Project SARF004: Vaccine performance/efficacy in gadoids measured by cell mediated immune responses

FINAL REPORT

By

Yolanda Corripio-Miyar and Chris Secombes

Scottish Fish Immunology Research Centre,

University of Aberdeen.

PROJECT OUTCOMES

The following are the main outcomes achieved in the project:

- *A possible marker for disease resistance for gadoids has been found.* Study of cytokines has shown that immunised fish show increased production of particular cytokines rapidly post challenge, relative to non-immunised fish.
- *A challenge model for optimal and suboptimal vaccines has been developed*. We have demonstrated the value of comparing optimal and suboptimal vaccination regimes in studies of relative disease resistance/susceptibility. In addition to gaining information on host responses in such experiments, the break-point of vaccines in terms of the limits on doses to be administered, can be defined.
- *A Bacterial Artificial Chromosome (BAC) library for cod has been produced*. This is an extremely useful tool that can be used for screening to obtain novel immune genes in future projects.
- *Key immune genes have been cloned and sequenced both in cod and haddock*. Genes representing cytokines known to be released from separate T helper cell populations in mammals, that direct immune responses to protect against intracellular and extracellular pathogens, have been cloned. Alongside these, several other genes related to IFN-γ activity have been sequenced in haddock, that have value to fish immune biologists and vaccinologists.

Executive Summary

Gadoids are currently the second most important fish being farmed in Northern European countries such as Norway, Iceland and Scotland. However, the rapid development of marine species in aquaculture has resulted in a high demand for new vaccines to target emergent diseases. To date, very few vaccines have been developed for diseases in marine species and more specifically for the serotypes of certain bacterial diseases that affect gadoids. Experimental analysis carried out in cod and haddock have shown that, despite the lack of induction of a specific immune response, high levels of protection can still be achieved following vaccination. The lack of production of specific antibodies impedes the use of non lethal techniques to determine vaccine efficacy and as a result, the only effective method is bacterial challenge. The aim of this project was to develop an alternative method to assess vaccine performance, by measuring the cell mediated immune responses after vaccination, as defined by the release of particular cytokine molecules that direct immune responses one way or another. The following summarises the milestones achieved in this project.

The main core of the project was the cloning and sequencing of two cytokine genes, interferon-gamma (IFN-γ) and Interleukin-2 (IL-2), that are released from a particular leucocyte subtype called Th1 cells, that drive responses to intracellular pathogens. However, during the course of this project further cytokines were added to the list of those to study, since they define new leucocyte subsets in mammals crucial for responses to extracellular pathogens. Following their identification and cloning, the cytokine response after immunisation was subsequently assessed in a vaccination experiment.

The sequencing of the two Th1 cytokines was attempted using a variety of approaches, such as homology cloning, gene walking and synteny. Since homology cloning was not successful, work was focused on the use of synteny, the conservation of the gene order over wide evolutionary distances. Different genes surrounding IL-2 or IFN-γ with a high level of conservation were successfully identified and sequenced using homology cloning. Neighbouring IL-2 are bFGF-2 and Centrin, and surrounding IFN-γ are IL-22 and DYRK2. Centrin and IL-22 were cloned both in cod and haddock, while bFGF-2 and DYRK2 were cloned in haddock. Probes were then designed to Centrin and DYRK2 to be used with haddock. Gene Walking libraries were produced during the project to allow the sequencing of IL-2 and IFN-γ respectively. This was arduous work, since the size of the DNA amplified in each step was relatively small (500-700bp) and the distances between the cloned genes and the cytokine of interest relatively large (up to 50Kb for IFN-γ and 8Kb for IL-2).

During the project, further funding was obtained to generate a BAC library, as another potentially more efficient means to screen for cod genes. Once the BAC library for cod was produced, and using probes for Centrin designed for this purpose, the libraries were screened for IL-2. A positive BAC clone was obtained from the screening and confirmed by PCR that the Centrin was indeed in the clone. After shotgun sequencing, the results showed that the clone size was smaller than initially expected, and whilst it contained neighbouring genes including SPATA5, NUDT6, FGF-2, FJ35630 and Centrin, it did not include IL-21 or IL-2.

However, we were able to find a fragment that corresponded to the other cytokine of interest, IFN-γ, by mining the recently created cod EST database. Primers were designed based on the predicted sequence and the full sequence for cod IFN- γ was obtained. After thorough analysis to confirm the obtained sequence was indeed cod

IFN- γ, the production of the recombinant protein started. After purification of the *E .coli* produced recombinant cod IFN- γ under native and denaturing conditions, the activity of the recombinant protein (rIFN- γ) was tested in primary cell cultures from head kidney and spleen. This was considered an important step prior to antibody generation, since lack of activity could reflect improper folding of the molecule. The experiment included three different concentrations of the rIFN- γ (1ng/ml, 10ng/ml and 100ng/ml) and a control containing the elution buffer in which the protein was eluted, with cells stimulated for 6h. A further experiment looked at the stimulatory effect of an individual dose of rIFN- γ, 10ng/ml, after 24h of culture stimulation. The bioactivity of the protein was then studied in terms of its ability to affect expression of TAP-1, a gene known to be induced by IFN-γ.

