DAFINET
Workshop

IMMUNE RESPONSE IN EARLY DEVELOPMENTAL STAGES OF FISH

November 2nd & 3rd, 2011

Venue:

Lecture Theatre 1-01
Bülowsvej 17
1870 Frederiksberg
Denmark

Organised by:

Danish Fish Immunology Research Centre and Network
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University of Copenhagen
Faculty of Life Science

Book of abstracts

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DAFINET WORKSHOP
Danish Fish Immunology Research Centre and Network

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A TWO DAY WORKSHOP

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Denmark

Programme

Wednesday November 2nd, 2011

10.00 DAFINET Board meeting 10.00-12.00. Only for board members

Scientific program

13.00 Welcome address by DAFINET leader Kurt Buchmann

13.15 Research director Scott LaPatra, Clear Springs Foods, Inc., Idaho, USA
Vaccination strategies in the trout farming industries

14.00 Post doc Lars Holten-Andersen, KU-LIFE, Copenhagen, Denmark
Resistance of salmonids against *Aeromonas salmonicida*: Host genetics as a main player

14.30 Ph.D. student Jiwan Kumar Chettri, KU-LIFE, Copenhagen, Denmark
Immune response of rainbow trout (*Oncorhynchus mykiss*) at early developmental stages (larvae and fry) against the bacterial pathogen *Yersinia ruckeri*

15.00 Coffee break

15.30 Professor **Chris Secombes**, School of Biological Sciences, University of Aberdeen, Scotland
The cytokine families

16.00 Ph.D. student Qusay Bahlool, KU-LIFE, Copenhagen, Denmark
Microhabitat preference of *Anisakis simplex* in three salmonid species: Immunological implications

16.20 Ph.D. student Dennis Bela-Ong, DTU-VET, Denmark
Correlation of mRNA and micro-RNA profiles and functional immune response in rainbow trout (*Oncorhynchus mykiss*) during infection with viral hemorrhagic septicemia virus (VHS) and in fish vaccinated with a DNA vaccine against VHSV

16.40 Ph.D. student Jakob Skov, KU-LIFE, Denmark
Immunostimulants in fish feed: Do they improve vaccination success?

17. 00 Wrap up by Kurt Buchmann

18.00 Dinner at Falconer Center, Falkoner Alle 9, 1870 Frederiksberg

Programme
Thursday November 3rd, 2011

10.00	Senior research scientist Bertrand Collet, Marine Laboratory, Aberdeen, Scotland In vitro models for immunity
10.30	Senior research scientist Katja Einer-Jensen, DTU-VET, Denmark Temperature influences the expression profiling of immune response genes in rainbow trout following DNA vaccination and VHS virus infection
11.00	Post doc Anders Østergaard, Wageningen University, The Netherlands Molecular characterization and genomic organisation of Novel Immunoglobulin-Like Transcripts (NILTs) in teleost fish
11.30	Post doc Mikkel-Ole Skjødt, University Hospital of Copenhagen, Denmark Crystalstructure and interaction properties of the novel MBL/Ficolin associated protein-1
12.00	**Lunch at Gimle**
13.00	Research assistant Ellen Lorenzen, DTU-VET, Denmark Insufficient protection of rainbow trout against furunculosis by commercial vaccines under experimental conditions
13.30	Ph.D. student Sekar Larashati, DTU-VET, Denmark Inhibition of reporter genes by small interfering RNAs in cell culture and living fish
13.50	Ph.D. Anders Stegmann, DTU-VET, Denmark Search for genetic virulence markers in viral haemorrhagic septicaemia virus (VHS) using a reverse genetics approach
14.10	Ph.D. student Anna Amanda Schönherz, DTU-VET, Denmark Oral transmission of viral haemorrhagic septicaemia virus in juvenile rainbow trout
14.30	**Coffee break**
15.00	Associate professor Martin Kristian Raida, KU-LIFE, Copenhagen, Denmark Vaccination against enteric red mouth disease caused by the bacterium *Yersinia ruckeri*
15.20	**Final discussion and conclusions of the workshop**

Abstracts

VACCINATION STRATEGIES IN THE TROUT FARMING INDUSTRIES

Scott E. LaPatra

Clear Springs Foods, Inc., Research Division,PO Box 712, Buhl, Idaho, 83316 USA

Vaccination is the most effective method of combating disease and currently there are a number of vaccines commercially available for use in fish. The majority of aquatic vaccines are delivered by injection, which is by far the most effective method when compared to oral or immersion deliveries. However it is labor intensive, costly and not feasible for large numbers of fish under 20 g. It may be possible to improve upon the efficacy of injection delivery. The highly efficacious DNA vaccines against the fish Novirhabdoviruses require intramuscular (i.m.) delivery to be effective, all other potential delivery strategies do not elicit the level of protection seen when these vaccines are delivered i.m.. It appears that the injection site within the muscle is not limited to the epaxial muscle around the dorsal fin as three alternative intramuscular delivery sites have also been shown to be equally efficacious. Additionally, an attenuated infectious hematopoietic necrosis virus (IHNV) vaccine was found to be equally efficacious as the IHNV DNA vaccine when delivered intramuscularly. This suggests that the muscle site generally may provide enhanced efficacy for other types of vaccines too and may warrant further investigation. Currently effective immersion vaccines are limited to only a handful of pathogens but ultrasound could offer some improvements. Advances have been made in oral delivery of vaccines to fish but to date there are still no effective methods commonly available. Attempts to develop novel oral and immersion delivery methods have resulted in varying degrees of success but may have great potential for the future.

Recently, a series of studies were undertaken in rainbow trout (mean weight, 2 -3 g) to assess if vaccination against a specific pathogen may induce some level of protection against other pathogens of rainbow trout known to cause acute disease. Live and killed vaccines against viral and bacterial pathogens were examined after delivery by immersion, intraperitoneal injection and orally on top coated feed. Additionally, challenge evaluations were conducted primarily with *Flavobacterium psychrophilum*, the causative agent of bacterial cold water disease (BCWD) and IHNV. Standardized challenges were conducted at various intervals post-vaccination including ~100, 400 and 800 degree days (dd) which equates to 7, 28 and 56 days post-vaccination at a water temperature of 14.5^{o} C.

