Pichia pastoris yeast as a vehicle for oral vaccination of larval and adult teleosts

Oral vaccination is of major interest because it can be used for mass vaccination of fish of various size and age. Given that their administration is relatively easy and stress-free, oral vaccines have both economic and animal welfare benefits. Yet, mostly due to their limited efficacy, only very few oral vaccines are available to aquaculture industry. Here we present a method for oral vaccine delivery based on the yeast Pichia pastoris. We could express a model antigen, green fluorescent protein (GFP), in this yeast and subsequently show delivery of the GFP protein to the intestine of juvenile flounder or adult carp and trout. We tested this approach in several commercially-relevant fish species, from juvenile to adult stage. To test the oral delivery of antigen to larval fish, the GFP-expressing Pichia pastoris was first fed to planktonic crustacean Daphnia or rotifers that served as ‘bioencapsulation vehicles’ and afterwards, fed to flounder larvae. Again, we could show delivery of intact GFP protein to the intestine. In rainbow trout, the orally-administered GFP-expressing yeast elicited a rapid local innate immune response in the intestine and a subsequent systemic response in the spleen. Our results show that Pichia pastoris is a good vehicle for oral antigen delivery and that it can be used in non-encapsulated form for older fish or in bioencapsulated form for larval fish. We discuss the immunomodulatory properties of the yeast itself, and its potential to enhance local immune responses and act as an adjuvant.
A pentavalent vaccine for rainbow trout in Danish aquaculture

Mariculture in Denmark is based on production of rainbow trout grown two years in fresh water followed by one growth season in sea cages. Although the majority of rainbow trout are vaccinated against the most serious bacterial pathogens - Aeromonas salmonicida subsp. salmonicida, Vibrio anguillarum and Yersinia ruckeri, by the use of commercially available vaccines, disease outbreaks requiring treatment with antibiotics still occur. The present study tested the potential of a new experimental multicomponent vaccine that is based on local bacterial strains, isolated from rainbow trout in Danish waters, and thus custom-designed for Danish rainbow trout mariculture. The vaccination with the multicomponent vaccine resulted in protection against three relevant bacterial diseases (yersiniosis, furunculosis, vibriosis) under experimental conditions. We showed that i.p. injection of the vaccine induced specific antibody responses in trout against the different bacterial antigens and regulated expression of genes encoding SAA, C3, IL-1β, IL-6, IL-8, IgD and MHCII.

A recombinant vaccine targeting the parasitic ciliate Ichthyophthirius multifiliis

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A recombinant vaccine targeting the parasitic ciliate Ichthyophthirius multifiliis

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Demonstration of herd immunity effects in DNA vaccinated rainbow trout

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DNA vaccination for finfish aquaculture
In fish, DNA vaccines have been shown to give very high protection in experimental facilities against a number of viral diseases, particularly diseases caused by rhabdoviruses. However, their efficacy in generating protection against other families of fish viral pathogens is less clear. One DNA vaccine is currently in use commercially in fish farms in Canada and the commercialisation of another one was authorised in Europe in 2017. The mechanism of action of DNA vaccines, including the role of the innate immune responses induced shortly after DNA vaccination in the activation of the adaptive immunity providing longer term specific protection, is still not fully understood. In Europe the procedure for the commercialisation of a veterinary DNA vaccine requires the resolution of certain concerns particularly about safety for the host vaccinated fish, the consumer and the environment. Relating to consumer acceptance and particularly environmental safety, a key question is whether a DNA vaccinated fish is considered a Genetically Modified Organism (GMO). In the present opinion paper these key aspects relating to the mechanisms of action, the development and the use of DNA vaccines in farmed fish are reviewed and discussed.

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Recombinant immunotherapy against Ichthyophthirius multifiliis in Oncorhynchus mykiss

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**Time-course study of the protection induced by an interferon-inducible DNA vaccine against viral haemorrhagic septicaemia in rainbow trout**