A vaccination experiment in haddock designed to study the Th1 responses (IFN-γ) was carried out. The vaccines used consisted of two doses of formalin-killed bacteria (*Vibrio anguillarum* serotype O2) and a control group vaccinated with PBS. The high dose (*HVang*) contained 10^7cfu/ml, a dose known to elicit a good level of protection (Corripio-Miyar et al., 2007b), and the low dose (*LVang*, 10^3cfu/ml), with a low count of bacteria, intended to act as low efficiency vaccine. Efficacy of vaccine preparations was then compared to a control group of non-immunised fish in terms of immune gene expression analysed by PCR. Samples from head kidney and gill were taken at four time points after immunisation and at three time points post-challenge. These were then processed for RNA extraction and cDNA production. The obtained cDNA was then used in real time PCR analysis to look at the expression of the Th1 responses in which IFN-γ is involved. This analysis included transcription factors involved in the activation of Th1 cells, IFN-γ itself, and factors that inhibit IFN-γ production (which should show an inverted correlation) or are turned on by IFN-γ. These

molecules (LMP2, TAP1 and GATA3) were cloned from haddock for use. These genes, alongside IFN-γ, were then used for PCR analysis. The samples post-challenge, which give a good indication of the secondary response, were analysed by real-time PCR to look at the level of up/down regulation during bacterial challenge of vaccinated fish. However, no clear cut correlations between gene expression and disease resistance were apparent. As *V. anguillarum* was the pathogen used in this study, it was possible that IFN-γ, and associated genes, might not be as relevant a marker for this disease, although potentially very relevant for others that occur in gadoids (eg Franciscella, nodavirus). However, a new cytokine discovered in this project, was shown to undergo rapid and significant levels of gene expression post-challenge of optimally vaccinated fish, and thus this molecule has value as a potential marker of disease resistance.

Final report

Introduction

Gadoids are known for their lack of specific antibody responses after vaccination (Espelid et al., 1991; Corripio-Miyar et al., 2007b), which impedes the measurement of vaccine performance by serological methods. In addition to this, these methods are not always reliable when used in other species, since even in salmonids the antibody response is not always correlated with protection. The development of an assay that would show a good correlation with protection will be the key in future vaccine developments for these important fish species.

The knowledge of gadoid immunology is very limited and only few cytokines have been discovered to date (Corripio-Miyar et al., 2007a; Seppola et al., 2008). Prior to this project, no T-helper 1 cytokine had been sequenced in cod or haddock, making this the priority of the project. However, due to the difficulty in sequencing these two cytokines, a parallel avenue to the project was agreed. This involved the study of factors involved in the expression of IFN-γ and its downstream effects in a vaccination study in haddock. The following summarises the milestones achieved during this project.

Project milestones

1.1. Searching for cod IFN-γ and IL-2 (Milestone 1)

Homology cloning

IFN-γ and IL-2 are two cytokines involved in the T-helper 1 responses which are commonly used as markers of cell-mediated immune responses in mammals (Dahmen et al., 2002; Smith et al., 2003). Thus, measurement of either IL-2 or IFN-γ, or the

cells producing them, could be used as a marker of vaccine performance, making this a goal of this project.

IFN-γ and IL-2 have been cloned in only a few teleost species such as trout, fugu or zebrafish (Zou et al., 2005; Bird et al., 2005a; Igawa et al., 2006). This, alongside the low level of conservation between fish species, makes the homology cloning of these cytokines relatively difficult. Nevertheless, this method of gene discovery can be highly effective when the target gene has been cloned from other closely related species or when there are high areas of conservation in the gene. Despite designing of primers in areas of high homology, this approach proved unsuccessful for cloning of cod or haddock IL-2 and IFN-γ, and a different approach was adopted.

Synteny

Gene order in chromosomes may be conserved over wide evolutionary distances. This is known as ***synteny***, and to date it has been used on a variety of occasions to facilitate the characterisation of cytokines and other genes from fish species like zebrafish or *Fugu*. By exploiting this conservation between the human and *Fugu* chromosomes, IL-2 and IFN-γ have recently been sequenced (Bird et al., 2005a; Igawa et al., 2006), and could also be a helpful tool for cod/haddock. A variety of genes with a high degree of conservation, located in the surroundings of our target genes, were identified and their sequencing attempted by homology cloning (see Fig. 1). Surrounding IFN-γ the dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2 (DYRK-2), Interleukin-22 (IL-22) and Ubiquitin-protein ligase E3 (MDM2) were targeted. And near IL-2, the testis nuclear RNA-binding protein (TENR), Interleukin-21, and basic fibroblast growth factor-2 (bFGF-2), were identified as good targets to use in this new approach.