As an example of the type of results that were observed, intraperitoneal injection of separate groups of rainbow trout with killed vaccines against *Vibrio angullarum* and *Yersinia ruckeri* reduced BCWD challenge mortality but increased the susceptibility of fish to IHN at 100 dd post-vaccination. When the challenge was repeated at 400 dd on additional *V. angullarum* and *Y. ruckeri* vaccinated fish, the IHN mortality was reduced in the vaccinates compared to the mock vaccinated controls while no differences in susceptibility were observed with BCWD.

Model systems were developed using immersion and oral vaccinations with live and killed vaccines that could be used to further analyze the functionality of mucosal immunity. Recently it was reported that in rainbow trout IgT is an immunoglobulin specialized in gut mucosal immunity. Studies carried out to date have not examined the specific contribution of IgT in protecting fish and it may be an important indicator of the potential efficacy of oral and immersion delivered vaccines.

E-mail: scottl@clearsprings.com

RESISTANCE OF SALMONIDS AGAINST *AEROMONAS SALMONICIDA*: HOST GENETICS AS A MAIN PLAYER

Lars Holten-Andersen[1,2], Inger Dalsgaard[2], Kurt Buchmann[1]

[1] *Laboratory of Aquatic Pathobiology, Section of Biomedicine, Department of Veterinary Disease Biology Faculty of Life Sciences, University of Copenhagen, Frederiksberg, Denmark*

[2]*National Veterinary Institute, Technical University of Denmark, Copenhagen V, Denmark*

Furunculosis, caused by *Aeromonas salmonicida*, continues to be a health problem for the growing salmonid aquaculture. Despite effective vaccination programs regular outbreaks occur at Danish trout farms calling for repeated antibiotic treatment. We hypothesized that a difference in natural susceptibility to this disease might exist between Baltic salmon and the widely used rainbow trout. Hence, a cohabitation challenge model was applied to investigate the relative susceptibility to infection with *Aeromonas salmonicida* in rainbow trout and Baltic salmon. The course of infection was monitored daily over a 30-day period post challenge and the results were summarized in mortality curves.

A. salmonicida was recovered from mortalities during the entire test period. At day 30 the survival was 6.2 % and 34.0 % for rainbow trout and Baltic salmon, respectively. Significant differences in susceptibility to *A. salmonicida* were demonstrated between the two salmonids and hazard ratio estimation between rainbow trout and Baltic salmon showed a 3.36 higher risk of dying from the infection in the former.

The finding that Baltic salmon carries a high level of natural resistance to furunculosis might raise new possibilities for salmonid aquaculture in terms of minimizing disease outbreaks and the use of antibiotics.

E-mail: lhoa@life.ku.dk

IMMUNE RESPONSE OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) AT EARLY DEVELOPMENTAL STAGES (LARVAE AND FRY) AGAINST THE BACTERIAL PATHOGEN *YERSINIA RUCKERI*

Jiwan K. Chettri, Martin K. Raida, Per W. Kania and Kurt Buchmann

Laboratory of Aquatic Pathobiology, Section of Biomedicine, Department of Veterinary Disease Biology Faculty of Life Sciences, University of Copenhagen, Frederiksberg, Denmark

Innate immune factors play a crucial role in survival of young fish especially during early stages of life when adaptive immunity is not fully developed. In the present study, we investigated the immune response of rainbow trout (Oncorhynchus mykiss) larvae and fry at an early stage of development. We exposed 17 and 87 days post hatch larvae and fry (152 and 1118 degree days post hatch; avg. wt. 70 and 770 mg, respectively) to the bacterial pathogen, Yersinia ruckeri for 4 h by bath challenge. Samples were taken at 4, 24, 72 and 96 h post exposure for qPCR and immunohistochemical analyses to elucidate the immune response mounted by these young fish.

Larvae showed no mortality, although infected larvae at 48 h post exposure showed hyperaemia in the mouth region and inflammation on the dorsal side of the body. Gene expression studies showed an up-regulation of iNOS and IL-22 in infected larvae 24 h post exposure but most of the investigated genes did not show any difference between infected and uninfected larvae. Immunohistochemical studies demonstrated a high expression of IgT molecules in gills and CD8 positive cells in thymus of both infected and uninfected larvae. Infection of rainbow trout fry with Y. ruckeri, in contrast, induced a cumulative mortality of 74%. A high expression of cytokines (IL-1β, TNF-α, IL-22, IL-8 and IL-10), acute phase proteins (SAA, hepcidin, transferrin and precerebellin), complement factors (C3, C5 and factor B), antimicrobial peptide (cathelicidin-2) and iNOS was found in infected fry when compared to the uninfected control. IgT molecules and mannose binding lectins in gills of both infected and uninfected fry were detected by immunohistochemistry. The study indicated that early life stages (yolk-sac larvae), merely up-regulate a few genes and suggests a limited capacity of larvae to mount an immune response by gene regulation at the transcriptional level. Based on the observed clearance of bacteria and lack of mortality it could be speculated that larvae may be covered by protective shield of different immune factors providing protection against broad range of pathogens. However, the increased susceptibility of older fry suggests that Y. ruckeri may utilize some of the immune elements to enter the naïve fish. The up-regulation of iNOS and IL-22 in the infected larvae implicates an important role of these molecules in immune response at early developmental stages. A dense covering of surfaces of gill filaments by IgT antibody in the young fish suggest a role of this antibody as innate immune factor at early developmental stages.

E-mail: jkc@life.ku.dk

A RE-EXAMINATION OF PROINFLAMMATORY CYTOKINE FAMILIES IN SALMONID FISH

Chris Secombes,

Scottish Fish Immunology Research Centre, University of Aberdeen, Aberdeen, UK.