The highly effective DNA vaccines against diseases caused by fish rhabdoviruses in farmed fish consist of a DNA plasmid vector encoding the viral glycoprotein under the control of a constitutive cytomegalovirus promoter (CMV). Among others, attempts to improve efficacy and safety of these DNA vaccines have focused on regulatory elements of plasmid vectors, which play a major role in controlling expression levels of vaccine antigens. Depending on the context, use of a fish-derived promoter with minimal activity in mammalian cells could be preferable. Another aspect related to the CMV promoter is that constitutive expression of the vaccine antigen may lead to rapid elimination of antigen expressing cells in the fish and thereby potentially reduce the long-term effects of the vaccine. In this study, we compared DNA vaccines with the interferon-inducible Mx promoter from rainbow trout and the CMV promoter, respectively. Plasmid constructs encoding the enhanced green fluorescent protein (EGFP) were used for the in vitro analysis, whereas DNA vaccines encoding the glycoprotein (G) of the viral haemorrhagic septicaemia virus (VHSV) were applied for the in vivo examination. The in vitro analysis showed that while the DNA vaccine with the CMV promoter constitutively drove the expression of EGFP in both fish and human cell lines, the DNA vaccine with the Mx promoter inducibly enhanced the expression of EGFP in the fish cell line. To address the impact on protection, a time-course model was followed as suggested by Kurath et al. (2006), where vaccinated fish were challenged with VHSV at 2, 8 and 78 weeks post-vaccination (wpv). The DNA vaccine with the CMV promoter protected at all times, while vaccination with the DNA vaccine containing the Mx promoter only protected the fish at 8 wpv. However, following induction with Poly (I:C) one week before the challenge, high protection was also evident at 2 wpv. In conclusion, the results revealed a more fish host dependent activity of the trout Mx promoter compared to the traditionally used cross species-active CMV promoter, but improvements will be needed for its application in DNA vaccines to ensure long term protection.

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**Virulence marker candidates in N-protein of viral haemorrhagic septicaemia virus (VHSV): virulence variability within VHSV Ib clones**

Four major genotypes of viral haemorrhagic septicaemia virus (VHSV), which have been isolated from many marine and freshwater fish species, are known to differ in virulence. While fast and low-cost genotyping systems based on monoclonal antibodies (MAbs) have been developed for typing of VHSV virulence, there is a need for supplementing the knowledge. In particular, 2 field isolates from viral haemorrhagic septicaemia (VHS) outbreaks in sea-reared rainbow trout Oncorhynchus mykiss in Sweden, SE-SVA-14 and SE-SVA-1033 (both genotype Ib), have yielded contradictory reactions. In the present study, upon cloning by limited dilution, both isolates appeared to be heterogeneous in terms of reactivity with nucleo (N)-protein-specific MAbs as well their gene sequences. Infection trials in rainbow trout further revealed differences in the virulence of these virus clones derived from the same primary isolate. Based on a comparative analysis of the entire genome of the clones tested, we suggest that the differences in virulence are tentatively linked to substitutions of amino acids (aa) in the N-protein region covered by aa 43-46 and aa position 168, or a combination of the two. The fact that such minor naturally occurring genetic differences affect the virulence implies that even low-virulent VHSV isolates in the marine environment should be considered as a potential threat for the trout farming industry. The described MAbs can represent useful tools for initial risk assessment of disease outbreaks in farmed trout by marine
Intramuscular DNA Vaccination of Juvenile Carp against Spring Viremia of Carp Virus Induces Full Protection and Establishes a Virus-Specific B and T Cell Response

Although spring viremia of carp virus (SVCV) can cause high mortalities in common carp, a commercial vaccine is not available for worldwide use. Here, we report a DNA vaccine based on the expression of the SVCV glycoprotein (G) which, when injected in the muscle even at a single low dose of 0.1 μg DNA/g of fish, confers up to 100% protection against a subsequent bath challenge with SVCV. Importantly, to best validate vaccine efficacy, we also optimized a reliable bath challenge model closely mimicking a natural infection, based on a prolonged exposure of carp to SVCV at 15°C. Using this optimized bath challenge, we showed a strong age-dependent susceptibility of carp to SVCV, with high susceptibility at young age (3 months) and a full resistance at 9 months. We visualized local expression of the G protein and associated early inflammatory response by immunohistochemistry and described changes in the gene expression of pro-inflammatory cytokines, chemokines, and antiviral genes in the muscle of vaccinated fish. Adaptive immune responses were investigated by analyzing neutralizing titers against SVCV in the serum of vaccinated fish and the in vitro proliferation capacity of peripheral SVCV-specific T cells. We show significantly higher serum neutralizing titers and the presence of SVCV-specific T cells in the blood of vaccinated fish, which proliferated upon stimulation with SVCV. Altogether, this is the first study reporting on a protective DNA vaccine against SVCV in carp and the first to provide a detailed characterization of local innate as well as systemic adaptive immune responses elicited upon DNA vaccination that suggest a role not only of B cells but also of T cells in the protection conferred by the SVCV-G DNA vaccine.
Involvement of two microRNAs in the early immune response to DNA vaccination against a fish rhabdovirus

