted vaccine (MULTIVaCC$^{3}_{TM}$, Microtek International Inc., Saanichton, Canada) against *Vibrio anguillarum* and *Aeromonas salmonicida* using the dose (0.4 ml) recommended for the immunization of Atlantic salmon (*Salmo salar*). An equal number of control fish were injected with an equivalent volume of phosphate buffered saline (PBS). Immunostimulated and control fish both fed equally throughout experiment. In order to obtain the highest number of transcripts related to both innate and adaptive immune systems, liver and anterior kidney tissues were obtained from 3 individuals at 1, 2, 7, and 14 days post-immunostimulation (DPI). These samples were preserved in RNA Later (Ambion, Austin) for RNA extraction.

Equal amounts (100 mg) of tissue from 3 individual fish at each time point were combined to isolate tissue-specific total RNA. Total RNA was purified from individuals using TRIZOL (life technologies, Carlsbad) according to the manufacturer's instructions, and then pooled. Messenger RNA was purified from pooled total RNA using a commercial mRNA purification kit (Stratagene, La Jolla). The integrity and quantity of mRNA were determined using formaldehyde RNA gel and the Ultra Spec 2000 spectrophotometer (Phamacia Biotech, Piscataway) respectively.

Construction of cDNA Microarray

The Atlantic halibut cDNA microarray was constructed using clones obtained from an EST (expressed sequence tag) library from vaccinated Atlantic halibut. Three hundred and eighty one clones that included inserts of genes with a wide range of known and unknown functions were selected from a total of 1072 clones (Table 1) [5]. Selected bacterial clones were grown in Luria-Bertani (LB) broth overnight at 30^{0}C, shaking at 200 rpm and one microlitre of the overnight growth was used for PCR-based amplification, using vector arm primers: T3 and T7. PCR cycling parameters included an initial denaturation step of 15 min. at 95 ^{0}C followed by 35 cycles of a denaturation/annealing/extension (94 ^{0}C, 30 sec/55 ^{0}C, 30sec/72 ^{0}C, 2.5 min.) and a final extension at 72 ^{0}C for 5 min. Amplified

Table 1. Differential expression of Atlantic halibut genes after immunostimulation with a commercial vaccine against *Vibrio anguillarum* and *Aeromonas salmonicida*. The shaded box indicates more than 2-fold changes in gene expressions. The symbol (‡) indicates that values are statistically not significant.

Accession No	Description	Transcription Fold Change							
		Liver				Kidney			
	Days post-vaccination	1	2	7	14	1	2	7	14
Cell/Organism defence									
CF931746	antitrypsin-alpha 1 precursor	-1.4	-2.2	-1.4	-2.0	-1.2	1.2	1.5	1.5
CN655597	Carbonmonoxy Hemoglobin, chain A	1.1	-1.3	1.3	1.0	1.5	1.2	2.0	1.4
CN655696	complement regulatory plasma protein SB1	2.0	1.4	1.2	-1.1	1.2	-1.3	1.0	-1.2
CN655701	cytochrome P450 monooxygenase	1.4	3.3	10.2	11.7	1.0	1.0	1.1	-1.3
CN655580	haptoglobin fragment 1	18.5	9.0	‡3.3	2.9	1.3	1.5	1.9	1.3
CF931737	hepcidin antibacterial peptide type I	157.9	9.2	‡2.0	14.6	2.2	1.8	2.0	1.5
CN655667	hepcidin antibacterial peptide type II	2.5	1.1	1.3	1.2	1.2	1.6	1.4	1.0
CN655588	Major Histocompatibility Complex class I A	4.4	1.8	-1.2	1.4	2.3	1.2	1.3	1.2
CN655606	Major Histocompatibility Complex class I	2.2	1.0	1.0	1.1	3.7	1.4	1.2	1.6
CN655656	transferrin	2.0	1.3	1.5	1.5	-1.2	1.5	1.7	1.2
Cell/Organism defence (no significant differential expression)									
CF931881	alpha-2-macroglobulin receptor (LRP1)	-1.2	1.0	-1.2	1.2	1.0	-1.2	1.0	-1.2
CF931751	alpha-2-macroglobulin-1	-1.3	-1.4	-1.5	1.2	1.5	1.3	1.5	1.1
CN655585	Apolipoprotein A-I precursor	-1.4	1.0	-1.8	-1.3	1.5	1.9	1.7	1.1
CF931943	B-cell receptor-associated protein 37	1.1	-1.3	-1.1	1.0	-1.2	1.3	-1.5	-1.1
CN655665	C1q-like adipose specific protein	1.3	-1.4	-1.4	-1.1	1.7	1.1	1.4	1.1
CF931817	ceruloplasmin	1.7	1.3	1.1	1.1	-1.3	1.3	1.5	1.4
CN655672	ceruloplasmin	1.7	1.2	1.5	1.1	1.1	1.8	-1.2	-1.2
CF931876	complement BF/C2	1.8	1.7	1.0	1.1	1.2	1.1	1.2	-1.1
CN655676	complement binding protein	1.4	1.2	1.0	1.3	1.0	1.5	1.2	-1.3
CN655645	complement component C5	1.5	1.4	1.7	1.6	1.0	1.2	1.4	1.2
CN655652	complement component C5	1.2	1.2	1.3	1.2	1.5	1.3	1.9	1.4
CN655688	complement component C3	1.2	-1.2	-1.2	-1.2	1.0	-1.6	1.0	1.0
CN655723	complement control protein factor I-B	1.0	-1.2	-1.1	-1.3	-1.3	-1.4	-1.2	1.0
CN655729	complement control protein factor I-B	1.5	1.7	1.6	1.0	-1.2	1.2	1.2	1.1
CF931877	complement factor B/C2-B	1.4	1.1	-1.1	1.3	1.9	1.6	-1.1	-1.3
CN655618	complement regulatory plasma protein	1.8	1.5	-1.3	1.0	1.2	1.2	1.5	1.0
CN655668	complement regulatory plasma protein SB1	1.8	1.2	1.4	1.1	1.9	1.4	1.2	1.0
CN655687	early B-cell factor	-1.2	-1.3	-1.1	-1.1	1.0	-1.2	-1.2	1.2
CN655623	Ferritin, heavy subunit	1.0	-1.2	-1.1	1.2	1.4	1.2	1.2	1.0
CF931932	Heat shock cognate 71 kDa protein (Hsc70.1)	-1.4	-1.1	-1.1	-1.1	1.0	1.0	1.1	-1.3
CN655590	heat shock protein 70	-1.3	-1.4	-1.4	-1.3	1.5	1.0	1.3	1.1
CN655682	heat shock protein 90 beta	-1.4	-1.7	-1.5	1.4	1.4	1.4	-1.2	-1.3
CN655718	heat shock protein 90 beta	-1.1	-1.3	-1.4	-1.3	1.6	1.3	-1.1	1.1
CF931951	immunoglobulin light chain constant region	1.0	-1.1	1.0	-1.3	1.0	-1.1	1.1	1.0
CN655619	immunoglobulin light chain L2	-1.4	-1.2	-1.3	1.2	1.6	1.2	1.5	1.3
CF931977	immunoglobulin light chain precursor	1.0	1.1	1.4	1.6	1.6	1.0	1.3	1.1
CN655722	interferon consensus sequence binding protein	-1.1	-1.2	-1.1	-1.1	1.0	1.4	-1.1	1.0
CN655712	Kininogen, HMW I precursor	-1.3	-1.6	-1.5	1.0	-1.2	1.0	-1.3	1.2
CF931747	Kininogen, LMW II precursor	1.0	-1.6	-1.2	-1.3	1.1	1.3	1.6	1.1
CF931782	macrophage lectin 2 (calcium dependent)	1.0	-1.1	1.0	1.1	1.7	1.8	1.6	1.2