The classical cytokine cascade in response to Gram negative bacteria consists of induction of IL-1β, followed by TNF-α and then IL-6, which serve to induce downstream antimicrobial defences. The expression and function of IL-1β and TNF-α in salmonids has been well known for some time, and duplicated genes, probably as a consequence of recent genome duplication events, are known. However, it now seems likely that the more ancient teleost wide genome duplication event may have also led to additional genes in both of these important proinflammatory cytokine families. Recent advances in the discovery of additional IL-1β and TNF-α genes, together with recent studies of IL-6 and IL-6R expression and function in rainbow trout will be outlined in this talk.

E-mail: c.secombes@abdn.ac.uk

MICROHABITAT PREFERENCE OF *ANISAKIS SIMPLEX* IN THREE SALMONID SPECIES: IMMUNOLOGICAL IMPLICATIONS

Qusay Z. M. Bahlool, Kurt Buchmann

Laboratory of Aquatic Pathobiology, Section of Biomedicine, Department of Veterinary Disease Biology Faculty of Life Sciences, University of Copenhagen, Frederiksberg, Denmark

Third stage larvae of *Anisakis simplex* nematodes are considered to have a low host-specificity and are able to infect a wide range of fish species. However, the physiological and immunological status of the fish species may affect the fate of the worm following infection. We selected three different salmonid species to investigate the in vivo behavioural difference of experimentally inoculated *Anisakis* parasite inside these fishes. Rainbow trout (*Oncorhynchus mykiss*), Baltic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) were used in this experiment. Infection success differed between species. Baltic salmon showed a higher number of nematodes successfully established, whereas brown trout and rainbow trout showed a higher natural resistance. Microhabitat results were also different according to the fish species. *Anisakis simplex* found in brown trout were attached to the digestive tract (stomach, intestine), while the majority of larvae found in rainbow trout were located between the pyloric caeca. In Baltic salmon, nematodes were dispersed and attached to different organs (e.g. spleen, swim bladder) and partially penetrating others (liver, muscles). Encapsulating and inflammatory cellular reactions differed accordingly. *Anisakis* larvae found both in rainbow trout and brown trout were not fully encapsulated until day 28 post infection but merely partially encapsulated at day 21 post-infection. In contrast, all nematodes larvae retrieved from Baltic salmon were fully encapsulated already at day 21 post infection. Immunohistochemical studies using monoclonal antibodies raised against IgT, IgM, CD8 and MHCII were used to detect the presence of immune cells around the infecting nematodes. None of the three fish species showed positive reactions for IgT or IgM bearing cells located in the inflammatory tissue found in connection with the worms. CD8+ cells were detected in all three species but MHC II bearing cells were only found associated with encapsulated *Anisakis* in rainbow trout and brown trout, but not in Baltic salmon. In this study we have shown that *Anisakis* nematodes show a site predilection following infection depending on the host species and the immunological/physiological implications will be discussed.

E-mail: qusay@life.ku.dk

CORRELATION OF MRNA AND MICRO-RNA PROFILES AND FUNCTIONAL IMMUNE RESPONSE IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) DURING INFECTION WITH VIRAL HEMORRHAGIC SEPTICEMIA VIRUS (VHS) AND IN FISH VACCINATED WITH A DNA VACCINE AGAINST VHSV

Dennis B. Bela-ong*[1], Brian Dall Schyth[1], Hanne Jørgensen[2], Mette H. Hansen[2], Mark Henryon[2], Peer Berg[2], and Niels Lorenzen[1]

[1]*Department of Poultry, Fish, and Fur Animals, National Veterinary Institute, Technical University of Denmark, Denmark*

[2]*Department of Genetics and Biotechnology, Faculty of Agricultural Sciences, University of Århus, Denmark*

Micro ribonucleic acids (miRNAs) are a diverse class of small (18-22 nucleotides) endogenous RNAs that potently mediate post-transcriptional silencing of a wide range of genes and are emerging as critical regulators of cellular processes. They are transcribed and processed from larger precursors and are incorporated into the RNA-Induced Silencing Complex (RISC), which target specific mRNA sequences, causing either mRNA degradation or translation repression. This results in altered mRNA and protein profiles characteristic of a particular cellular phenotype or physiological state. By targeting immune relevant mRNAs, miRNAs could be involved in controlling the expression of fish immune response genes.

This project aims to analyze mRNA and miRNA expression in organs of vaccinated and non-vaccinated rainbow trout (*Oncorhynchus mykiss*) families showing differential mortality in previous infection trials with the highly pathogenic fish rhabdovirus *Viral hemorrhagic septicemia virus* (VHSV). This talk will discuss our overall strategy and present preliminary data on the expression of miRNAs and the type I interferon-inducible Mx gene in the liver and the skeletal muscle tissue of fish injected with a DNA vaccine encoding the VHSV glycoprotein gene.

We will link mRNA and miRNA profiles with phenotypic, genotypic, and immunological data, which will provide an integrated view of the mechanisms of resistance and the strong protective immune responses provided by vaccination. This information is important in designing effective strategies to mitigate the danger of potential VHS disease outbreaks.

E-mail: debo@vet.dtu.dk

IMMUNOSTIMULANTS IN FISH FEED: DO THEY IMPROVE VACCINATION SUCCESS?

Jakob Skov, Per Walther Kania and Kurt Buchmann

Laboratory of Aquatic Pathobiology, Section of Biomedicine, Department of Veterinary Disease Biology Faculty of Life Sciences, University of Copenhagen, Frederiksberg, Denmark

β-Glucan is represented by a diverse group of linear and branched polysaccharides from a variety of sources (i.e. bacteria, fungi, algae and plants) and has been extensively studied as an immunostimulatory additive in fish feed. Several studies have demonstrated an immunostimulatory effect of orally administered β-glucan resulting in both increased innate and adaptive responses as well as increased resistance to experimental infection. However, the potential of β-glucan for improving vaccination success has received limited attention.