Mechanisms that account for the high protective efficacy in teleost fish of a DNA vaccine expressing the glycoprotein (G) of Viral hemorrhagic septicemia virus (VHSV) are thought to involve early innate immune responses mediated by interferons (IFNs). Microribonucleic 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, including immune responses. We have recently reported that miR-462 and miR-731 were strongly induced in rainbow trout infected with VHSV. In this study, we analyzed the expression of these miRNAs in fish following administration of the DNA vaccine and their potential functions. Quantitative RT-PCR analysis revealed the increased levels of miR-462, and miR-731 in the skeletal muscle tissue at the site of vaccine administration and in the liver of vaccinated fish relative to empty plasmid backbone-injected controls. The increased expression of these miRNAs in the skeletal muscle correlated with the increased levels of the type I interferon (IFN)-inducible gene Mx, type I IFN and IFN-γ genes at the vaccination site. Intramuscular injection of fish with either type I IFN or IFN-γ plasmid construct resulted in the upregulation of miR-462 and miR-731 at the site of injection, suggesting that the induction of these miRNAs is elicited by IFNs. To analyze the function of miR-462 and miR-731, specific silencing of these miRNAs using anti-miRNA oligonucleotides was conducted in poly I:C-treated rainbow trout fingerlings. Following VHSV challenge, anti-miRNA-injected fish had faster development of disease and higher mortalities than control fish, indicating that miR-462/731 may be involved in IFN-mediated protection conferred by poly I:C.
High virulence differences among phylogenetically distinct isolates of the fish rhabdovirus viral hemorrhagic septicaemia virus are not explained by variability of the surface glycoprotein G or the non-virion protein Nv

Viral hemorrhagic septicaemia virus (VHSV) is an important viral pathogen in European rainbow trout farming. Isolates from wild marine fish and freshwater trout farms show highly different virulence profiles: isolates from marine fish species cause little or no mortality in rainbow trout following experimental waterborne challenge, whilst challenge with rainbow trout isolates results in high levels of mortality. Phylogenetic analyses have revealed that the highly virulent trout-derived isolates from freshwater farms have evolved from VHSV isolates from marine fish host species over the past 60 years. Recent isolates from rainbow trout reared in marine zones show intermediate virulence. The present study aimed to identify molecular virulence markers that could be used to classify VHSV isolates according to their ability to cause disease in rainbow trout. By a reverse genetics approach using a VHSV-related novirhabdovirus [infectious hematopoietic necrosis virus (IHNV)], four chimaeric IHNV–VHSV recombinant viruses were generated. These chimaeric viruses included substitution of the IHNV glyco- (G) or non-structural (Nv) protein with their counterparts from either a trout-derived or a marine VHSV strain. Comparative challenge experiments in rainbow trout fingerlings revealed similar levels of survival induced by the recombinant (r)IHNV–VHSV chimaeric viruses regardless of whether the G or Nv genes originated from VHSV isolated from a marine fish species or from rainbow trout. Interestingly, recombinant IHNV gained higher virulence following substitution of the G gene with those of the VHSV strains, whilst the opposite was the case following substitution of the Nv genes.

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Antiviral immunity in fish – functional analysis using DNA vaccination as a tool
In fish, DNA vaccines encoding the glycoproteins (G proteins) of the salmonid rhabdoviruses VHSV and IHNV have proved very efficient under experimental conditions. Nano-gram amounts of plasmid DNA can induce long-lasting protective immunity when delivered by intramuscular injection in rainbow trout fingerlings. Vaccination of fish at an early stage appears advantageous, since larger fish require higher doses of vaccine to be protected. Even in fish with an average size of 0.5 g at the time of vaccination, good protection can be obtained. Interestingly, immunity is established already a few days after vaccination and cross-challenge experiments have revealed that protection in the early phase is non-specific. Later on, protection becomes very specific in terms of virus species. The protection in the early non-specific phase is related to interferon induced defence mechanisms whereas specific antibodies and cellular components both play a role in the long lasting protection. The similarity of the functional immune response profile to that induced by a natural virus infection is striking and is most likely one of the major reasons for the efficacy of the rhabdovirus DNA vaccines. Although other elements like CpG motifs in the plasmid backbone sequence might play a role, the viral G protein appears to have an inherent ability to stimulate innate immune mechanisms by receptors and pathways that still remain to be characterized in detail. Immunity to VHS in rainbow trout can be induced by DNA vaccination across a temperature range of at least 5-15°C. Interestingly, the initial non-specific phase is significantly prolonged at lower temperatures, thereby ensuring protection despite a slow activation of adaptive mechanisms. Expression of the rhabdovirus G protein on the surface of transfected muscle cells induces a histologically visible local inflammatory reaction with higher doses of VHSV G DNA vaccine. Cell surface expression may be important for a proper activation of the fish immune system, since blocking of the intracellular trafficking of the expressed glycoprotein G-gene interferes with protection. It may be anticipated that the viral G protein acts like a PAMP (pathogen associated molecular pattern), but it remains to be determined which PRRs (pattern recognition receptors) that may be involved in the recognition of the G protein. Recent data from DNA vaccination trials with variant forms of the G protein gene suggest that the structural requirements for
DNA vaccination in fish promotes an early chemokine-related recruitment of B cells to the muscle