CF931837	MHC class II alpha	-1.6	-1.1	1.0	1.0	1.2	1.2	1.6	1.2
CN655713	MHC class II beta	-1.2	-1.4	-1.1	1.2	1.4	1.4	-1.3	-1.1
CF931778	MHC, class II-associated invariant chain	1.0	-1.1	1.0	1.2	1.8	1.4	1.3	1.5
CF931982	Macrophage Inflammatory Protein-1beta	-1.1	1.0	-1.1	1.1	1.0	1.0	1.1	1.0
CN655671	molluscan defence molecule precursor	-1.3	1.0	1.0	1.0	1.4	1.0	1.0	1.2
CN655655	plasma protease (C1) inhibitor precursor	1.1	-1.2	1.0	1.0	-1.5	1.3	1.7	1.3
CN655646	precerebellin-like protein	1.2	-1.6	-1.3	-1.4	1.0	1.1	1.6	1.2
CF931940	proteasome activator subunit 2	1.0	1.0	1.0	1.0	1.4	-1.1	1.1	-1.3
[c]N/A	recombination activating gene 1	1.0	-1.1	1.0	1.5	-1.1	1.1	1.3	1.5
CN655629	serine (or cysteine) proteinase inhibitor	-1.2	1.0	-1.1	1.0	1.4	1.2	1.7	1.3
CF931900	small inducible cytokine subfamily A	1.5	1.0	1.1	1.3	1.3	1.4	1.0	1.0
CF931748	suppression of tumorigenicity 13[a]	-1.3	1.1	1.6	1.3	1.3	1.3	1.6	1.0
[c]N/A	Interleukin-1β	1.0	1.2	1.1	-1.1	-1.3	1.0	1.0	1.1
[c]N/A	Transforming Growth Factor-β	1.0	1.1	1.0	1.0	1.0	1.0	1.2	1.0
[c]N/A	Tumor Necrosis Factor-α	-1.4	1.0	-1.1	-1.1	1.0	1.0	1.0	1.1
Cell signaling/Cell communication									
CN655603	alpha-2-HS-glycoprotein (Fetuin-A)	-2.0	-1.7	-1.4	1.2	1.3	1.4	1.7	1.4
CF931851	alpha-2-HS-glycoprotein (Fetuin-B)	-1.7	-1.9	-1.2	1.0	1.2	1.4	1.1	-1.2
CN655605	Cadherin 1-D (CDH1-D)	-2.7	1.0	1.0	1.2	2.5	1.5	‡2.7	‡2.5
CF931970	leucine-rich alpha-2-glycoprotein	3.6	2.0	1.4	1.5	1.2	-1.4	-1.1	-1.1
CF931886	S100-type calcium binding protein A14	-1.1	-1.1	-1.4	1.0	2.1	1.4	1.2	1.1
CF931878	Tetraspanin 47F	1.3	1.0	-1.2	1.3	1.2	1.2	-1.5	-2.2
CN655608	thyroid receptor interacting protein 12	5.8	2.2	-1.3	1.3	1.1	1.9	-3.1	1.0
Cell structure/Motility									
CN655630	beta-cytoplasmic actin2	1.0	1.3	1.1	1.1	2.4	‡2.0	‡3.3	2.0
CF931753	Fibrinogen-like protein A precursor	2.2	1.2	1.3	-1.3	1.5	1.3	1.5	1.2
Gene/Protein expression									
CN655651	18S ribosomal RNA gene	-1.3	1.2	1.8	2.1	1.9	1.4	1.1	1.1
CF931820	28S ribosomal RNA	‡2.0	17.1	27.9	34.5	-1.1	1.1	1.4	1.4
CN655631	40S ribosomal protein S17	-1.2	1.0	1.0	1.4	2.5	‡2.5	3.9	2.3
CN655706	cathepsin D precursor	-1.3	-1.4	-1.3	-1.2	-2.2	-1.8	-2.3	-1.8
CF931800	cathepsin L	1.3	-1.2	-1.5	-1.4	1.2	1.5	2.1	1.7
CF931835	cathepsin L-like cysteine protease	-1.8	-1.6	-2.7	-2.0	1.7	1.6	1.6	1.4
CF931811	chymotrypsinogen 2	1.3	-1.6	-1.3	-1.8	2.1	1.1	1.8	1.2
CN655709	similar to Cystatin C	1.4	1.6	1.0	1.1	-2.1	-2.6	-3.7	-2.1
CF931917	KDEL receptor 2 (ERD-2-like protein)	1.6	1.8	3.6	10.2	1.0	1.0	-1.2	-1.2
CF931891	leukocyte elastase inhibitor (LEI)	1.0	1.1	1.1	-1.1	2.1	-1.2	2.2	2.3
CF931964	Polyadenylate-binding protein 2	1.1	-1.2	1.0	-1.1	2.3	1.2	1.8	1.4
CF931794	ribosomal protein L38 mRNA	1.1	-1.4	-1.1	-1.4	-2.2	1.1	1.5	1.1
CN655610	ribosomal protein L44	1.4	2.6	-1.3	1.0	1.2	1.6	-1.2	1.1
CN655642	tax-responsive element binding protein 107	-1.2	-2.1	-1.5	-1.6	1.9	1.2	1.4	1.2
CN655715	thrombin	1.0	1.0	-1.1	1.0	-2.6	-2.1	-3.7	-1.8
CN655661	translation elongation factor G	9.6	8.3	1.7	3.3	-1.7	1.1	1.8	1.1
CF931773	U2 small nuclear RNA	-2.0	1.0	-1.1	1.1	1.3	1.6	1.8	1.3
CF931749	Unknown	-1.1	-1.4	-2.0	-1.4	1.4	1.3	1.4	1.2
CF931981	valyl-tRNA synthetase	-1.1	-1.4	-1.2	-1.1	-2.3	-2.1	-2.1	-1.2
Metabolism									
CN655628	1-phosphatidylinositol-4-phosphate 5-kinase isoform C	1.0	1.1	1.0	1.1	1.2	1.6	2.0	1.5
CN655581	Betaine-Homocysteine S-Methyltransferase	3.2	1.8	10.8	1.6	1.0	1.5	1.8	1.4
CN655621	chicken-type lysozyme	1.0	-1.5	-1.1	1.0	1.3	1.7	2.2	1.2