We have examined the effect a linear, pure (purity ≥ 98%) β-glucan from an alga (Euglena gracilis) included in feed for rainbow trout (Oncorhynchus mykiss) in combination with an immersion vaccine against enteric redmouth disease (ERM) caused by the bacterium, Yersinia ruckeri. The inclusion level of β-glucan in feed was 1% and fish were fed 1% their biomass every day for 84 days. Fish were immersion vaccinated after 2 weeks of feeding (day 14) and bath challenged with live Y. ruckeri 6 weeks later (day 56). Blood and head kidney were sampled at day 0, 13 (1 day pre-vacc.), 15, 55, 59 (day 3 post challenge (p.c.)), 70 and 84. The vaccine proved to be effective resulting in increased survival p.c. ($p = 0.0002$). No significant effect of the β-glucan in vaccinated fish was observed for the following parameters: survival p.c., and lysozyme activity and antibodies in plasma. Quantitative PCR gene expression analysis of head kidney tissue showed barely detectable levels of complement factor 3 (C3) and IL-6 and no regulation of IgM, IgT, MHC I, MHC II, TCR-β, CD4 and CD8. Pronounced and significant up-regulations of IL-1β, INF-γ, TNF-α, SAA, precerebellin and hepcidin were observed in unvaccinated fish post-challenge, mainly restricted to day 3 p.c. ($p < 0.01$). However, these up-regulations in unvaccinated fish were not significantly affected by β-glucan. In contrast, a significant effect of β-glucan was observed in vaccinated fish post-challenge in the regulation of IL-1β (day 3 p.c.), INF-γ (day 14 p.c.), SAA (day 3, 14 and 28 p.c.), precerebellin (day 3 p.c.), hepcidin (day 3 p.c.), and the gene encoding lysozyme (day 14 p.c.) ($p < 0.001$ for all differences observed). Common for these significant differences was a lack of regulation or a down-regulation in vaccinated fish receiving β-glucan compared to vaccinated controls. As an example, the pro-inflammatory cytokine IL-1β was down-regulated (4.0 fold) in vaccinated fish receiving β-glucan (day 3 p.c.) compared to an up-regulation (5.1 fold) in vaccinated controls, whereas unvaccinated fish showed a pronounced up-regulation of 91.9-96.9 fold. Interestingly, the lysozyme gene was the only gene significantly up-regulated in head kidney before challenge. This up-regulation occurred in β-glucan fed fish at day 13 (1 day before vaccination).

In conclusion, the present data showed no improving effect of β-glucan on vaccination success in terms of the most important parameter, i.e. survival following infection. Nevertheless, the significant differences apparently induced by β-glucan on gene expression level in vaccinated fish points towards a potential positive effect of orally administered β-glucan in fish combined with vaccination. However, further experiments are required to elucidate the effect of β-glucan on vaccination.

E-mail: jask@life.ku.dk

IN VITRO MODELS FOR IMMUNITY

Bertrand Collet, Katy Urquhart, Catherine Collins, Katherine Lester, [*]Steve Bird

Marine Scotland, Marine Laboratory, Aberdeen, UK.
[*] University of Aberdeen, Aberdeen, UK

The ability to modify the genome of fish cell lines is a valuable technology that can lead to the development of novel tools for the diagnostic of fish diseases, the monitoring of immune response and the characterisation of pathogens. Over the years we have engineered several stable transgenic cells lines derived from the rainbow trout gonads RTG-2, the Atlantic salmon kidney ASK-1 or the embryonic Chinook salmon CHSE cell lines. Four projects involving the development of stable recombinant fish cell lines with application ranging from viral diagnostic to monitoring the immune response will be presented.

The RTG-P1 cell line, isolated in 2003, expresses a reporter gene (firefly luciferase) under the control of the promoter for the interferon (IFN)-induced antiviral Mx gene. It was initially developed to measure the IFN activity in blood but we have found recently that this cell line responds to infection with a wide range of fish viruses and can be used to detect the presence of viral agents in serum or tissue homogenates. This is particularly useful for the detection of emerging viral disease or the characterisation of disease with unknown aetiology.

The CHSE-TOF5-MX8 cell line over-expresses the antiviral protein Mx1 and was used to measure the sensitivity of different strains of different categories of fish viruses to Mx as predictors of virulence.

To measure the fusion activity of some surface viral proteins, a dual inducible expression plasmid have been constructed and tested in fish cells. Two recombinant CHSE cell lines expressing two surface proteins isolated from Infectious Salmon Anaemia Virus (ISAV): F isolated from a virulent strain and the HE protein from either an avirulent or virulent strain. The comparison of the fusion abilities of these two cell lines will inform on the ability of the avirulent HE protein to induce membrane fusion and achieve efficient viral entry.

The development of an efficient antibody response relies on the interaction between antigen presenting cells and helper 2 T lymphocytes as well as the production of a set of specific Th2 cytokines. These cytokines will act on specific effectors cells by a signalling pathway involving the nuclear translocation of a transcription factor Signal Transducer and Activator of Transcription (STAT)-6. Therefore (STAT)-6 translocation can be used to detect the presence of active Th2 cytokines in the serum of salmon (non-lethal assays) and can improve the ability to evaluate potential vaccine efficacy prior to testing with large scale fish experiments. Collaboration with Dr Steve Bird (University of Aberdeen) who has identified a partial sequence encoding for the salmon STAT6 will be the basis for a non-lethal assay for fish Th2 that will be developed and used in a project aiming at monitoring the immune response and progression of disease on the same animal.

E-mail: Bertrand.Collet@scotland.gsi.gov.uk

TEMPERATURE INFLUENCES THE EXPRESSION PROFILING OF IMMUNE RESPONSE GENES IN RAINBOW TROUT FOLLOWING DNA VACCINATION AND VHS VIRUS INFECTION

Katja Einer-Jensen[1], Laurent Gautier[2], Jesper S. Rasmussen[1], Ellen Lorenzen[1], Mikkel B. Christensen[1], Sara A. Villanueva[3], Samuel Martin[4], Uwe Fischer[5], Øystein Evensen[6], Brian D. Schyth[1], Niels Lorenzen[1]

[1] National Veterinary Institute, Technical University of Denmark, Århus, Denmark.

[2] The Multi-Assay Core (DMAC), Center for Biological Sequence Analysis, Technical University of Denmark, Denmark.