In fish, intramuscular injection of plasmid DNA encoding viral proteins has proved as the most effective vaccination strategy against many viral pathogens. The efficacy of DNA vaccination in teleost fish is based on a high level of viral antigen expression in muscle cells inducing a strong and long-lasting protection. However, the mechanisms through which this protection is conferred in fish are still not understood. Moreover, similarities to mammalian models can not be established since DNA vaccination in mammals induces much lower responses. In this work, we have focused on the characterization of immune cells that infiltrate the muscle at the site of DNA delivery in vaccinated fish and the chemokines that may be involved in their infiltration. It was observed that B lymphocytes, both IgM+ and IgT+, represent a major infiltrating cell type in fish vaccinated with a viral hemorrhagic septicemia virus (VHSV) DNA vaccine, whereas in control fish injected with an oil adjuvant mainly granulocytes were attracted. While IgM+ cells were the major B cell population at early time points post vaccination, IgT+ cells represented the predominant cell type later on. Among twelve chemokine genes studied in the injected muscle tissues, only CXCL10, CK5B and CK6 were more strongly transcribed in DNA vaccinated fish compared to control fish injected with the corresponding vector backbone. In vitro tests performed with recombinant trout CK5B and CK6 revealed that these chemokines have chemotactic capacities which might explain the recruitment of immune cells to the site of DNA injection. Our results suggest that B cells are involved in the initial phase of the immune response to intramuscular DNA vaccination against VHSV. This appears to be a major difference to what we know from mammalian models where T cells play a major role.
Do microRNAs induced by Viral Hemorrhagic Septicemia virus in rainbow trout (Oncorhynchus mykiss) possess anti-viral activity?

Micro ribonucleic acids (miRNAs) are small (18-22 nucleotides) endogenous RNAs that potently mediate post-transcriptional silencing of a wide range of genes. They are emerging as critical regulators of cellular processes and some miRNAs have been demonstrated to possess direct antiviral effects. We have previously observed and validated that the fish-specific miRNAs, miR-462 and miR-731, were among the most highly expressed miRNAs in rainbow trout liver following Viral hemorrhagic septicemia virus (VHSV) infection. These miRNAs were also upregulated in the liver and muscle (vaccination site) of fish vaccinated with a DNA vaccine expressing the VHSV glycoprotein gene.

Recent studies further suggest that the expression of these miRNAs is induced by interferons. In order to analyze if miRNA-462 and miRNA-731 have any antiviral effects, we designed inhibitory synthetic oligonucleotides called antagomiRs or anti-miRNAs. These saline-formulated 2'-O-methylated Locked Nucleic Acid (LNA)-based antagomiRs were injected intraperitoneally into rainbow trout fingerlings followed by exposure of the fish to VHSV. Development of disease and levels of infection will be analyzed and compared to data from fish treated with control miRNAs.

Evaluation of the potential anti-viral activity of microRNAs in rainbow trout (Oncorhynchus mykiss)

Micro ribonucleic acids (miRNAs) are small (18-22 nucleotides) endogenous RNAs that potently mediate post-transcriptional silencing of a wide range of genes. They are emerging as critical regulators of cellular processes and some miRNAs have been demonstrated to possess direct antiviral effects. We have previously observed and validated that the fish-specific miRNAs, miR-462 and miR-731, were among the most highly expressed miRNAs in rainbow trout liver following Viral hemorrhagic septicemia virus (VHSV) infection. These miRNAs were also upregulated in the liver and muscle (vaccination site) of fish vaccinated with a DNA vaccine encoding the glycoprotein gene of VHSV. Recent studies further suggest that the expression of these miRNAs is induced by interferons. In order to analyze if miRNA-462 and miRNA-731 have any antiviral effects, we designed inhibitory synthetic oligonucleotides called antagomiRs or anti-miRNAs. These antagomiRs were injected intraperitoneally into rainbow trout fingerlings followed by exposure of the fish to VHSV. Development of disease and levels of infection will be analyzed and compared to data from fish treated with control miRNAs.
Evaluation of the potential roles of microribonucleic acids in the interaction of rainbow trout (Oncorhynchus mykiss) with Viral hemorrhagic septicemia virus