CF931895	Dihydrodipicolinate synthase	-1.1	-1.4	-1.3	1.5	-1.1	-1.3	-2.7	-1.9
CN655693	erythroid 5-aminolevulinate synthase	1.3	1.4	-1.1	1.1	-2.2	-1.3	1.0	-1.4
CN655680	Glutamate dehydrogenase (GDH)	-1.8	-2.1	-1.2	1.0	1.0	1.1	1.0	1.1
CN655594	glyceraldehyde-3-phosphate dehydrogenase	-1.7	-3.2	-1.4	-1.5	1.4	1.1	1.3	1.1
CF931980	N-terminal Asn amidase	-1.4	-1.5	-1.2	-1.2	-2.8	-2.5	-3.4	-1.9
CF931812	O-methyltransferase containing protein	7.2	3.5	1.4	1.3	1.0	1.2	1.3	1.2
CF931813	phenylalanine hydroxylase	1.0	-3.4	1.2	-1.5	1.2	1.4	1.7	1.3
CN655728	pKU-beta protein kinase	-1.3	-1.4	1.0	1.0	-2.6	-1.9	1.1	-1.1
CF931935	thymidine kinase 1, soluble	-1.4	-1.4	-1.4	-1.1	-2.2	-2.1	-1.4	-1.2
Unclassified function									
CF931868	78 KD Glucose-regulated protein precursor	3.5	3.5	-1.4	1.0	1.5	2.2	-1.1	1.3
CN655662	calumenin	‡2.5	2.2	1.1	-1.2	1.2	1.4	1.6	1.2
CN655613	chromosome 17 open reading frame 27	1.0	1.3	1.0	1.3	-1.2	2.1	1.3	1.3
CF931863	Similar to cDNA clone JFConA705R reverse	1.8	1.1	-1.4	1.0	2.8	1.7	-1.3	1.1
CF931850	DIPB protein	2.2	1.9	2.1	1.5	1.0	1.1	-1.2	-1.1
CF931985	hypothetical protein	-1.7	-2.6	-1.5	-1.5	-1.5	-1.2	1.0	-1.2
CF931798	Japanese flounder liver cDNA clone LE11(9)	1.0	-2.0	1.1	-1.6	1.2	1.2	1.3	1.2
CN655582	Japanese flounder liver cDNA clone LF1(3)	-1.7	-2.4	1.0	-1.4	-1.3	1.1	1.6	1.0
CF931856	Similar to cDNA clone JFConA12F	-1.4	1.0	1.5	1.7	-2.0	-1.6	-1.9	-1.3
CN655584	Multifunctional protein ADE2	1.3	2.3	1.1	1.0	1.3	1.7	1.9	1.4
CF931939	Similar to cDNA clone MF01FSA047J19 3'	1.3	1.0	1.0	1.0	4.1	4.0	1.9	1.3
CF931927	Similar to cDNA clone MF01SSA128A09 3'	1.0	-2.1	-1.6	1.0	-1.1	1.0	1.5	-1.3
CF931929	Similar to cDNA clone MF01SSB027M02 5'	-1.4	-1.5	-1.3	-1.1	-1.2	1.0	2.3	-1.1
CF931942	Similar to cDNA clone JFSK-5-38	1.0	1.4	1.1	1.1	-1.7	-2.0	-3.2	-1.5
CF931818	protein disulfide isomerase-related protein P5	5.3	5.9	-1.6	1.2	-1.3	1.9	1.8	1.4
CF931913	RIKEN cDNA 2210021G21	-1.1	-1.3	1.0	1.1	2.3	1.4	-1.2	1.1
CN655666	similar to cytochrome b5	-1.5	-1.8	-1.1	-1.4	2.0	1.5	1.4	1.4
CF931873	Son of sevenless protein hoomolog 2 (SOS-2)	-2.0	-2.1	-1.7	-1.2	-1.8	-1.5	-1.1	-1.6
CN655719	ssalrga076376 mixed_tissue Salmo salar cDNA	1.2	1.1	1.2	-1.1	-2.3	-2.5	-3.7	-1.7
CN655612	TBT-binding protein	-1.2	-1.9	-1.3	-2.0	2.3	1.3	1.8	1.3
CN655717	translational inhibitor protein	6.9	3.3	1.2	‡2.6	1.1	-1.3	1.0	-1.3
CF931846	vitronectin	-1.1	-1.3	1.2	-1.1	1.0	2.5	-1.1	1.0
CF931849	wap65[b]	4.3	2.7	‡2.4	‡2.0	-1.2	-1.1	-1.1	-1.1
CF931955	wap65	4.5	2.3	3.2	1.4	-1.2	‡2.1	1.1	1.1
CF931967	Winter flounder spleen cDNA clone Sp177f 5'	1.3	1.3	1.1	-1.1	-3.3	-2.2	1.9	1.1
CF931871	Similar to Zebrafish cDNA clone 4962032 5'	1.3	-1.1	-1.1	1.2	3.1	‡2.0	-1.1	1.3
CF931907	Unknown protein, IMAGE:3635549	1.1	-1.2	-1.2	-1.2	1.0	-2.0	1.3	-1.2
CN655593	Similar to hypothetical protein XP_159532	-1.5	1.0	1.5	1.3	1.4	-1.1	‡2.5	2.4
CF931758	Unknown	1.0	3.0	-1.1	1.1	1.5	1.1	1.6	1.1
CF931761	Unknown	-1.4	-1.1	-1.5	-1.4	-2.1	1.3	1.7	1.4
CF931763	Unknown	1.6	2.1	1.8	1.3	1.1	1.2	1.2	1.1
CF931828	Unknown	-1.5	-1.2	1.0	-1.1	-2.6	-1.3	1.5	1.2
CF931832	Unknown	-1.6	-1.3	1.1	-1.2	-3.2	1.9	1.7	-1.1
CF931844	Unknown	-1.2	-1.3	-1.2	1.1	1.0	1.0	1.1	-2.0
CF931903	Unknown	1.6	1.5	1.7	1.3	2.5	‡2.3	-1.2	1.8

DNA fragments were purified using a 96-well filtration system (Millipore, MA). To increase the concentration of template before spotting, the samples were dried in 96 well plates for 2.5h. This concentrated template was resuspended in 50% dimethylsulfoxide (DMSO) and gently shaken for 20 min. to give a final concentration of 150 ng/ul. These selected genes were

Table 2. Nucleotide sequences of primers used in reverse transcription PCR. Primer names were abbreviated following as: W*ap65* = warm-temperature-acclimation-related-65 kDa-protein-like-protein; BHM = betaine-homocysteine methyltransferase; GAPDH = glyceraldehydes-3-phosphate dehydrogenase; O-MCP = O-methyltransferase containing protein; PAH = phenylalanine hydroxylase; CYP2A = cytochrome P450 monooxygenase; PDIPP5 = protein disulfide isomerase-related protein P5; LEI = leukocyte elastase inhibitor; MHC I = major histocompatibility complex class I; RAG 1 = recombination activating gene 1; Ig L-constant = immunoglobulin light chain constant region; HSP70 = heat shock protein 70 ;TGF-β = Transforming Growth Factor- β ; TNF-α = Tumor Necrosis Factor-α; IL1- β; Interleukin-1β.

Primer name	GenBank Number	Nucleotide sequence (5′ → 3′)	PCR conditions
Actin F	CF931650	TAGCAGCTCTCGTTGTTGAC	94 °C, 30 sec/51 °C, 30 sec/
Actin R	CF931650	GGAGCCTCGGTCAGCAGGA	72 °C, 1 min; 25 cycles
Wap65 F	CF931849	AGACGGTGAAGACCAAGACG	94 °C, 30 sec/55 °C, 45 sec/
Wap65 R	CF931849	TCAGTGGTGATGGCGTCTAA	72 °C, 1 min; 30 cycles
BHM F	CF931569	GGGCATCTTCAAGAAACAGC	94 °C, 30 sec/55 °C, 45 sec/
BHM R	CF931569	CCTTCTCCACTCCCTCCTTC	72 °C, 1 min; 25 cycles
GAPDH F	CF931620	CCAACGTGTCAGTGGTTGAC	94 °C, 30 sec/55 °C, 45 sec/
GAPDH R	CF931620	TGGCTGCTTGGTTTACTCCT	72 °C, 1 min; 27 cycles
O-MCP F	CF931812	CCAGTGAACAAGCCCAGTTT	94 °C, 30 sec/55 °C, 45 sec/
O-MCP R	CF931812	CCGGCTGCTATCAGTTCATT	72 °C, 1 min; 30 cycles
CYP2A F	CF931662	CGAGATTCCCACTGTCCCTA	94 °C, 30 sec/55 °C, 45 sec/
CYP2A R	CF931662	TCCCCAGTGATTTTCACCTC	72 °C, 1 min; 30 cycles
Haptoglobin F	CF931649	GCATCCCCAAAACAAACATT	94 °C, 30 sec/55 °C, 45 sec/
Haptoglobin R	CF931649	GACAACGACCTGGCTTTGAT	72 °C, 1 min; 25 cycles
Hepcidin type I F	CF931737	GCCACCTTTCCTGAGGTACA	94 °C, 30 sec/55 °C, 45 sec/
Hepcidin type I R	CF931737	TTTGATGGTTGTTGGAGCAG	72 °C, 1 min; 25 cycles
Complement C3 F	CF931592	GGTTTAAAGTCGGCCTCCTC	94 °C, 30 sec/55 °C, 45 sec/
Complement C3 R	CF931592	CCTTCACCTGGAAAACATGG	72 °C, 1 min; 30 cycles
Complement C5 F	CF931601	CGTGTTACTCAAGGGGAGGA	94 °C, 30 sec/55 °C, 45 sec/
Complement C5 R	CF931601	CTCGAGGGAGAGTTTTCCAG	72 °C, 1 min; 30 cycles
PAH F	CF931813	GGGACGGACAAAGACCACTA	94 °C, 30 sec/55 °C, 45 sec/
PAH R	CF931813	CGAGCCGTAACTGAGGATCT	72 °C, 1 min; 27 cycles
Cathepsin L F	CF931835	GTTTCGACCCTGACACCATC	94 °C, 30 sec/55 °C, 45 sec/
Cathepsin L R	CF931835	TGAGTACACCGTGGTCCAAA	72 °C, 1 min; 30 cycles
PDIPP5 F	CF931818	AGGCCTCTCTGGGTATTGGT	94 °C, 30 sec/55 °C, 45 sec/
PDIPP5 R	CF931818	GGGTTCAACAGCGTGAATCT	72 °C, 1 min; 30 cycles
LEI F	CF931891	CGCTTTGATGAAGCAAACAC	94 °C, 30 sec/55 °C, 45 sec/
LEI R	CF931891	TCCATTCATTCAGCCTCTCC	72 °C, 1 min; 30 cycles
MHC I F	CF931621	CTTCATGAGGACGTGGACCT	94 °C, 30 sec/55 °C, 45 sec/
MHC I R	CF931621	GTGGGCTTCTCTCTGTTGGA	72 °C, 1 min; 30 cycles
RAG 1 F	AY454396	GCCCACAATGGATGCGTTACAC	94 °C, 30 sec/59 °C, 30 sec/
RAG 1 R	AY454396	CAGTTTGTTCCCCGACTCGTTC	72 °C, 1 min; 35 cycles
Ig L-constant F	CF931951	TGCTTCTGCAGCATGAAAAC	94 °C, 30 sec/55 °C, 45 sec/
Ig L-constant R	CF931951	GCTGGTAGAGATCCCACTGC	72 °C, 1 min; 30 cycles
HSP70 F	CF931574	TGAAGCCTACCTCGGAAAAA	94 °C, 30 sec/55 °C, 45 sec/
HSP70 R	CF931574	ATCTTCCCCACCAAGATGTG	72 °C, 1 min; 30 cycles
HSP90 F	CF931680	ACAAGGCTGTGAAGGACCTG	94 °C, 30 sec/55 °C, 45 sec/
HSP90 R	CF931680	AGGGGAGGAATCTCATCTGG	72 °C, 1 min; 30 cycles
TGF-β F	[a]N/A	TGGGTGTCTTTTGATGTTACCG	94 °C, 30 sec/60 °C, 30 sec/
TGF-β R	[a]N/A	ACGCAGCAGGGTGAGGCG	72 °C, 1 min; 30 cycles
TNF-α F	[a]N/A	GGCCTCTACTTCGTCTAC	94 °C, 1 min/55 °C, 1 min/
TNF-α R	[a]N/A	CCTTCGTTCAGCTGGAACAC	72 °C, 1 min; 35 cycles
IL1-β F	[a]N/A	GGATTCACAAGAACTAAGGAC	94 °C, 45 sec/55 °C, 45sec/
IL1-β R	[a]N/A	ACTGTGATGTACTGCTGAAC	72 °C, 1 min; 35 cycles