[3]Bionostra Biotechnology Applications, S.L.U. Bionostra Group, Spain

[4] Scottish Fish Immunology Research Centre, University of Aberdeen, Scotland, United Kingdom

[5] Laboratory for Fish Immunology, Friedrich Loeffler Institute, Germany

[6] Basic Sciences and Aquatic Medicine, Norwegian School of Veterinary Science, Norway

A DNA vaccine encoding the glycoprotein (G) genes of the salmonid rhabdovirus viral haemorrhagic septicaemia virus (VHSV) has proven highly efficient against the disease caused by this virus in rainbow trout (*Oncorhynchus mykiss*). Several studies have demonstrated that this vaccine induces both an early unspecific antiviral response as well as a long-lasting specific protection. However, temperature appears to influence immune response with respect to the nature and duration of the protective mechanisms. In this study, groups of fish were temperature acclimated, vaccinated and challenged at three different temperatures (5, 10 and 15ºC). Tissue and organ samples were collected at numerous time points post vaccination (pv) and post viral challenge (pch). Then, gene expression levels of a two immune genes (Vig-1 and Mx3) involved in unspecific antiviral response mechanisms were determined by Q-PCR. The expression profiles appeared similar for the two genes in terms of temperature dependency with a faster induction and shorter duration at the higher temperature. In order to analyze the temperature effect on the relative expression profiles across a larger set of immune genes time points displaying similar Mx3 expression levels post vaccination were identified: 4 days pv (15ºC); 7 days pv (10ºC); 23 days pv (5ºC), respectively. A targeted trout immune gene array including probes for VHSV genes was used for analysis of vaccinated vs control fish per temperature (4-5 replicates). Temperature altered the transcriptome response to the viral challenge, and the number of responsive genes increased at higher temperature: 51 (5ºC), 64 (10ºC) and 72 (15ºC), respectively, indicating that fish kept at higher temperature may have an enhanced immune response.

Additional correlation analysis allowed identification of genes that were significantly up- or down regulated synchronous with expression of the vaccine G gene. These findings encompass that for example in fish kept at 5ºC , putative CD3, CD4, CD9, CD28, CD53, CD63, CD83, CD84 were up regulated, while CD59, CD83 were down regulated, potentially indicating balancing mechanism of the immune system.

An experimental VHSV challenge was performed 7 weeks pv. Similar protection levels of approximately 10% mortality were found for the vaccinated fish, regardless of temperature during immunisation and challenge, whereas the course and level of mortality among the controls were temperature dependent. At day 5 post infection, the number of differentially regulated genes in the livers of vaccinated versus control fish was highest in fish kept at 10ºC and lowest in fish at 5ºC. This difference presumably reflects the temperature dependent progression of the disease in the controls. Further analysis of the obtained data with respect to gene regulation pathways as a result of DNA vaccination and/or viral infection will be presented.

This work was supported by the EC FP6 project IMAQUANIM. *E-mail:* kaei@vet.dtu.dk

MOLECULAR CHARACTERIZATION AND GENOMIC ORGANISATION OF NOVEL IMMUNOGLOBULIN-LIKE TRANSCRIPTS (NILTS) IN TELEOST FISH

A. E. Østergaard[1*], S. A. M. Martin[1], K. P. Lubieniecki[2], T. Wang[1], W. S. Davidson[2], R. J. M. Stet[1] and C. J. Secombes[1]

[1]*Scottish Fish Immunology Research Centre, University of Aberdeen, UK.*

[2]*Department of Molecular Biology and Biochemistry, Simon Fraser University, Canada.*

[*]*Current: Cell Biology & Immunology Group, Department of Animal Sciences,Wageningen University, The Netherlands*

The recognition of pathogens by the innate immune system relies on a wide range of inhibitory and activating receptors. Some of these non-rearranging receptors belong to the immunoglobulin superfamily (IgSF). Members of this family include the immunoglobulin (Ig) molecules produced by B cells, and also T cell receptors (TCR) consist of Ig domains. Furthermore, Ig domains are found in the major histocompatibility complex (MHC) class I and II molecules, the killer cell Ig-like receptors (KIR), adhesion molecules and several cytokine and growth factor receptors.

A hallmark of the innate Ig-like receptors is their ability to initiate activation of the immune system, but at the same time prevent an excessive response. These complex interactions, which regulate both the quality and magnitude of the ultimate response, depend on two short, loosely conserved motifs found in the intracellular domain of various signalling proteins including IgSF receptors. To transmit positive signals, the immune system employs the immunoreceptor tyrosine-based activation motif (ITAM). For opposing signalling the immune system employs negative regulators in the form of receptors bearing one or more immunoreceptor tyrosine-based inhibitory motifs (ITIM).

In teleosts, there are a number of different IgSF members; one group contains the novel immune-type receptors (NITR), which represent one of the largest groups of activating and inhibitory immune receptor genes reported to date. A new receptor family of the IgSF has been identified in carp, termed novel immunoglobulin-like transcripts (NILT).

In this study, three new NILT genes were successfully cloned and characterized from rainbow trout, with one NILT alternatively spliced into a long isoform containing two Ig domains and a short isoform containing one Ig domain. NILTs were found to be expressed in immunologically important tissues. The genomic organisation and structure of the multigene family of NILTs in Atlantic salmon was investigated using a BAC sequencing approach. This revealed the presence of six novel NILT genes, which either contained one or two Ig domains and several ITIMs in the cytoplasmic region.

By homology search two NILT-like genes in zebrafish (Danio rerio) located on chromosome 1 have been obtained. Chromosome 1 in zebrafish also contains the Dare-ZE genes, which are equivalent to the human MHC class I genes located on chromosome 6. In addition, two NILT-like Ig domains were obtained from the green spotted pufferfish (Tetraodon nigroviridis), putatively part of the same receptor family.

Future experiments are needed to reveal the function of these receptors. One might speculate whether they perform a function in cell surveillance, pathogen recognition, immune regulation or another immune related function. As these receptors are found as a multigene family conserved across several fish species they could be involved in a general inflammatory process in teleost fish.