Micro ribonucleic acids (miRNAs) are endogenous, 18-22-nucleotide non-coding RNAs that potently mediate post-transcriptional silencing of a broad range of genes. They are emerging as critical regulators of a broad spectrum of biological processes, including immune responses and host-pathogen interactions. Some miRNAs in mammals have been shown to directly inhibit viruses, whereas other cellular miRNAs can be co-opted by viruses to promote viral replication or evade host immune responses. We have previously observed that two miRNAs known from zebrafish, miR-462 and miR-731, were the most highly expressed miRNAs in rainbow trout liver following Viral hemorrhagic septicemia virus (VHSV) infection. These miRNAs were also upregulated in the liver and muscle (vaccination site) of fish vaccinated with a DNA vaccine encoding the glycoprotein of VHSV. Recent studies further suggest that the expression of these miRNAs is induced by interferons. In order to investigate the potential role(s) of miRNA-462 and miRNA-731 in host-pathogen interactions, we designed synthetic oligonucleotides called antagomiRs or anti-miRNAs to silence these two miRNAs. These antagomiRs were injected intraperitonealy into rainbow trout fingerlings followed by exposure of the fish to VHSV. Development of disease and levels of infection were analyzed and compared to data from fish treated with control miRNAs. Further analysis of the effect of anti-miRNAs in cell culture will be performed.

Inter-species transmission of viral hemorrhagic septicemia virus (VHSV) from turbot (Scophthalmus maximus) to rainbow trout (Oncorhynchus mykiss)

Successful viral infection is a complex mechanism, involving many host–pathogen interactions that developed during coevolution of host and pathogen, and often result in host-species specificity. Nevertheless, many viruses are able to infect several host species and sporadically cross species barriers. The viral hemorrhagic septicemia virus (VHSV), a rhabdovirus with high economic impact on the aquaculture industry, has developed an exceptionally wide host range across marine and freshwater environments. Transmission of VHSV between host species therefore represents a potential risk for aquaculture, which currently is not addressed in biosecurity managements. The objective of this study was to investigate the inter-species transmission potential of VHSV and evaluate whether infected marine wild fish pose a potential risk on marine cultured rainbow trout. A cohabitation infection trial with turbot as donor and rainbow trout as recipient host species was conducted. Turbot were intraperitoneally injected with either a marine-adapted (MA) or a trout-adapted (TA) VHSV isolate and subsequently grouped with naïve rainbow trout. Both VHSV isolates were able to replicate and cause mortality in turbot, while only the TA isolate was able to cross the species barrier and infect rainbow trout with fatal outcome. The results demonstrate that a marine fish species can function as reservoir and transmitter of TA VHSV isolates.
MicroRNA expression in rainbow trout (Oncorhynchus mykiss) vaccinated with a DNA vaccine encoding the glycoprotein gene of Viral hemorrhagic septicemia virus

Viral hemorrhagic septicemia caused by a fish rhabdovirus, Viral hemorrhagic septicemia virus (VHSV), results in significant mortality in farmed rainbow trout (Oncorhynchus mykiss Walbaum). Although the disease had been eradicated in Denmark, wildlife marine reservoir of VHSV poses a threat particularly to sea-farmed rainbow trout and thus necessitates strategies to mitigate potential disease outbreaks. A DNA vaccine encoding the glycoprotein gene of VHSV has been developed and shown to elicit protective immune responses in laboratory trials. It is important to identify key factors as biomarkers during infection and vaccination in order to understand the complex web of interactions involved in the underlying host immune response. 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, including immune responses. A microarray experiment in our lab revealed that miR-155, miR-462, and miR-731 were upregulated in fish liver following VHSV infection. Therefore, we analysed the expression of these miRNAs together with that of the type I interferon (IFN)-inducible Mx gene in rainbow trout in response to DNA vaccination.

Using quantitative RT-PCR, we found that miR-155, miR-462, and miR-731 were upregulated in the skeletal muscle tissue at the site of injection and the liver of vaccinated fish relative to saline- and empty plasmid-injected controls. The increased expression of these miRNAs in the skeletal muscle correlated with the increased levels of the type I IFN-inducible Mx gene, the vaccine gene, and immune marker genes (CD4, CD8, sec-IgM, TCR, MHCI, and MHCII) at the vaccination site, indicating infiltration with immune cells. Since the expression of these miRNAs correlated with the increased expression of the Mx gene, we then determined whether this induction depends on interferons. Injecting fish with IFN 1-13 (a type I IFN) construct resulted in increased expression of miR-155, miR-462, and miR-731 in the skeletal muscle tissue relative to controls. The same response was obtained from injection with the general IFN stimulator and Toll-like receptor (TLR) 3 agonist, polyinosinic: polycytidylic acid (poly I:C). These suggest that the induction of these miRNAs is elicited by interferons, which are major mediators of immune responses.