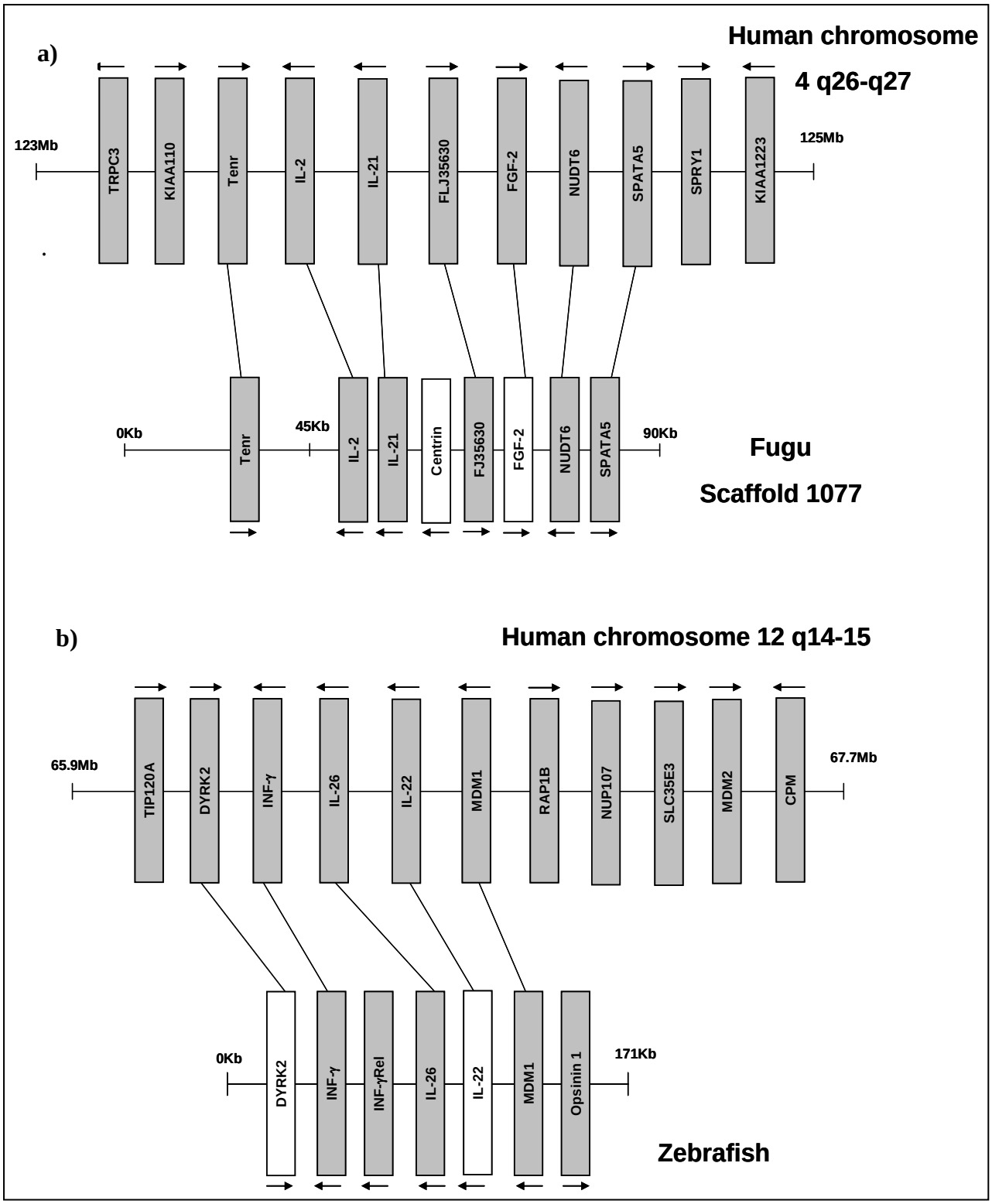


Figure 1. Comparative location map (synteny) between Human and fugu (a) and zebrafish (b) genomes. Arrows represent gene orientation. White blocks represent genes cloned in haddock and/or cod.

FGF2 and DYRK2 were cloned and sequenced in haddock, while Centrin and IL-22 were sequenced in both cod and haddock (see Fig.1). Probes were then designed for Centrin and DYRK2 to be used in haddock Gene Walking libraries produced earlier in the project. These libraries were constructed with high molecular weight haddock genomic DNA, which was then digested using 7 different enzymes, therefore producing 7 libraries. These libraries were then used with probes designed specifically for Centrin and DYRK2 to walk the genome towards the targeted cytokine. Using this method, a total of 2,770 bp were walked from DYRK2 and 2,054bp from Centrin. This was a slow procedure, since only around 500-700bp products were obtained for sequencing at a time, and the distances between the cloned genes and the cytokine of interest were predicted up to 50Kb for IFN-γ and 8Kb for IL-2.

During the project, further funding was allocated for the production of a BAC (Bacterial Artificial Chromosome) library, a system based on *E. coli* F factor (Shizuya et al., 1992). The replication in bacteria containing this F factor is highly controlled, maintaining the plasmid to a minimum number, 1 or 2, so as to minimise the recombination between DNA fragments in the cell. This type of plasmid is also capable of accepting and maintaining large fragments of DNA of up to 300kb, which makes it an ideal method for the construction of DNA libraries for genome analysis. BAC libraries offer the benefit of sequencing fragments of between 100-150kb and faithfully represent the organism's genome, therefore increasing the chances of sequencing targeted genes.

The Cod BAC library was commercially constructed by Amplicon Express, delivering the libraries with a matrix technology, a system that uses pools and superpools for easier screening by PCR. The gene Centrin, only one gene along from IL-2, was used as a marker to identify a clone which will potentially contain IL-2. Two primers were

designed and used as a probe to screen the Pools and Superpools of the BAC library by PCR, where one individual BAC clone was identified. An aliquot of this clone was grown overnight in agar plates and individual colonies were screened by PCR to reconfirm this clone contained Centrin, and therefore potentially IL-2. Glycerol stocks of the BAC clone were then sent for shotgun sequencing. This type of sequencing consists of the breaking of the inserted DNA of the BAC clone, and then the ends of these fragments are overlapped into various continuous sequences called contigs. The analysis of the sequenced contigs showed the presence of Centrin and four more genes downstream, SPATA5, NUDT6, FGF-2, FJ35630 (see Fig. 2). Unfortunately, the sequence of IL-2, upstream of Centrin, was not present in any of the sequenced contigs.