E-mail: anders.oestergaard@wur.nl

CRYSTAL STRUCTURE AND INTERACTION PROPERTIES OF THE NOVEL MBL/FICOLIN ASSOCIATED PROTEIN-1 (MAP-1)

Mikkel-Ole Skjoedt*, Pietro Roversi†, Tina Hummelshøj*, Yaseelan Palarasah‡, Steven Johnson†,, Peter Garred*, Susan M Lea†

**Laboratory of Molecular Medicine, Department of Clinical Immunology Rigshospitalet, Copenhagen, Denmark.*

†Sir William Dunn School of Pathology, University of Oxford, Oxford, UK.

‡Research Unit of Cancer and Inflammation, University of Southern Denmark.

Background

The lectin complement pathway comprises MBL, Ficolin-1, -2 and –3 and probably also collectin-11 in complex with associated serine proteases MASP-1, -2 and -3 and the non-enzymatic sMAP. Recently, a novel serum protein named MBL/Ficolin associated protein-1 (MAP-1) was identified. This protein is a product of differential splicing of the MASP1 gene and contains the major part of the heavy chain but lacks the entire serine protease domain.

Methods

Association, regulatory and structural properties of MAP-1 were investigated by surface plasmon resonance, ELISA, size-exclusion chromatography, multiple angle laser light scattering (MALLS) and X-ray crystallographic methods.

Results

The interactions between MAP-1, Ficolin-2, -3, MBL and collectin-11 were found to be in the range of 3-15 nM. MAP-1 showed a strong inhibitory effect in concentrations above 1 nM on complement activation, evaluated as C4, C3 and terminal complement complex depositions. Although MAP-1 migrated with a major peak corresponding to ~250kDa (equivalent to a pentamer/hexamer) in size-exclusion chromatography we found upon further analysis using multiple angle laser light scattering that MAP-1 has a molecular mass equivalent to a dimer under physiological conditions and is present in a monomer form in absence of calcium. Additionally, we present a high-resolution crystal structure of MAP-1.

Conclusion

We present the crystal structure of a MAP-1 dimer and show that it binds strongly to the lectin pathway initiator molecules. Furthermore, we show a profound down-regulating effect of MAP-1 on central complement components.

E-mail: moskjoedt@health.sdu.dk

INSUFFICIENT PROTECTION OF RAINBOW TROUT AGAINS FURUNCULOSIS BY COMMERCIAL VACCINES UNDER EXPERIMENTAL CONDITIONS.

E. Lorenzen[1], T. E. Kjær[1], N. H. Henriksen[2], I. Dalsgaard[1], L. H. Andersen[1,3], J. Nylen[4], S. B. Madsen[5], K. Buchmann[3] and N. Lorenzen[1].

[1]National Veterinary Institute, DTU, Denmark

[2]Danish Aquaculture, Silkeborg, Denmark

[3]Laboratory of Aquatic Pathobiology, Section of Biomedicine, Department of Veterinary Pathobiology Faculty of Life Sciences, University of Copenhagen, Denmark

[4]Schering-Plough Animal Health, Denmark

[5]Simon B. Madsen, Veterinary Services, Denmark

Despite vaccination with oil-adjuvanted vaccines against vibriosis and furunculosis, sea reared rainbow trout in Denmark often encounter outbreaks of furunculosis during warm summer periods. This implies an excessive use of antibiotics and has also decreased the fish farmers' confidence in the commercially available vaccines. To address this issue under experimental conditions, two groups of rainbow trout were vaccinated by i.p. injection with two different commercial vaccines, both comprising Vibrio anguillarum serotype O1 and O2, and Aeromonas salmonicida subspecies salmonicida bacterins based on cultures of bacteria originally isolated from Atlantic salmon. The experiment also included a third group of non-vaccinated controls. All fish were individually chip-tagged. After 6 months at 10°C, half of the fish were challenged by i.p. injection of 106 cells of a recent Danish field isolate of A. salmonicida.

While the non-vaccinated fish all died within 2 weeks, a certain level of protection was evident among the vaccinated groups although high mortality also occurred here. No mortality/clinical disease was evident among the non-injected cohabitants. However, when the water temperature was subsequently raised to 17°C, the cohabitants started to die. Some variability was evident between replicate tanks, but the overall outcome was a superior performance of the non vaccinated fish. The mortality among the vaccinated groups was thus either higher or equal to the mortality of the non vaccinated controls. The results demonstrate the importance of the challenge procedure for evaluation of vaccine efficacy under experimental conditions. Batch release testing of vaccine potency based on ip challenge may thus be of limited value. Although it may be anticipated that the vaccination can confer some protection against furunculosis under field conditions, the results also indicate that there is a need for tailoring the vaccines to the needs of sea reared rainbow trout in Denmark.

E_mail: ello@vet.dtu.dk

INHIBITION OF REPORTER GENES BY SMALL INTERFERING RNAS IN CELL CULTURE AND LIVING FISH

S. Larashati, B. D. Schyth and N. Lorenzen

Department of Poultry, Fish and Fur Animals, Veterinary Institute-Denmark
Technical University, Århus N, Denmark

RNA interference is a mechanism for silencing specific genes. It has been applied in cell culture to inhibit expression of genes involved in disease including viral genes as recently shown for the fish pathogenic rhabdovirus viral haemorrhagic septicaemia virus or VHSV (Bohle et al., 2011). But evidence of specific siRNA inhibition in living fish is still needed. Using the small interfering RNAs (siRNAs), messenger RNA (mRNA) can be targeted resulting in degradation of targeted transcript or translational repression. Reporter genes such as luciferase and green fluorescence protein (GFP) can be used to observe the knock down effect by siRNAs designed to target these reporters.