[a]N/A indicates the sequence is not submitted to database.

Table 3. Primers used in quantitative RT-PCR and cycling conditions.

Genes Primers	GenBank Number	Nucleotide sequence (5′ → 3′)	qPCR conditions (annealing temps and time, cycles)	Amplicon size (bp)
EF-1a F	EU561357	TACCTGTCGGTCGTGTTGAG	57°C for 30S, 40X	190
EF-1a R	EU561357	ACGTATCCACGACGGATCTC		
RAG 1 F	AY454396	CAGCTGTCCTGAGGCCTATC	55°C for 30S, 40X	133
RAG 1 R	AY454396	CGCACCATCTTCTCATCGTA		
Ig L-constant F	CF931951	AGACAGAGCAGAGTGGCTTTGTTG	55°C for 30S, 40X	95
Ig L-constant R	CF931951	ATATTTCCAGGCTCCAGCCTCTCT		
TNF-α F	[a]N/A	CTGATTCCATGGGCGACAAAGTGT	58°C for 30S, 40X	104
TNF-α R	[a]N/A	GTCTCTGAAGGCGTAATCCTGAGT		

[a]N/A indicates the sequence is not submitted to database.

manually spotted in duplicate on each of two GAPS II coated slides (Corning, NY) using MicroCASTer™ (Schleicher & Schuell, Germany) that is economical, entry-level manual microarray system. This spotter was originally designed for protein array, resulting in larger spot size (2 mm) than cDNA microarray (100 µm). Ribosomal protein genes were included for adjusting PMT power. Array pins were cleaned between each spotting, according to the manufacturer's directions. Slides were dried for 30 min then baked in an oven at 80°C for 2 hours to immobilise the spotted cDNA.

Microarray procedures and data analysis

Messenger RNA samples (3 µg) from the control and immunostimulated fish were labeled with either Cy3 dCTP or Cy5 dCTP (Amersham Biosciences, Piscataway, NJ) by a direct labeling method using SuperScript II reverse transciptase (GIBCO, Carlsbad). To control for bias due to differences in labeling efficiencies of Cy3 and Cy5, mRNA from both groups were labeled with each of the dyes. Hybridization for the first set of two slides consisted of Cy3-labeled control cDNA and Cy5-labeled experimental cDNA; hybridization for the second set of two slides consisted of Cy5-labeled control cDNA and Cy3-labeled experimental cDNA.

After purification of labelled cDNA using a PCR Purification Kit (Qiagen), each samples were concentrated to a final volume of approximately 8 µl in a speed vacuum and combined. One microlitre of poly (dA) DNA (20 mg/ml) was added to the sample to block non-specific hybridization. The combined sample was denatured at 95 ^{0}C for 3 min. and followed by the addition of 20 µl of hybridization buffer (Amersham Biosciences, Piscataway) and 40 µl of deionized formamide (Sigma, St. Louis). This sample was hybridized overnight at 42^{0}C to the halibut microarray. Slides were washed in the following order: 1x SSC (150 mM NaCl, 15 mM Sodium Citrate) with 0.2% SDS (sodium dodecyl sulphate) for 10 min., 0.1x SSC with 0.2% SDS for 10 min. (2 times repeat), 0.1x SSC for 5 min., and 0.1x SSC for 1-2 sec. (3 times). Microarrays were scanned (50 µm resolution) immediately using ScanArray 5000 XL reader and its proprietary software (Packard Bioscience) after drying by spinning in a plate centrifuge for 5 min. at 500 x g. The Cy3 and Cy5 signal intensities were adjusted with the signal intensities of internal controls such as ribosomal protein genes. The resulting images were imported into QuantArray software (Packard Bioscience, Boston) for quantitative analysis. These data were normalized using background subtraction and global median normalization via the QuantArray software. Only those spots with a hybridization signal intensity of greater than 500 fluorescence units for both Cy3 and Cy5 were used for calculating expression ratios. Genes with an expression ratio ≥2-fold at the $p<0.05$ significance level were regarded as differentially expressed genes. For the technical statistical analysis, we used a t-test in the GraphPad program (GraphPad Software, Inc. San Diego, CA); while, no biological statistic was analyzed because mRNA was pooled together from 3 fish. The differentially expressed genes were categorized into functional groups as described in Adams et al. [6].

Analysis of mRNA expression by RT-PCR and qRT-PCR

Reverse transcription PCR and qRT-PCR were used to confirm the microarray data. Fifteen genes for RT-PCR and three genes for qRT-PCR from the microarray analysis were selected if they showed the most significant change or if they were deemed to be important immune genes. Primers specific to these genes were constructed (Table 2 and 3) based on previous EST study [5].

The cDNA templates for RT-PCR were synthesized from pooled 500 ng poly(A) RNA from 3 control or 3 immunostimulated fish at each time points, using SuperScript II reverse transcriptase because the isolated RNA was not sufficient to perform microarray RT-PCR, and qRT-PCR analyses. For qRT-PCR analysis of three selected genes, two-step reverse transcription-Real-Time PCR was conducted using the Superscript III qRT-PCR kit with SYBR green (Invitrogen) on an iCycler iQTM Real-Time PCR detection system (Bio-Rad). All detail steps was followed by manufacturer's instruction. As a control, elongation factor (EF)-1A was chosen and cloned into a TA-cloning vector (pCR-TOPO; Invitrogen). A cloned EF-1A in a plasmid vector

was sequenced for the confirmation of a PCR product and used as a standard for qPCR. The statistical significance in qPCR was assessed using one-way analysis of variance ($p<0.05$). PCR/qPCR amplification was optimized for each of these genes and these conditions are given in Table 2 and 3.

Results

Liver gene expression

Of the 381 genes on the microarray, we identified a total of 44 different genes that were differentially up or down-regulated (≥2-fold change, $p<0.05$) in at least one of the time points (Tables 1,4). Twenty eight genes were significantly up-regulated showing up to 158-fold changes in expression, while 16 genes were down regulated having from 2 to 3-fold changes. The highest numbers of genes were differentially expressed at 2 days post-immunostimulation (DPI) (Table 4).