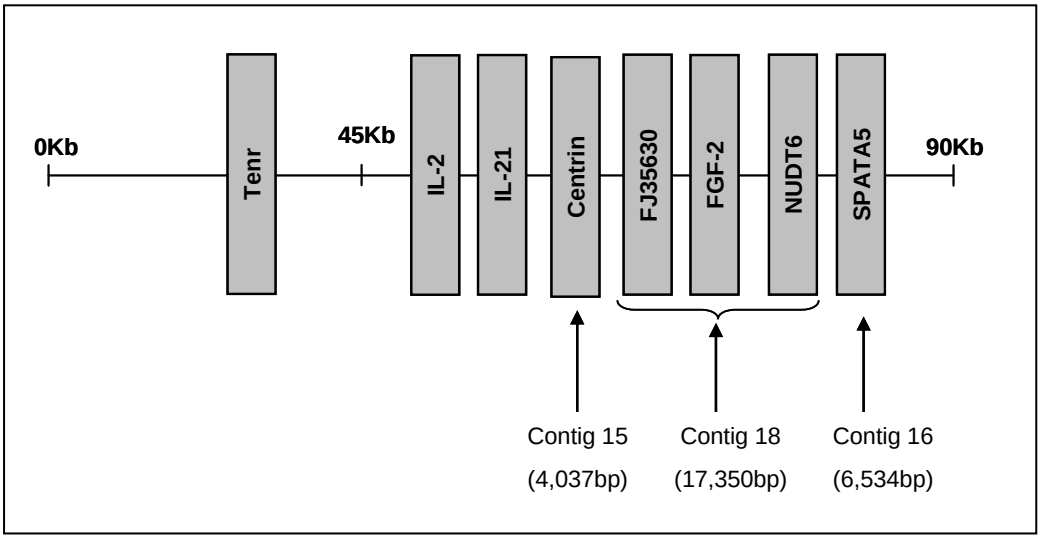


Figure 2. Cod BAC clone sequencing. Using Fugu scaffold 1077 as a template, the sequenced contigs containing the genes in the proximity of IL-2 are indicated with an arrow.

Cod Interferon-gamma (IFN-γ)

As for IL-2, IL-22 probes were designed for the screening of the BAC libraries. However, while these probes were being optimised, a fragment encoding cod IFN-γ

was identified by mining the recently created cod EST database (Cod Genome and Broodstock Project; http://ri.imb.nrc.ca/codgene/). Primers were then designed to obtain the complete transcript of the gene by RACE-PCR and further analysis was carried out to confirm the identification of the gene. The full-length cod IFN-γ cDNA contained 1139bp, of which 549bp are the open reading frame of the gene. The predicted protein of 182aa, contains 6 identifiable alpha helixes, typical of IFN-γ's and the family signature motif present in all other vertebrate IFN-γ molecules. Downstream from this motif was also found a high level of conservation in the nuclear localisation signal (NLS), which is involved in the mediation of the transport of nuclear proteins into the nucleus.

Probes for cod IFN-γ were then designed to screen the BAC libraries alongside probes for IL-22 to further verify this sequence was indeed cod IFN-γ, based on co-localisation of these two genes.

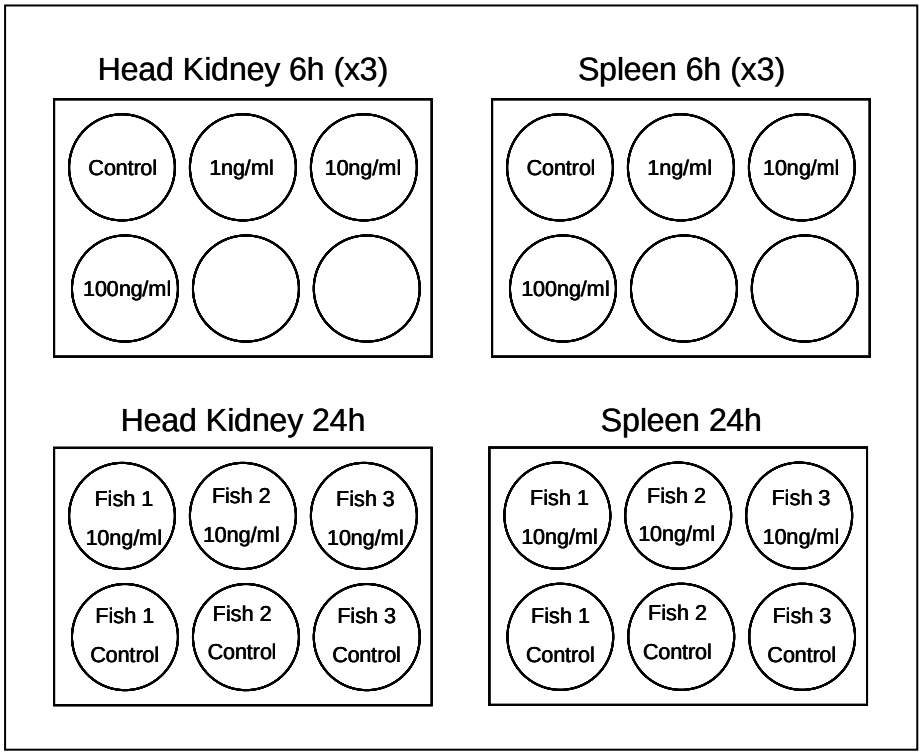
1.2. Production of Recombinant IFN-γ, rIFNγ (Milestone2)