One aim of this project is to verify the specific knock down effect of siRNAs in cell culture and in living fish and to establish easy-read out models for testing the effect especially in vivo. Cell culture from human embryonic kidney HEK293t cells was used because they are easy to transfect and generally show high expression of transfected genes. Two types of fish including albino trout were used as animal models to get better visualization of reporter gene expression. The luciferase gene was used as reporter gene as it provides low background compared to other reporter genes such as green fluorescence protein (GFP). In cell culture, the luciferase can be used as reporter gene to see the effect of gene silencing. In the living fish, the bioluminescence signal detected is influenced by the melanin pigment. Timing between coinjection and the assay is important in order to detect knock down by siRNA. Our experiment reveal in vivo knock down at 72 hours post injection of reporter gene and siRNA, but further dose-response experiments are required to confirm specificity.

Bohle, H., Lorenzen, N. & Schyth, B. D. 2011. Species specific inhibition of viral replication using dicer substrate siRNAs (DsiRNAs) targeting the viral nucleoprotein of the fish pathogenic rhabdovirus viral haemorrhagic septicaemia virus (VHSV). *Antiviral Research,* 90(3): 187-194.

E-mail: selar@vet.dtu.dk

SEARCH FOR GENETIC VIRULENCE MARKERS IN VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS (VHS) USING A REVERSE GENETICS APPROACH

A. Stegmann[1], S. Biacchesi[2], M. Bremont[2], N. Lorenzen[1] and K. Einer-Jensen[1]

[1]National Veterinary Institute, DTU, Aarhus, Denmark. [2]INRA, Jouy, France

VHSV is a negative strand RNA virus causing serious disease in farmed rainbow trout. A wildlife marine reservoir represents a threat against rainbow trout farming. In Scandinavia, outbreaks of VHS in sea reared rainbow trout have demonstrated, that although marine variants of VHSV are considered to be non pathogenic to rainbow trout, the virus is potentially able to adapt to this host and cause disease. The VHSV genotypes occurring in the marine environment vary depending geographical location, and some genotypes might have a higher chance of changing into rainbow trout virulent forms than others. However, the limited knowledge about the genetic background for virulence to rainbow trout makes it difficult to differentiate between dangerous and harmless VHSV variants and to classify marine locations according to their suitability for rainbow trout farming. With the aim of genetic virulence marker identification, we have implemented reverse genetic technology for generation of hybrid virus variants. By substituting different regions in the genome of a pathogenic VHSV variant with the homologous regions from the genome of a marine non pathogenic variant, a set of chimeric viral genomes were generated. Following rescue of the corresponding viral chimeras, comparative challenge experiments using rainbow trout fingerlings were carried out in order to assess, which substitutions affect the virulence of the virus.

E-mail: anders.oestergaard@wur.nl

ORAL TRANSMISSION OF VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS IN JUVENILE RAINBOW TROUT

A.A. Schönherz[1], M.M.H. Hansen[1], H.B.H. Jørgensen[1], P. Berg[1], K. Einer-Jensen[2]

[1]University of Aarhus, Faculty of Science and Technology, Department of Molecular Biology and Genetics, Denmark. [2]Technical University of Denmark, National Veterinary Institute, Denmark

Viral haemorrhagic septicaemia virus (VHSV) is an important pathogen of cultured rainbow trout in continental Europe. In recent years the virus has been isolated from increasing numbers of marine fish species. VHSV strains isolated from marine fish are of no or very low pathogenicity to rainbow trout following immersion challenge. The phylogeny of VHSV, however, indicates that initial introduction of VHSV to cultured rainbow trout most likely originated from a marine ancestor. In marine aquaculture systems for rainbow trout invasion of wild fish cannot be prevented totally. Preying on invading wild fish thus might represent a risk factor for new introduction and adaptation of VHSV to rainbow trout.

The objective of this study was to demonstrate the existence of an oral transmission route for VHSV in rainbow trout. Juvenile trout (n = 165) were infected through oral, waterborne, and cohabitation transmission routes, using a recombinant virus strain that contained a reporter gene, Renilla luciferase. Viral replication in stomach and kidney tissue was analyzed through detection of bioluminescence activity of Renilla luciferase and through qRT-PCR. Viral replication was detected in both tissues, irrespective of transmission route. Replication patterns, however, differed among transmission routes. In trout infected through oral transmission, replication was detected in stomach prior to kidney tissue. In trout infected through waterborne or cohabitation transmission, replication was detected in kidney prior to stomach or in both tissues simultaneously. We thus demonstrate the existence of an oral transmission route for VHSV in rainbow trout and thereby show that viral introduction and adaptation may lead to disease outbreaks in marine cultured rainbow trout where trout are able to prey on invading wild fish.

E-mail: Anna.Schonherz@agrsci.dk

VACCINATION AGAINST ENTERIC RED MOUTH DISEASE CAUSED BY THE BACTERIUM *YERSINIA RUCKERI*

Martin K. Raida

Laboratory of Aquatic Pathobiology, Section of Biomedicine, Department of Veterinary Disease Biology Faculty of Life Sciences, University of Copenhagen, Frederiksberg, Denmark

Recently the Danish research council has granted the project:"Understanding key mechanisms in mucosal immunity: Development of a new immersion vaccine".

The main objective of the project is to develop a new vaccine which is long lasting and protective against both *Y.ruckeri* biotype 1 and a European isolate of biotype 2. Recently a new biotype (biotype 2) of *Y. ruckeri* has been isolated from farmed trout diagnosed with ERM across Europe although the fish had been vaccinated against ERM with the commercially available ERM-vaccine.

Further, we will develop a new vaccination strategy based on the hypothesis that booster vaccinations will improve and prolong the protection of the rainbow trout against ERM. The vaccine induced protection will be tested in laboratory bath challenge infections before and after booster vaccination. It is the goal that the effect of the experimental vaccine and the most effect full booster method will be confirmed in a field experiment at a commercial fish farm with ERM biotype 2 disease problems.

We will in this project investigate the mucosal and systemic immune mechanisms behind the improved protective response by new molecular and immunological tools as well as a novel 3D bioimaging method. Since it was recently shown that rainbow trout is mainly infected with *Y. ruckeri* through the gills, will our research focus especially on vaccine induced development of immunity in gill tissue, since only one cell layer separate the bacteria containing water and the blood stream.