Table 4. Summary of microarray-based analysis of differentially expressed genes in the liver and kidney of Atlantic halibut immunostimulated with a commercial vaccine against Aeromonas salmonicida and Vibrio anguillarium. In total 381 genes were analysed of which 335 were genes with significant sequence similarity with known genes. Genes of expression ratio ≥ 2-fold at $P<0.05$ significant level were considered differentially expressed.

Days post-immunostimulation	1	2	7	14
Liver				
Up-regulated genes (28 genes)				
Known	13	9	3	4
Unknown	6	9	3	2
Total	19	18	6	6
Down-regulated genes (16 genes)				
Known	3	6	2	2
Unknown	1	4	0	1
Total	4	10	2	3
Kidney				
Up-regulated genes (27 genes)				
Known	10	0	7	4
Unknown	7	4	1	0
Total	17	4	8	4
Down-regulated genes (21 genes)				
Known	9	5	7	2
Unknown	6	4	2	1
Total	15	9	9	3

Up-regulated genes

Of the 28 differentially up-regulated genes, hepcidin type I, an antibacterial peptide, showed the highest level of up-regulation with an approximately 158-fold increase at 1 DPI (Table 1). With exception of 7 DPI this gene was highly expressed throughout this study. The results of RT-PCR for this gene support the results of the microarray analysis with higher levels of gene expression on all days relative to the control and a general decline in expression seen over time (Figure 1). A second antimicrobial peptide gene, hepcidin type II, showed a significant 2.5-fold increase in expression only at 1 DPI.

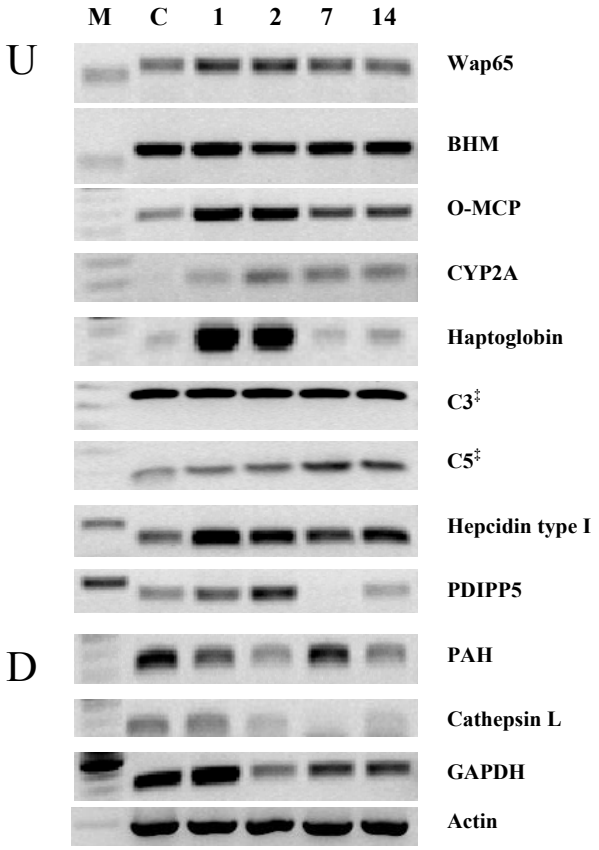

Figure 1. Reverse transcription PCR (RT-PCR) analysis of mRNA expression for selected genes in the liver. U = up-regulated genes; D = down-regulated genes; M = marker having 100 bp ladder (MBI Fermentas); C = control fish; 1,2,7,14 = 1, 2, 7, 14 days post-immunostimulation. The symbol (‡) indicates the gene that was not differentially (≥2-fold at the $P<0.05$) expressed in the microarray analysis. Actin gene was used as an internal control in order to ensure equal loading of template and PCR condition. Gene names were abbreviated following as: W*ap65* = warm-temperature-acclimation-related-65 kDa-protein-like-protein; BHM = betaine-homocysteine methyltransferase; GAPDH = glyceraldehydes-3-phosphate dehydrogenase; O-MCP = O-methyltransferase containing protein; PAH = phenylalanine hydroxylase; CYP2A = cytochrome P450 monooxygenase; C3 = complement component 3.

Other genes that were highly up-regulated (≥5-fold) in at least

1 sample include: haptoglobin fragment 1, betaine-homosysteine S-methyltransferase, thyroid receptor interacting protein 12, O-methyltransferase containing protein, protein disulfide isomerase-related protein P5 precursor, KDEL receptor 2 (ERD-2 like protein), cytochrome P450 monooxygenase, translational inhibitor protein, 28S ribosomal RNA, and translation elongation factor G (Table 1).

A number of genes that encode components of the acute phase-response showed significant up-regulation expression following immunostimulation. Transferrin expression was significantly up-regulated at 1 DPI. Microarray analysis showed that haptoglobin gene expression was significantly up-regulated at all-time points (Table 1). Analysis of this genes expression by RT-PCR revealed a similar pattern of expression with very high levels of expression at 1 and 2 DPI when compared to the controls and lower levels of expression at 7 and 14 DPI (Figure 1).

Other genes classified as cell/organism defense genes which showed significant up-regulation included: one complement pathway-related gene (complement regulatory plasma protein SB1, cytochrome P450 monooxygenase and MHC class I (2 sequences) (Table 1). There was no evidence for increase C3 expression by either microarray analysis or RT-PCR. RT-PCR analysis of C5 suggested a gradual increase in the level of expression in immunostimulated fish over time; however, changes in the levels of expression were not significant by microarray analysis (Figure 1). The two sequences that share identity with MHC class I showed significant up-regulation (4.4 and 2.2-fold) at 1 DPI by microarray analysis (Table 1). This result was not supported by RT-PCR analysis (data for the liver is not shown). Two genes encoding proteins with roles in cell signaling and cell communication were significantly up-regulated at 1 and 2 DPI (Table 1). These included genes with sequence similarity to the thyroid hormone receptor interacting protein 12 and leucine-rich alpha-2-glycoprotein. Two genes encoding products involved in metabolism were homologous to betaine-homocysteine methyltransferase and O-methyltransferase ; these were also highly up-regulated at 1 and 7 and 1 and 2 DPI, respectively. Betaine-homocysteine methyltransferase had a 10.8-fold increase at 7 DPI (Table 1). Ten genes of unknown function were seen to be significantly up-regulated in immunostimulated animals by microarray analysis (Table 1). These included two genes that are homologous to warm-temperature-acclimation-associated 65-kDa protein (wap65). Significant increases in expression of these genes were confirmed by RT-PCR analysis (Figure 1).

Down-regulated genes

Sixteen genes showed significant down-regulation in the liver at least at 1 time point post-immunostimulation (Tables 1,4). These included genes with sequence similarity to the negative acute-phase proteins such as anti-trypsin alpha 1 and alpha-2-HS-glycoprotein (fetuin A) (Table 1). Anti-trypsin alpha 1 gene expression was generally down-regulated after immunostimulation with significant reduction in expression at 2 and 14 DPI. Alpha-2-HS-glycoprotein (Fetuin-A) had a significant 2-fold decrease in gene expression at 1 DPS. Three genes related to metabolism including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phenylalanine hydroxylase and glutamate dehydrogenase (GDH) were significantly down-regulated at 2 DPI (Table 1). The RT-PCR expression data for GAPDH and phenylalanine hydroxylase also showed decreased expression at 2 DPI (Figure 1). The gene for cathepsin L-like cysteine protease, which is involved in gene and protein expression [6], was significantly down-regulated following immunostimulation at 7 and 14 DPI. Expression data for this gene obtained by RT-PCR showed a similar pattern of expression (Figure 1). Six genes of unknown function were also differentially down-regulated including a gene homologous to TBT-binding protein.