Regulated miRNAs could serve as molecular signatures of responses to infection and vaccination and could provide suitable selection criteria for identifying disease-resistant fish under production conditions during resistance breeding as fish do not show visible signs of resistance.
Of Fish and Micrornas

Fish is an important small vertebrate multidisciplinary model for investigating various aspects of reproduction, development, disease (immunology, toxicology, carcinogenesis), and aging. It is also an important model for comparative and evolutionary studies because it represents the lower vertebrates and serves as an essential link to early vertebrate evolution. Microribonucleic acids (miRNAs) are 18-22 nucleotide-long endogenous RNAs that bind to specific mRNAs, usually at the 3'-untranslated region, (UTR), thereby potently regulating a wide spectrum of target mRNAs. This adds a new layer to the mechanisms of control of gene expression, impacting a broad range of biological processes. Thus far, >25,000 miRNA sequences have been identified in 193 species, including fish. In fish, the interest on miRNAs started with the analysis of their expression and function during embryonic development. In our lab, we investigate miRNA regulation during viral infection and vaccination in rainbow trout. We aim to identify miRNA biomarkers during infection and vaccination in order to understand the complex web of interactions involved in the underlying host immune responses. They may also be used as suitable selection markers to identify disease-resistant fish.

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Rhabdovirus-Induced Fish-Specific Micrornucleic Acids In Rainbow Trout (Oncorhynchus Mykiss)

The fish rhabdovirus, Viral hemorrhagic septicemia virus (VHSV), causes significant mortality in farmed fish. The potential threat from wildlife marine reservoir of VHSV to sea-farmed rainbow trout (Oncorhynchus mykiss) demands disease protection measures. Identification of biomarkers during infection is important to understand the complex web of interactions involved in the underlying host response, which is needed to develop effective disease control strategies. Micrornucleic acids (miRNAs) are important regulators of biological processes, including responses to pathogens, while some miRNAs have been demonstrated to possess direct antiviral effects. We have observed and validated that miR-462 and miR-731, miRNAs which to date, has been described only in fish, were among the most highly expressed miRNAs in rainbow trout liver following VHSV infection and in the liver and muscle of fish intramuscularly injected with a DNA vaccine encoding the VHSV glycoprotein gene. The two miRNAs were further shown to be induced in fish intramuscularly injected with a type I interferon (IFN) construct and the general IFN stimulator and TLR-3 agonist, poly I:C, suggesting that the increased levels of the these miRNAs at the site of administration is associated with type I IFNs. In order to investigate the potential role(s) of miR-462 and miR-731 in host-pathogen interactions, we designed synthetic oligonucleotides called antagomiRs or anti-miRNAs to silence the two miRNAs. These antagomiRs were injected intraperitoneally into rainbow trout fingerlings followed by exposure of fish to VHSV. Development of disease and levels of infection were analyzed and compared to data from fish treated with control anti-miRNAs. Further analysis of the effect of anti-miRNA treatment in cell culture is underway.

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Testing the ability of viral haemorrhagic septicaemia virus to evade the protective immune response induced in rainbow trout by DNA vaccination

Viral haemorrhagic septicaemia virus, a negative strand RNA virus belonging to the genus Novirhabdovirus within the family Rhabdoviridae, is the causative agent of VHS, which is a serious disease in rainbow trout and other economically important fish species. The DNA vaccine encoding the viral glycoprotein, the only surface protein of the VHSV, has been successful as an experimental prophylactic treatment against this disease, because it induces a strong innate (interferon) and adaptive (cellular and humoral) immune response. However, since RNA viruses are known to possess high variability, this work aims to evaluate whether VHSV is able to evade the protective immune response induced by the DNA vaccination. Earlier studies have demonstrated that VHSV can evade the neutralizing effect of monoclonal antibodies by mutations in the glycoprotein gene. One approach of the present study is therefore to try to isolate VHSV variants which can escape the neutralizing activity of serum from fish immunized with the DNA vaccine. To do so, a highly pathogenic VHSV isolate (DK3592B) will be repeatedly passaged in fish cell cultures in the presence of neutralizing fish serum. Another approach comprises repeated passaging of VHSV in vaccinated fish. The study was initiated recently. Current results will be presented and discussed.