Once the identity of the sequence was confirmed, the mature peptide was predicted and primers spanning this peptide were designed for the production of the recombinant protein. The product obtained with these primers was then ligated into the pQE-30 UA vector (Qiagen) containing an N-terminal 6-Histidine tag necessary to facilitate the purification of the protein. After growing the JM109 competent cells containing the plasmids, an aliquot of the culture was plated in agar plates. Individual colonies were then screened and positive cells selected and grown overnight, alongside a colony containing an empty vector (negative control). Cultures were stimulated with IPTG for 2h to induce the production of the protein, after which a lysate of the stimulated cells was run in an 4-12% SDS-PAGE gel to identify the

induced IFN-γ protein. The colonies containing the rIFNγ were then sequenced to confirm the vector contained the full pQE30-IFNγ. Two of these positive colonies were then chosen for purification under denaturing conditions (in JM109cells) and native conditions (in M15[pREP4] cells). The protein was purified by both methods; however, the best results were obtained under denaturing conditions. Once the protein was refolded and its concentration measured using a BCA protein assay, its bioactivity was assessed.

To determine if the cod-rIFNγ was active, an experiment using primary cultures of head kidney and spleen was set up. This experiment was considered an important step prior to antibody generation, since lack of activity could reflect improper folding of the molecule. The experiment (see Fig. 3A) included three different concentrations of the rIFN- γ (1ng/ml, 10ng/ml and 100ng/ml) and a control containing the elution buffer in which the protein was eluted, where cells were stimulated for 6h. A further experiment looked at the stimulatory effect of an individual dose of rIFN- γ, 10ng/ml, after 24h of stimulation. The bioactivity of the protein was then studied in terms of its ability to affect expression of TAP1, a gene know to be up-regulated by IFN-γ . RT-PCR analysis from the head kidney cells stimulated with different doses of rIFN-γ, showed a small increase in the expression of TAP1 when 10ng/ml and 100ng/ml of the recombinant protein was added to the cells (Fig 3B), indicating that the rIFN-γ is most likely correctly folded and active. However, further analysis will be required to fully prove its activity.

A)



B)

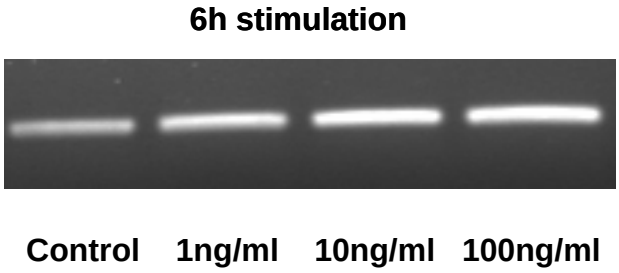


Figure 3. Bioactivity of cod-rIFN-γ experiment. Three doses of cod-rIFN-γ were set up for the 6h experiment, 1ng/ml, 10ng/ml, 100ng/ml alongside a control containing the same volume of Elution buffer in which the protein was eluted. An individual dose of 10ng/ml was used for an incubation of 24h. Each experiment was replicated using 3 fish. Activity was studied in head kidney in terms of expression of TAP-1 gene by RT-PCR.

As agreed during the project and due to the difficulties in cloning IL-2 and IFN-γ at the time, a parallel avenue to the projected work was carried out. The overall goal to develop immunological markers that enable identification of efficacious vaccines was unchanged. However, since it was considered that the development of an ELISpot

would not be possible due to time limitations, this change involved the study of factors involved in the expression of IFN-γ and its downstream effects during a vaccination experiment.

The cell-mediated immune responses (i.e., IFN-γ expression) can be measured indirectly using a variety of transcription factors known to be important for Th cell differentiation, or genes turned on by IFN-γ. In both cases relevant genes have already been identified in fish. For example, T-bet is a Th1 cell-specific transcription factor that controls the expression of IFN-γ whilst GATA-3 regulates Th2 cell differentiation, and inhibits IFN-γ expression. Since these transcription factors regulate gene expression by binding to the promoter elements upstream of genes and either facilitate or inhibit transcription, they have a relatively high level of conservation. This conservation allowed the use of homology cloning to obtain GATA-3 in haddock from the known fish and mammalian sequences. Following induction of IFN-γ, two genes which are part of the MHC class I pathway, LMP2 and TAP1, are known to be up-regulated. LMP2 along with LMP7 form a proteosome which degrades proteins into peptides. These peptides are then transported from the cytosol to the endoplasmic reticulum by the complex formed by TAP1/TAP2 and then associate to the MHC class I molecule. These two genes, as in the case of the transcription factors, have a high degree of conservation. Therefore homology cloning was also used to sequence these genes in haddock.

The designed vaccination trial was aimed at the study of the expression of GATA-3, TAP1 and LMP2. Once IFN-γ was sequenced in cod, this cytokine was also included as part of the study. Since this experiment was carried out with haddock, primers were designed against cod IFN-γ and the gene was sequenced in haddock. However, in addition it became apparent during the course of these studies that other populations

exists in mammals that drives responses to extracellular pathogens. Therefore analysis of the expression of further genes was also included in this study.