Kidney gene expression

We identified 48 genes which were differentially regulated (≥2-fold change) in at least one of the kidney samples (Tables 1,4). Of these, 27 genes were up-regulated and 21 genes were down-regulated. Levels of differential expression seen in the kidney were often lower than those seen in the liver with most up-regulated genes having 2 to 5-fold increases and down-regulated genes 2 to 3-fold decreases (Table 1). Three immunologically important genes, showing less than 2-fold up-regulation in the microarray analysis, were confirmed by qRT-PCR. These genes were significantly up-regulated (up to 7-fold) in the kidney.

Up-regulated genes

Of the 27 differentially up-regulated genes the following had more than a 3-fold change in at least one of samples: MHC class I receptor, 40S ribosomal protein S17, beta-cytoplasmic actin 2, and two unknown genes (Table 1). Other significantly up-regulated genes (2-3-fold changes) included leukocyte elastase inhibitor, CDH1-D, C-type lysozyme, vitronectin, S100-type binding protein A14, cathepsin L, and chymotrypsinogen 2 (Table 1). Of the acute phase proteins only c-type lysozyme showed a significant 2.2-fold up-regulation significantly at 7 DPI.

Several genes with cell/host defence functions were also up-regulated. Hepcidin antimicrobial peptide I was up-regulated significantly at 1 and 7 DPI but at a much lower level than seen in the liver. Two clones of MHC class I related genes were significantly up-regulated with 2.3- and 3.7-fold increases in expression at 1 DPI (Table 1). This increase in expression was supported by RT-PCR results (Figure 2). Leukocyte elastase inhibitor (LEI) was generally up-regulated after immunostim-

ulation and this up-regulation was significant with approximately 2-fold at 1, 7 and 14 DPI. RT-PCR results for MHC I and LEI revealed similar patterns of expression to the microarray analysis. Based on 2-fold change criteria, none of the genes related to the complement system showed differential expression in the kidney.

Two genes involved in cell signaling and cell communication were significantly up-regulated. CDH1-D was up-regulated approximately 2-fold at 1, 7 and 14 DPS. S 100-type calcium binding protein was also up-regulated at 1 DPI. One of two actin genes; beta-cytoplasmic actin 2 was significantly up-regulated in the kidney at 1 and 14 DPI.

Six genes encoding proteins with roles in gene/protein expression function were up-regulated, including ribosomal proteins, cathepsin L, chymotrypsinogen 2, polyadenylate-binding protein. Cathepsin L was up-regulated 2.1-fold at 7 DPI and chymotrypsinogen 2 and polyadenylate-binding protein were up-regulated 2.1-and 2.3-fold respectively at 1 DPI. Twelve functionally unclassified genes were also up-regulated, including one homologous to TBT-binding protein, vitronectin and a 78 kDa glucose-regulated protein.

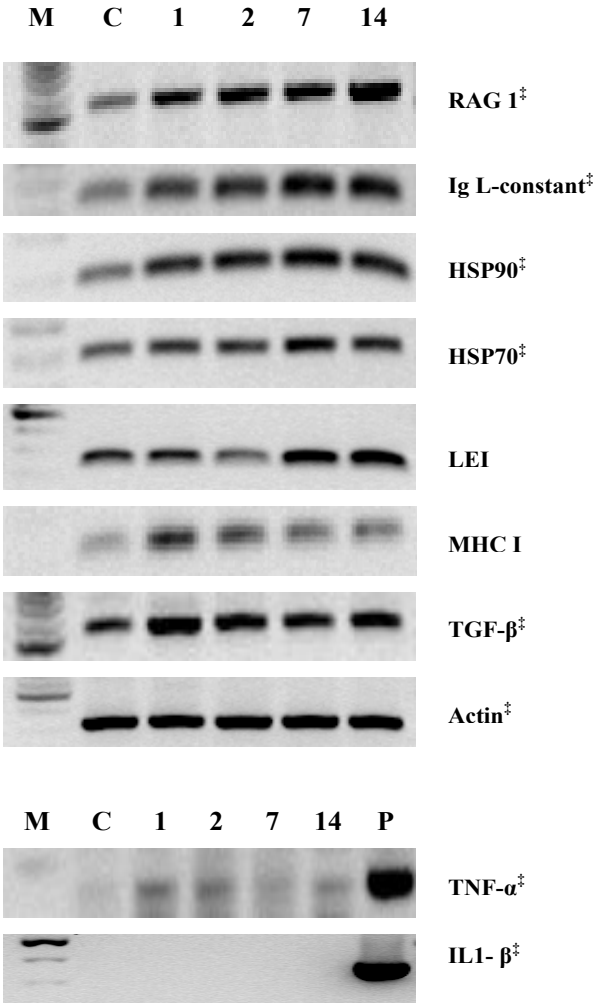

Figure 2. Reverse transcription PCR (RT-PCR) analysis of mRNA expression for selected genes in the kidney. M = marker having 100 bp ladder (MBI Fermentas); C = control fish; 1,2,7,14 = 1, 2, 7, 14 days post-immunostimulation; P = positive control. The symbol (‡) indicates the gene that was not differentially (≥2-fold at the P<0.05) expressed in the microarray analysis. Actin gene was used as an internal control in order to ensure equal loading of template and PCR condition. Gene names were abbreviated following as: LEI = leukocyte elastase inhibitor; MHC I = major histocompatibility complex class I; RAG 1 = recombination activating gene 1; Ig L-constant = immunoglobulin light chain constant region; HSP70 = heat shock protein 70; TGF-β = Transforming Growth Factor- β; TNF-α = Tumor Necrosis Factor-α; IL1- β; Interleukin-1β.

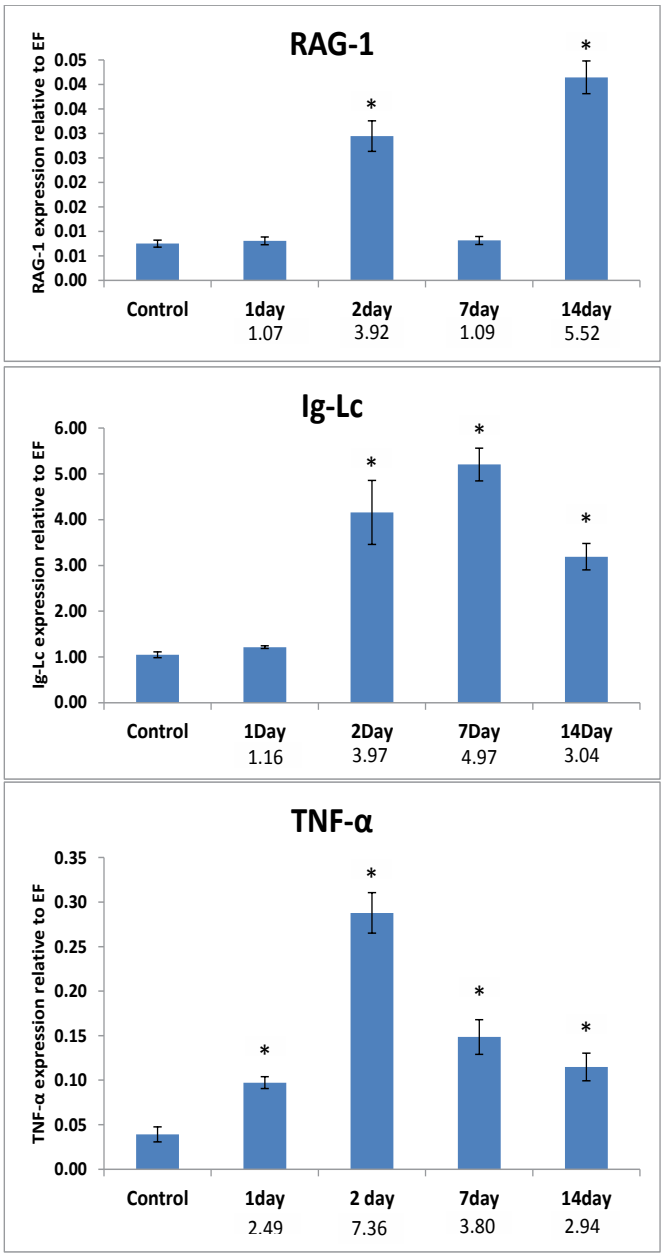

Figure 3. QRT-PCR analyses of three selected genes identified in microarray analysis. Gene expression data are presented as mean (±SE) expression relative to elongation factor-1A. *Asterisks denote significant difference in fold up-regulation (<0.05) relative to control fish at day 0. The numbers below days on X axis indicate fold up-regulation relative to expression from control fish. Gene names were abbreviat-

ed following as: RAG 1 = recombination activating gene 1; Ig L-constant = immunoglobulin light chain constant region; TNF-α = Tumor Necrosis Factor-α.