Use of DNA vaccination for determination of onset of adaptive immunity in rainbow trout fry

Vaccine producers often recommend a minimum size of 5g for vaccination of rainbow trout, but implementation of prophylactic vaccination in smaller sized fish would be an advantage for several infectious diseases. To implement a cost efficient vaccination strategy, it is important to know the duration and nature of the protective immunity induced by the vaccines in the fish. The present work aimed at determination of the smallest size at which specific immunity could be induced in rainbow trout fry by DNA vaccination against viral haemorrhagic septicaemia (VHS). Earlier experiments revealed that intramuscular injection of the DNA vaccine encoding the viral glycoprotein G induced protective immunity to VHS in rainbow trout fry of 0.5g. However, the vaccine is known to induce both innate and adaptive protection. The present work therefore aimed at determination of which type of protection the DNA vaccine induced in such early life stages of rainbow trout. Vaccination trials were performed with fry at average sizes of 0.25 g and 0.5 g respectively and included both the homologous VHSV G-gene vaccine and a heterologous DNA vaccine encoding the G-protein of infectious haematopoietic necrosis virus (IHNV). The fish were challenged by immersion at different times post vaccination. Protective immunity was induced in both sizes of fish, but whereas clear-cut specific protection was evident in
the fish vaccinated at 0.5g, the results suggested that the protection in the fish vaccinated at 0.25 g was mainly due to innate cross-reactive antiviral mechanisms of shorter duration. The critical size for induction of an adaptive immune response in rainbow trout to this type of vaccination thus appears to be between 0.25 and 0.5g. This work was supported by the “DAFINET” grant from the Danish Council for Strategic Research.

**Chemical modification of RNA-based medicine can be used to reduce its induction of the innate immune response**

Small interfering RNAs (siRNAs) are regarded as promising new active compounds in gene medicine. They are small 21-22bp long double stranded RNAs which act by targeting and inhibiting expression of specific mRNAs through base complementarity to one of their strands. But one serious problem with siRNA based treatment is the non-specific activities of double stranded RNAs when formulated in some effective delivery reagents. Cellular reactions upon double stranded RNAs include those of the 2’-5’ oligoadenylate synthetase system, the protein kinase R, RIG-I and Toll-like receptor activated pathways all resulting in innate antiviral defence mechanisms. Following injection of formulated siRNAs we have shown that we are able to detect the effect of such defence mechanisms as lowered mortality of rainbow trout infected with the fish pathogenic virus Viral Haemorrhagic Septicaemia Virus (VHSV).

We used the trout and VHSV to screen siRNAs containing various chemical modifications of the RNA backbone and found that was possible to modify the backbone so as to reduce the antiviral effect of the RNA. Antiviral protection was also dependent upon localisation of the modified nucleotide residues in the RNA strands and we found some evidence of an effect of both base composition and thermal stability of the double strands.

We conclude that our model is a potent tool for gaining insight into the triggering of antiviral cellular reactions towards
RNAs in living fish. The overall perspective is to learn how to avoid nonspecific antiviral responses of RNA-based gene medicine, but the knowledge gained also has a potential for use in the design of adjuvants (although adjuvance effect has not been tested for any of our siRNAs yet). The model can also be used for screening various commercial and noncommercial delivery reagents with the same perspective.

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Determining Vaccination Frequency In Farmed Rainbow Trout Using Vibrio anguillarum O1 Specific Serum Antibody Measurements

Background
Despite vaccination with a commercial vaccine with a documented protective effect against Vibrio anguillarum O1 disease outbreaks caused by this bacterium have been registered among rainbow trout at Danish fish farms. The present study examined specific serum antibody levels as a valid marker for assessing vaccination status in a fish population. For this purpose a highly sensitive enzyme-linked immunosorbent assay (ELISA) was developed and used to evaluate sera from farmed rainbow trout vaccinated against V. anguillarum O1.

Study Design
Immune sera from rainbow trout immunised with an experimental vaccine based on inactivated V. anguillarum O1 bacterin in Freund’s incomplete adjuvant were used for ELISA optimisation. Subsequently, sera from farmed rainbow trout vaccinated with a commercial vaccine against V. anguillarum were analysed with the ELISA. The measured serum antibody levels were compared with the vaccine status of the fish (vaccinated/unvaccinated) as evaluated through visual examination.

Results
Repeated immunisation with the experimental vaccine lead to increasing levels of specific serum antibodies in the vaccinated rainbow trout. The farmed rainbow trout responded with high antibody levels to a single injection with the commercial vaccine. However, the diversity in responses was more pronounced in the farmed fish. Primary visual examinations for vaccine status in rainbow trout from the commercial farm revealed a large pool of unvaccinated specimens (vaccination failure rate = 20%) among the otherwise vaccinated fish. Through serum analyses using the ELISA in a blinded set-up it was possible to separate samples collected from the farmed rainbow trout into vaccinated and unvaccinated fish.