Vaccination was carried out at the Ardtoe Marine Laboratory where fish were kept at their facilities during the immunisation period of 2 months. This experiment consisted of 180 haddock divided into 3 treatments. The first group was immunised with an intraperitoneal injection of 10^9cfu/ml of formalin-killed *Vibrio anguillarum* (*HVang*), a dose proven to induce protection to the fish after bacterial challenge in previous studies (Corripio-Miyar et al., 2007b). The remaining two groups, *LVang* and *Control*, consisted of an injection of a low dose of the formalin-killed vaccine (10^3cfu/ml) and phosphate buffer saline (PBS). Two months after starting the trial, fish were transported to Aberdeen. After a week of acclimatisation, fish were challenged. Bacterial challenge consisted of a bath containing 10^7cfu/ml *V. anguillarum* MT2582 for a period of 30 min. Samples from gill and head kidney were collected from 5 fish per treatment throughout the immunisation period, 48h, 1, 2 and 4 weeks post-vaccination and 24, 48 and 72 h post-challenge.

Once fish were challenged, they were divided into two tanks to avoid tank effect. Mortalities started to occur on day 4 post-challenge, and the experiment was terminated once mortalities ceased, on day 9. As expected, highest mortalities were found in the control group, which reached 48%. Vaccinated fish had lower levels of mortality, with a relative percentage of survival (RPS) of 75% in *LVang*, while none of the fish from the *HVang* group died, therefore reaching an 100% RPS (see Fig. 4).

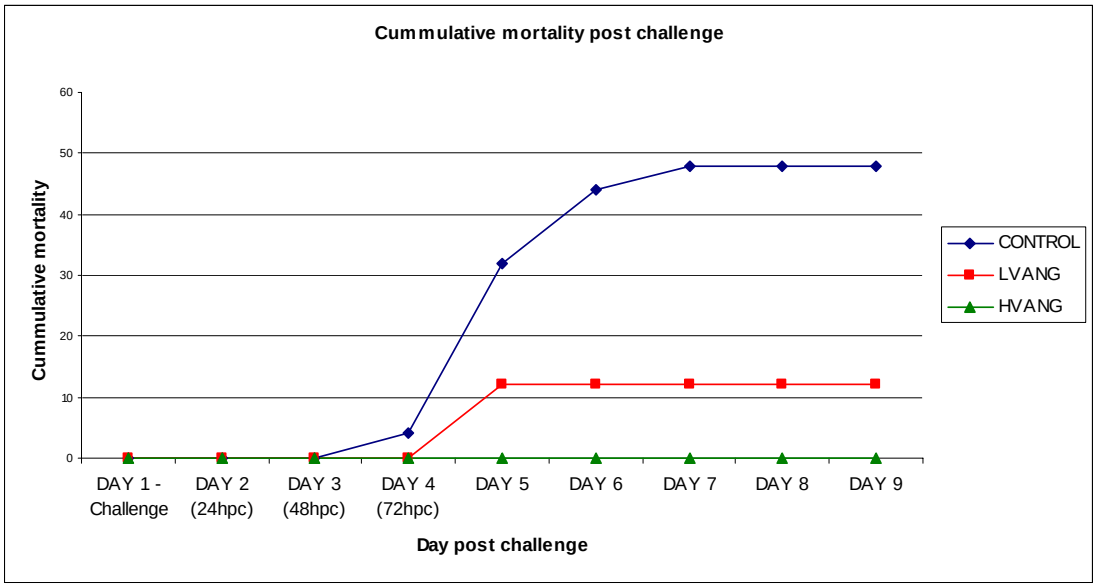
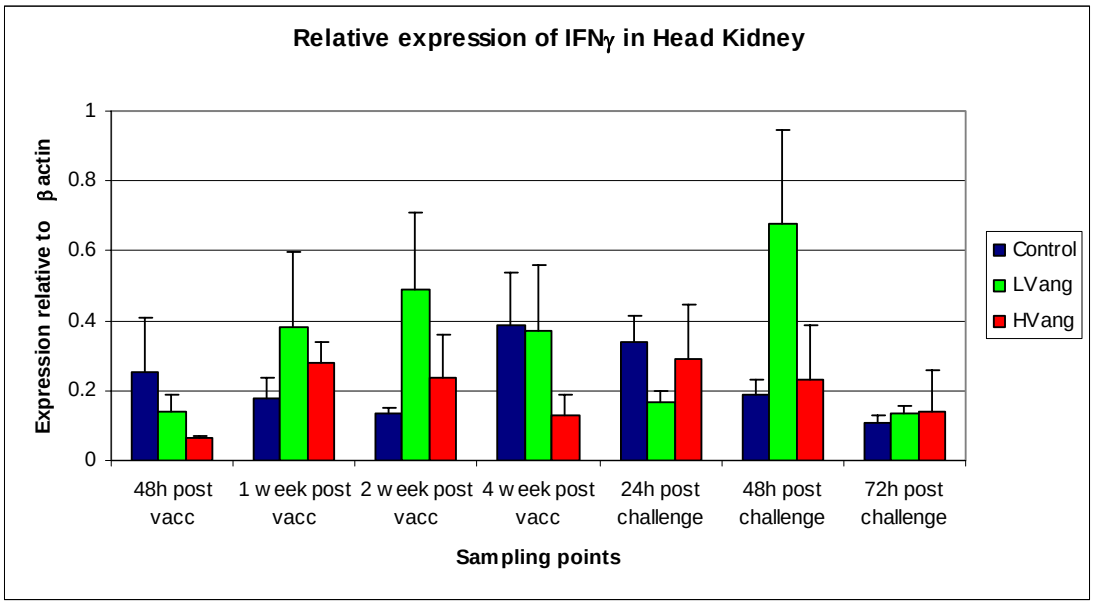