The project is currently in its preliminary phase and presentation will focus on future plans for the project.

E-mail: mkr@life.ku.dk

List of participants

First name	Surname	e-mail	Affilation
Azmi	Al-Jubury	aljubury@life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Jørn	Andreassen	jandreassen@webspeed.dk	Faculty of Science, University of Copenhagen, Denmark
Qusay	Bahlool	qusay@life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Dennis	Bela-Ong	debo@vet.dtu.dk	National Veterinary Institute, Technical University of Denmark
Peer	Berg	Peer.Berg@agrsci.dk	Faculty of Agricultural Sciences, Aarhus University, Denmark
Kurt	Buchmann	kub@life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Ejner	Børsting	ejner@borsting.dk	Bjæverskov, Denmark
Jiwan Kumar	Chettri	jkc@life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Bertrand	Collet	Bertrand.Collet@scotland.gsi.gov.uk	Marine Laboratory, Ellis Building, Aberdeen, Scotland
Catherine	Collins	C.Collins@marlab.ac.uk	Marine Laboratory, Ellis Building, Aberdeen, Scotland
Inger	Dalsgaard	inda@vet.dtu.dk	National Institute of Aquatic Resources, Technical University of Denmark, Denmark
Sidhartha	Deshmukh	sid@life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Katja	Einer-Jensen	kaei@vet.dtu.dk	National Veterinary Institute, Technical University of Denmark
Kirsten	Engell-Sørensen	kes@fishlab.dk	Fishlab, Denmark
Inge Rosenbek	Fink	inge.fink@wur.nl	Department of Animal Sciences, Wageningen University
Anette	Furevik	anette.furevik@pharmaq.no	PHARMAQ AS, Norway
Rasmus Demuth	Heinecke	rdh@life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Maya	Henriksen	mmah@vet.dtu.dk	Division of Veterinary Diagnostics and Research, Technical University of Denmark
Niels Henrik	Henriksen	niels@danskakvakultur.dk	Dansk Akvakultur, Denmark
Niels	Hjermitslev	nhh@biomar.dk	Biomar, Denmark

Lars	Holten-Andersen	lhoa@life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark

List of participants

First name	Surname	e-mail	Affilation
Simon	Haarder	simon.haarder@gmail.com	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Hans-Christian	Ingerslev	hain@vet.dtu.dk	Division of Veterinary Diagnostics and Research, Technical University of Denmark
Louise von Gersdorff	Jørgensen	lgj@life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Per	Kania	pwk@life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Jesper Andreas	Kuhn	jesperku@life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Gudny	Palsdottir	gudnyrp@dsr.life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Scott	LaPattra	scottl@clearsprings.com	Clear Springs Foods, Inc., Idaho, USA
Sekar	Larashati	selar@vet.dtu.dk	National Veterinary Institute, Technical University of Denmark
Anne Hjørngaard	Larsen	ahl@biomar.dk	Biomar, Denmark
Jens Laurits	Larsen	ellen@jeanty.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Ellen	Lorenzen	ello@vet.dtu.dk	National Veterinary Institute, Technical University of Denmark
Niels	Lorenzen	nilo@vet.dtu.dk	National Veterinary Institute, Technical University of Denmark
Lone	Madsen	loma@vet.dtu.dk	Division of Veterinary Diagnostics and Research, Technical University of Denmark
Sanaz	Mazaheri	sma@life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Foojan	Mehrdana	foojan@life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Bernt	Melgård	bernt.melgard@pharmaq.no	PHARMAQ AS, Norway
Rzgar Jaarfar	Mohammad	rezgarhadad@life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Lukas	Neumann	neumann@dsr.life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Torben	Nielsen	torben@aquasearch.dk	Aquasearch, Denmark
Michael Engelbrecht	Nielsen	mice@food.dtu.dk	National Food Institute, Technical University of Denmark, Denmark

List of participants

First name	Surname	e-mail	Affilation
Jørgen	Nylen	jorgen.nylen@sp.intervet.com	Intervet/Schering-Plough, Denmark
Alexandre	Pargana	Emailampa@aqua.dtu.dk	National Veterinary Institute, Technical University of Denmark
Martin	Raida	mkr@life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Jesper Skou	Rasmussen	jsra@vet.dtu.dk	National Veterinary Institute, Technical University of Denmark
Karina Juhl	Rasmussen	karina.juhl.rasmussen@gmail.com	Department of medical Biology, University of Southern Denmark, Denmark
Kasper	Rømer Villumsen	krv@life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Jacob	Schmidt	jacsc@food.dtu.dk	National Institute of Aquatic Resources, Technical University of Denmark
Brian Dall	Schyth	bdsc@vet.dtu.dk	National Veterinary Institute, Technical University of Denmark
Anna Amanda	Schönherz	Anna.Schonherz@agrsci.dk	Dept. of Molecular Biology and Genetics, Aarhus University, denmark
Chris	Secombes	c.secombes@abdn.ac.uk	School of Biological Sciences, University of Aberdeen, Scotland
Karsten	Skjødt	kskjoedt@health.sdu.dk	Department of medical Biology, University of Southern Denmark, Denmark
Mikkel-Ole	Skjødt	moskjoedt@health.sdu.dk	Department of medical Biology, University of Southern Denmark, Denmark
Jakob	Skov	jask@life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Alf	Skovgaard	alf@life.ku.dk	Laboratory of Aquatic Pathobiology, University of Copenhagen, Denmark
Anders	Stegmann	andst@vet.dtu.dk	National Veterinary Institute, Technical University of Denmark
Nils	Steine	Nils.Steine@pharmaq.no	PHARMAQ AS, Norway
Gry Hougaard	Svendsen	gsv@aqua.dtu.ku	Division of Veterinary Diagnostics and Research, Technical University of Denmark
Anders	Østergaard	anders.oestergaard@wur.nl	Department of Animal Sciences, Wageningen University
Bent	Aasted	bas@life.ku.dk	Department of Veterinary Disease Biology, University of Copenhagen, Denmark