Our microarray analysis showed no evidence of significant up-regulation of genes related to specific immunity (e.g. RAG 1 and Ig L-constant region), cytokines, or heat shock proteins (Figure 2). However our RT-PCR or qRT-PCR results suggest that expression levels of RAG 1, IgL-constant, HSP 70 and HSP 90 gradually increased following immunostimulation and that their expression levels in immunostimulated fish were significantly higher than the controls (Figure 2,3). Three cytokines were tested using RT-PCR or qRT-PCR (Figure 2,3). TGF-ß and TNF-α showed evidence of increased expression at 1 DPI when compared to the controls. There was no evidence of IL1-ß expression in control or immunostimulated fish. Based on qRT-PCR analysis, RAG1, Ig L-constant region and TNF- α were significantly up-regulated with up to 5.5, 4.0 and 7.0-fold over two-weeks period.

Down-regulated genes

Twenty-one genes showed significant down-regulation, including thyroid receptor interacting protein, tetraspanin 47F, erythroid 5-aminolevulinate synthase, cathepsin D, cystatin C, thrombin, pKU-beta protein kinase, N-terminal Asn amidase, dihydrodipicolinate synthase, and valyl-tRNA synthetase. Nine genes of unknown function were also significantly down-regulated (Table 1).

Two genes, thyroid receptor interacting protein and tetraspanin 47F, with roles in cell signaling/cell communication were significantly down-regulated. Thyroid receptor interacting protein was down-regulated 3-fold at 7 DPI and tetraspanin 47F was down-regulated 2-fold at 14 DPI. Cystatin C was significantly down-regulated 2-3-fold post-stimulation and this down-regulation was highest at 7 DPI. Cathepsin D, thrombin, and valyl-tRNA synthetase showed 2-3-fold decreases at 1, 2, 7 DPI. Erythroid 5-aminolevulinate synthase, pKU-beta protein kinase, and N-terminal Asn amidase were generally down-regulated in earlier samples, whereas dihydrodipicolinate synthase was down-regulated in later samples 7 and 14 DPI.

Discussion

We constructed a cDNA microarray using clones from the liver, kidney and spleen of Atlantic halibut that had been vaccinated against *Vibrio anguillarum* and *Aeromonas salmonicida* [5]. This microarray was used to investigate the transcriptional response of Atlantic halibut liver and kidney tissues over a 14-day period following immunostimulation with the same vaccine. Therefore the transcriptional response we report includes both responses to bacterial components, as well as responses, if any, to the commercial adjuvant. Compared to the information obtained from human and mouse [for review, 14,15], it is still unknown what effects adjuvants have on gene expression in fish.

With respect to genes involved in innate immunity, immunostimulation resulted in marked increases in the levels of expression of numerous genes. One of these genes, an antimicrobial peptide, hepcidin type I was highly expressed throughout this study in liver tissues of immunostimulated fish. This is one of two hepcidins that have been identified in Atlantic halibut, having > 85% amino acid identity to Type II and III hepcidin-like peptides of flounders [5]. A second antimicrobial peptide, hepcidin type II that was most similar to white bass hepcidin was significantly up-regulated only at 1DPI in liver. These two halibut hepcidins share a 42% amino acid identity. Hepcidins may have multiple biological functions in fish such as regulation of iron homeostasis during inflammation and modulation of genes involved in the acute phase response [16]. A variety of genes involved in the acute phase response showed significant changes in expression following immunostimulation. Haptoglobin gene expression was highly up-regulated in liver at all-time points. It appears that, as in mammals, haptoglobin plays an important role in the regulation of the immune system of fish. In higher vertebrates haptoglobin is important in the regulation of immunity through its actions as a potent anti-inflammatory agent, a regulator of a variety of macrophage functions, a suppressor of lectin and LPS-induced B cell and T lymphocyte proliferation, a regulator of cytokine production and a protector against endotoxin induced effects [17]. Haptoglobin has been previously reported to increase in the plasma following injection of rainbow trout with killed L. *anguillarum* in Freund's adjuvant [18]. In this study, we observed up regulation of haptoglobin only early after an immunostimulation (Figure 1), suggesting that its role in teleosts may be similar to that in mammal. Further work is required to elucidate these mechanisms.

In addition to its primary role as an iron-binding protein, transferrin is now recognized to have a variety of other functions in innate immunity. Transferrin expression in liver was significantly up-regulated in the liver at 1 DPI further supporting the view that transferrin is a positive acute phase protein in fish [19]. In addition to its role of limiting iron to bacterial pathogens, enzymatically cleaved transferrin has been found to be a endogenous activator of macrophage antimicrobial responses [20-22].

Fetuin is a negative acute-phase glycoprotein thought to suppress and/or play an important role in the resolution of inflammation by enhancing phagocytosis of apoptotic cells [23,24]. In this study fetuin A was significantly down regulated at 1 DPI. Expression of the protein is known to be down-regulated during the acute phase response in human and rat models [25]. It has also been reported that hemagglutination of *Ed-*

wardsiella tarda was strongly inhibited by fetuin suggesting that it plays a role in innate immunity [26]. Fetuin is also suggested to have other biological functions; for example, fetuin gene expression was significantly up-regulated in European flounder (*Platichthys flesus*) upon exposure to environmental pollutants [27].

Expression of α 1 anti-trypsin was significantly down-regulated in the liver at 2 and 14 DPI. Alpha 1 anti-trypsin is produced by primarily by hepatocytes but also by neutrophils, monocytes and some forms of macrophages. It is the main serine protease inhibitor in the blood regulating numerous proteolytic processes. In mammals, levels of this protein increase rapidly in the circulation during inflammation and infection where it protects tissues from neutrophil-derived proteases [28]. It also has other biological activities that are not associated with serine protease inhibition. For example, in human monocytes α 1 anti-trypsin has been reported to have several roles such as inhibition of LPS-stimulated production of TNF-α and IL-1β, as well as enhancing the release of IL-10 an anti-inflammatory cytokine [28]. This may be the case in teleosts, and may explain the reason for its down-regulation in liver.

There were significantly higher levels of fibrinogen expression in liver tissues at 1 DPI when compared to controls. In addition to its role in hemostasis, the importance of fibrin(ogen) as a regulator of the inflammatory response in mammals is now known [29]. The importance of fibrin in the control of inflammation response in halibut requires further study.

In this study two types of the warm-temperature-acclimation-related-65 kDa-protein (Wap65) were significantly up-regulated at 1,2 and 7 DPI in the liver. W*ap65* is a glycoprotein that was first identified in goldfish where it was found that both the transcription and protein levels increased markedly with temperature elevation and exposure to bacterial LPS [30,31]. A later study reported increased expression and protein levels in the goby (*Gillichthys mirabilis*) subjected to hypoxia [32]. In the medaka (*Oryzias latipes*) and the pufferfish (*Takifugu rubripes*), two types of W*ap65* have been reported [33,34]. Although the 5' flanking region of w*ap65* contains cytokine response elements in both of these species neither administration of LPS nor elevation of temperature resulted in increased accumulated levels of w*ap65* mRNA as determined by northern blot analysis. Based on genetic analysis and demonstrated binding of W*ap65* from some species to heme it is thought that the Wap65s are fish homologues of hemopexins [31,34]. Hemopexins are a class I acute phase protein that protects cells from heme-mediated oxidative stress and may play a role in bacteriostatic defence among other processes [35,36]. In higher vertebrates, hemopexin levels are known to be increased as the result of injury and inflammation. Our data supports a similar role for these genes in the innate immune response of Atlantic halibut.