Figure 4. Cumulative mortality of haddock, *Melanogrammus aeglefinus*. Bacterial challenge with *Vibrio anguillarum* (MT2582) delivered by immersion in a bath containing 10^7cfu/ml for 30min. Treatments consisted of Control: injection of 100ul/ml of PBS, LVANG: intraperitoneal injection with 10^3cfu/ml and HVANG intraperitoneal injection with 10^9cfu/ml.

All collected samples were used in real-time PCR analysis to look at the expression of IFN-γ. The expression of IFN-γ relative to the housekeeping gene, βactin, showed no significant effect of either of the treatments on the levels of expression of this Th1 cytokine in the head kidney (Fig. 5A) or the gill (Fig. 5B). These results indicate that this might not be the most appropriate cytokine to use as a marker of vaccine efficacy in case of *V. anguillarum*. However, it is not possible to rule out that increased expression of IFN-γ could be found at other tissue sites not studied here.

A)



B)

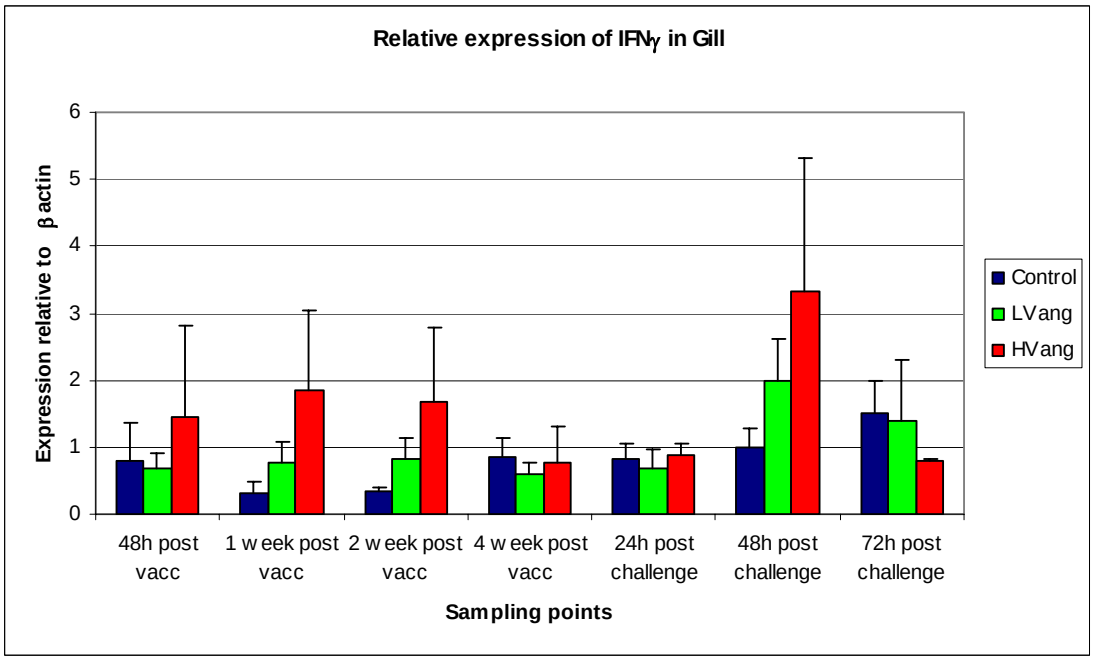


Fig 5. Real-time PCR analysis of the expression of haddock IFNγ relative to the housekeeping gene β-actin, in head kidney (A) and gill (B). Treatments consisted of Control: injection of 100ul/ml of PBS, LVang: intraperitoneal injection with 10^3cfu/ml and HVang: intraperitoneal injection with 10^9cfu/ml. sampling points indicated are 48h, 1week, 2weeks and 4 weeks post-vaccination and 24h, 48h, 72h post challenge. Bars indicate the mean + standard error (n=5).

1.4. Other cytokine responses to vaccination

As stated above, whilst this project was on-going the existence of a new cell population involved with immunity to extracellular microbes became apparent. Thus, samples from gill and head kidney taken during the vaccination experiment were analysed by real-time PCR using specific primers for further cytokines and whilst no significant difference was found between treatments using head kidney samples challenged fish had a four-fold increase in gene expression in the gills.

This result can be interpreted as showing the ability of fish vaccinated with the optimal dose (HVang) to mount an extremely quick response when encountered with the pathogen, allowing them to protect themselves from disease. Conversely, non-immunised fish appear to require a longer time to mount a response towards the pathogen, and by the time this happens, infection has already taken place and a proportion of the fish succumbed to the disease.