Of the 11 complement-related cDNAs on the microarray only complement regulatory plasma protein SB1 showed significant up-regulation in liver at 1 DPI by microarray analysis. The liver in fish is a major site of complement-related gene expression [37]. The RT-PCR results for complement component do not show any differences between the control and immunostimulated samples, whereas levels of C5 expression is higher, especially in the later samples when compared to the control. Cytokines play important roles in directing and controlling the immune system [38]. In our microarray, five genes homologous to cytokines were analyzed but none of the corresponding genes showed significant differential expression in either the liver or kidney tissue. When examined using RT-PCR, kidney tissue expression of IL-1ß was not detected in control or immunostimulated fish at any of the experimental time points, whileTGF-ß were up-regulated at 1 and 2 DPI. TNF-α was significantly up-regulated in the qRT-PCR analysis, ranging from 2.5-fold at 1 DPI to 7.4-fold at 2 DPI. The lack of expression of IL-1ß may be due to the type and the dose of the antigen and/or the time at which the samples were taken. The pattern of expression of cytokines such as IL-1ß or TNF-α depends on the type of the microbial pathogens and the host recognition pathways invoked [39-41]. In isolated kidney cells of Japanese flounder (*Paralichthys olivaceus*) stimulated with LPS, expression of IL-1ß was highly up-regulated 1 and 6-hour post stimulation having 9.9- and 14.9-fold changes, respectively [9]. Vaccination of common carp (*Cyprinus carpio* L.) against *Aeromonas salmonicida*, resulted in different expressions of IL-1ß and TNF-α, although these two pro-inflammatory cytokines are often co-stimulated [42]. Expression of IL-1ß was increased as early as 10 min post vaccination with the highest level seen at 3-hours post vaccination, while TNF-α expression was not increased at all time points. By 24-hours post vaccination IL-1ß expression had returned to its constitutive level. There are at least five isoforms of TGF-ß known from vertebrates with each of these isoforms playing specific roles in the immune response. In the plaice (*Pleuronectes platessa*), three isoforms have been identified [43]. The isoform in this study most closely resembles TGF- ß1 which is a cytokine that is known to be a primary regulator of inflammation in higher vertebrates [44]. Although our microarray study did not show significant up-regulation of TGF- ß1 our RT-PCR results suggest increased gene expression in kidney tissue at 1 DPI.

Vaccination can cause short-term stress and side-effects in fish [45,46]. In this study, we immunostimulated the animals with a commercial vaccine and report that a number of genes related to stress or toxin response were significantly differentially expressed. The cytochrome P_{450} superfamily contains a variety of enzymes that are involved in the oxidative metabolism of exogenous and endogenous compounds. Cytochrome P_{450} enzymes (CYP) have been most commonly reported in both fish and mammals to be down regulated in the liver during infection with viral, bacterial and parasites or after the adminis-

tration of immunoactive agents such as LPS and vaccines that cause inflammation [47,48]. However, there are some immunoactive agents known to induce some isoforms of CYP during the inflammatory responses [48-50]. In this study, we report significant up-regulation of gene that is homologous to CYP in the liver at 2, 7 and 14 DPI. The agent or agents in the vaccine that are responsible for this up-regulation are unknown.

We have also examined the expression of three heat shock proteins (HSPs): HSP 70, heat shock cognate 71 kDa protein, HSP 90. Our microarray analysis indicated that there was no significant differential expression of genes for these proteins between immunostimulated and control fish in either kidney or liver. However, our RT-PCR for HSP 70 and HSP 90 did show evidence for increased expression in vaccinated fish. In addition to their well-known role as molecular chaperones it is now reconginzed that HSPs play important roles in both the innate and adaptive immune response [51]. In higher vertebrates HSPs are highly expressed during bacterial infection [52,53] or in response to administration of bacterial LPS [54]. They are recognized to play an important role in the regulation of proinflammatory cytokines such as TNF-α and IL-1β [55,56]. In fish infection of Atlantic salmon with *Aeromonas salmonicida* resulted in up-regulation of HSP 90 transcripts at 7 and 13 days post infection [57]. Higher levels of HSP70 protein have been reported in the kidney and liver of coho salmon (*Oncorhynchus kisutch*) infected with *Renibacterium salmoninarum* [58]. It is likely that, as in higher vertebrates, HSPs play a number of important roles in the response of fish to bacteria or bacterial products. Our RT-PCR results provide preliminary evidence of this. Further study is required.

In this study we examined the expression of various adaptive immune system genes including Ig L-constant, MHC I and II, RAG 1 and early B cell factor over a 14 day period at a temperature of 10°C. With exception of MHC I, none of these genes were identified as significantly up-regulated (<2-fold) in our microarray analysis. However, RT-PCR and qRT-PCR results showed that expression of both RAG 1 and Ig L-constant genes gradually increased over time. RAG gene has been reported from many fish species [59-62], however these studies were focused on the development of immune system such as ontogeny. There is limited information how these genes respond to LPS stimulation, vaccination, and infection. A recent study [62] gives some insights how these genes respond to immune stimulation. In that study turbot were fed with nucleotide-supplement diet for 15 weeks and relative expression of immune genes was compared with control fish. The expression of RAG-1 was significantly increased with approximately 3-20 times from gill, spleen and kidney at 15 weeks post feeding with nucleotide-supplement diet.

On our microarray two MHC I and three MHC II related genes (class II α, β, and class II-associated invariant chain-like) were spotted. Using this array we found that two genes related to MHC I were significantly up-regulated at 1 DPI in the kidney and liver. This up-regulation was confirmed by RT-PCR in kidney. There was no evidence of significant up-regulation of MHC II genes in this study by microarray analysis. It has been known in higher vertebrates that expression of MHC class II-associated invariant chain is co-regulated with the MHC class II α and β expression. A recent study in Atlantic salmon (*Salmo salar* L.) head kidney has shown that expressions of MHC class I and II mRNA is closely linked [63]. Although our microarray data does not show any transcriptional correlation between MHC I and MHC II related genes at 2-fold criteria, the expression of MHC class II-associated invariant chain-like gene was increased by 1.8-fold at 1DPI when two MHC I alleles were also significantly up regulated. However, this is not clear if the increased expression of MHC class II-associated invariant chain-like gene was linked to MHC class II transport and peptide loading as MHC class II-associated invariant chain has multiple functions [for review, 64,65].

Although cDNA microarrays provide a powerful tool for examining patterns of transcript profiles, some technical constraints restrict facile analysis and comparison between data sets. The number of replicates in a microarray is an important issue and has been discussed in many publications [66]. In our experiment, we used pooled mRNA from three fish. Fish have been shown to have highly variable expression levels of mRNA or protein [4,12,67]. Under certain conditions, pooling samples have been advantageous in terms of cost and efficiency, especially when the biological variability is large relative to the technical variability [66]. We have found 83 genes that were differentially regulated in liver or kidney. The question remains as to how variable the expression of these genes is in other fish species or between individuals.

In this study, as has been reported for other microarray studies, there is not always complete correlation between the results obtained by microarray analysis and those obtained by qRT-PCR analysis [68,69]. It is readily accepted that cDNA microarray analysis provides a monitoring for a very large number of genes in parallel and that qRT-PCR provides greater accuracy and precision for confirmation of expressed genes. The results of three gnenes from qRT-PCR in this study indicates that the expressed levels of genes from microarray analysis were neutralized by some unknown reasons, which might reduce differential expression levels. Also, in this study, because of unavoidable technical constrains, the RNA for RT-PCR and qRT-PCR was not that used for microarray analysis although both RNA preparations were taken for the same tissue samples. This may explain why there are some expression level differences between microarray analysis and RT-PCR/qRT-PCR.

In conclusion, microarray analysis provides us with important information about the transcriptional immune response of Atlantic halibut following immunostimulation with a commercial

vaccine designed for Atlantic salmon. In the short-term, this stimulation results in far more up-regulated than down-regulated genes. This pattern of gene regulation demonstrates that the overall immune responses, such as acute phase, innate immune, and adaptive immune responses, in Atlantic halibut is similar to responses seen in other cold-water fish such as Atlantic salmon. However expression levels of adaptive immune-related genes in Atlantic halibut was low compared to that in Atlantic salmon, even though immunostimulation method in this study was different with that in other studies. We have also found many genes of unknown function that were significantly regulated following immunostimulation, leading us to consider further study on these genes. Some genes showed different expression profiles after immunostimulation compared to that reported in infection trials. We are currently analyzing the gene expression of Atlantic halibut after bacterial infection using another machine spotted cDNA array.

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