Future work

With the advances obtained in this project, we have now opened new avenues for the study of the gadoid immune system that would allow us to determine vaccine efficacy. The study of cell-mediated responses in gadoids is key to reducing the suffering of fish during vaccine trials. Using the cytokines cloned in this project, we could determine the presence of cells producing and secreting cytokines associated with protective responses. Furthermore, with a tool as powerful as the cod BAC library, we could attempt to sequence other cytokines associated with Th2 (IL-4) and Th17 (IL-17) cells to study responses to immunisation in cod and assess their production after vaccination in an attempt to correlate *in vitro* Th responses with protection in fish. The production of these key cytokines by fish can be measured by

the use of two techniques, flow cytometry and PCR, which could complement each other and give us a clear picture of the types of responses elicited in gadoid fish given efficacious vaccines. These types of studies are particularly important for fish species such as gadoids, for which no antibody based tests of vaccine efficacy are possible. However, this would serve more generally as proof in principle of this approach for fish, advancing the understanding of not only gadoid immunology but also fish immunology in general. By allowing the measurement of relevant protective immune responses it should be possible to undertake a great deal of vaccine optimisation without the need for lethal challenge experiments using virulent pathogens, thus reducing fish suffering during future vaccine development studies in accordance with the principal of the three 3R's.

Conclusions – Resources generated and Summary of deliverables

- Cloning and sequencing of the Th1 cytokine, IFN-γ in cod and haddock.
- Production of cod recombinant IFN-γ
- Cloning and sequencing of other relevant cytokines in cod and haddock
- Cloning and sequencing of haddock/cod:

    1) Th2 transcription factor GATA-3

    2) Genes associated with the downstream effects of IFN-g activation: TAP-1, LMP2.

    3) Interferon gamma inducible protein, γIP-10.

    4) The genes surrounding IL-2 and IFN-γ: Centrin, FGF-2 and DYRK2.

    5) Optimisation of real-time PCR for IFN-γ.

- Haddock vaccination experiment comparing a highly efficacious vaccine with a vaccine of low efficacy and non-vaccinated fish. Cytokine expression post immunisation and post bacterial challenge, and DNA/tissues samples stored.
- Production of haddock gene walking libraries and cod BAC library, highly useful tools for future projects on gadoid fish.
- Final outcome will be the submission of three scientific publications from the work carried out in this project.

References

Bird S., Zou J., Kono T., Sakai M., Dijkstra J. M. and Secombes C. (2005a) Characterisation and expression analysis of interleukin 2 (IL-2) and IL-21 homologues in the Japanese pufferfish, *Fugu rubripes*, following their discovery by synteny. *Immunogenetics* **56**, 909-923.

Corripio-Miyar Y., Bird S., Tsamopoulos K. and Secombes C. J. (2007a) Cloning and expression analysis of two pro-inflammatory cytokines, IL-1β and IL-8, in haddock (*Melanogrammus aeglefinus*). *Molecular Immunology* **44**, 1361-1373.

Corripio-Miyar Y., Mazorra de Quero C., Treasurer J. W., Ford L., Smith P. D. and Secombes C. J. (2007b) Vaccination experiments in the gadoid haddock, *Melanogrammus aeglefinus* L., against the bacterial pathogen *Vibrio anguillarum*. *Veterinary Immunology and Immunopathology* **118**, 147-153.

Dahmen A., Herzog-Hauff S., Böcher W. O., Galle P. R. and Löhr H. F. (2002) Clinical and immunological efficacy of intradermal vaccine plus lamivudine with or without interleukin-2 in patients with chronic hepatitis B. *Journal of Medical Virology* **66**, 452-460.

Espelid S., Rødseth O. M. and Jørgensen T. Ø. (1991) Vaccination experiments of the humoral immune responses in cod, *Gadus morhua* L., to four strains of monoclonal-defined *Vibrio anguillarum. Journal of Fish Diseases.* **14**, 185-197.

Igawa D., Sakai M. and Savan R. (2006) An unexpected discovery of two interferon gamma-like genes along with interleukin (IL)-22 and -26 from teleost: IL-22 and -26 genes have been described for the first time outside mammals. *Molecular Immunology* **43**, 999-1009.

Seppola M., Larsen A. N., Steiro K., Robertsen B. and Jensen I. (2008) Characterisation and expression analysis of the interleukin genes, IL-1[beta], IL-8 and IL-10, in Atlantic cod (Gadus morhua L.). *Molecular Immunology* **45**, 887-897.

Shizuya H., Birren B., Kim U., Mancino V., Slepak T., Tachiiri Y. and Simon M. (1992) Cloning and Stable Maintenance of 300-Kilobase-Pair Fragments of

Human DNA in Escherichia coli Using an F-Factor-Based Vector. *Proceedings of the National Academy of Sciences* **89**, 8794-8797.

Smith J. G., Levin M., Vessey R., Chan I. S. F., Hayward A. R., Liu X., Kaufhold R. M., Clair J., Chalikonda I., Chan C., Bernard M., Wang W. W., Keller P. and Caulfield M. J. (2003) Measurement of cell-mediated immunity with a varicella-zoster virus-specific interferon. *Journal of Medical Virology* **70**, S38-S41.

Zou J., Carrington A., Collet B., Dijkstra J. M., Yoshiura Y., Bols N. and Secombes C. (2005) Identification and bioactivities of IFN-gamma in Rainbow trout *Oncorhynchus mykiss*: The first Th1-type cytokine characterized functionally in fish. *J Immunol* **175**, 2484-2494.