Conclusions
Much attention has been devoted to development of new and more effective vaccines. Here we present a case from a Danish rainbow trout farm indicating that attention should also be directed to the vaccination procedure in order to secure high vaccination frequencies necessary for optimal protection with a reported effective vaccine.
DNA vaccination of small rainbow trout fry against VHSV

Small rainbow trout fry were DNA vaccinated by intramuscular injection at 0.25g and other fish later at 0.5g. Vaccine groups included pcDNA3-vhsG, heterologous vaccine (pcDNA3-ihnG), empty vector (pcDNA3) and unhandled fish. Fry vaccinated at 0.25g were challenged with VHSV by immersion at 3wpv, 11wpv and 21wpv. The challenge at 3wpv was started 1wpv, however as no mortality was observed, the fish were re-challenged 3wpv using a modified setup. Fry vaccinated at 0.5g were challenged with VHSV by immersion at 11wpv. By early challenge (3wpv) of fish vaccinated at 0.25g both homologous and heterologous vaccines induced unspecific protection (10 % mortality for both). Challenge 11wpv showed waning unspecific protection (60 % mortality) but also a poor specific protection (30 % mortality). By challenge 21wpv, hardly no specific (75 % mortality) or unspecific (81 % mortality) protection was observed. In contrast, fish vaccinated at 0.5 g and challenged at 11wpv showed good specific protection.

The results indicate that DNA vaccination of very small fry (0.25g) can induce an early innate response. However a late adaptive immune response is apparently not established. Vaccination of fry at 0.5g induces an adaptive response like in larger fish.

The experiment was repeated with same vaccination groups. Rainbow trout fry were vaccinated at 0.25g followed by challenge with homologous or heterologous virus at 13 dpv, 11 wpv and 21 wpv. At 13 dpv unspecific protection was induced with both homologous and heterologous challenge (5% mortality). At 11 wpv an unspecific protection with 30 % mortality was observed. At 21 wpv protection against VHSV had dropped further (50 % mortality). Protection against IHNV was better (10 % mortality) but equal for both homologous and heterologous vaccines confirming previous results, that vaccination of fry at 0.25g induces unspecific protection but no adaptive response.
Expression of micro-RNAs and immune-relevant genes in rainbow trout (Oncorhynchus mykiss Walbaum) upon vaccination with a Viral Haemorrhagic Septicemia Virus

Development of strategies to alleviate potential disease outbreaks in sea-farmed rainbow trout (Oncorhynchus mykiss Walbaum) due to wildlife marine reservoir of Viral hemorrhagic septicemia virus (VHSV) remains imperative. A DNA vaccine expressing VHSV glycoprotein (G) gene has been developed and shown to protect fish in VHSV challenge experiments. Identifying key factors as biomarkers during infection and vaccination will allow understanding of the complex web of interactions involved in the underlying host immune response. These molecular signatures of immune responses may also provide suitable selection criteria for identifying disease-resistant fish under production conditions during resistance breeding as fish do not show visible signs of resistance. 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, including immune responses. In a previous microarray experiment, we observed upregulation of miR-155, miR-462, and miR-731 in fish liver following VHSV infection. Therefore, we analysed the expression of these miRNAs and those of immune-related genes in rainbow trout in response to DNA vaccination.

Quantitative RT-PCR analysis revealed the increased levels of miR-155, miR-462, and miR-731 in the skeletal muscle tissue at the site of injection and in the liver of vaccinated fish relative to saline and empty plasmid-injected controls. The increased expression of these miRNAs in the skeletal muscle correlated with the increased levels of the type I interferon (IFN)-inducible Mx gene, the vaccine gene (G), type I IFN and IFN-γ genes, and immune cell marker genes (CD4, CD8, sec-IgM, TCR, MHCI, and MHCII) at the vaccination site. The increased expression of immune cell markers indicates infiltration of the vaccination site with activated immune cells. Since the expression of these miRNAs correlated with increased levels of the type 1 IFN gene, IFN-γ gene, and the type I IFN-inducible Mx gene, we then determined whether this induction depends on interferons. Injecting fish with IFN 1-13 (a type I IFN) and IFN-γ constructs resulted in the increased expression of miR-155, miR-462, and miR-731 in the skeletal muscle tissue relative to controls. The same response was obtained from injection with the general IFN stimulator and Toll-like receptor (TLR) 3 agonist, polyinosinic: polycytidilic acid (poly I:C). These suggest that the induction of these miRNAs is elicited by interferons, which are major mediators of immune responses.

These regulated microRNAs could potentially be used as biomarkers of immune responses and as suitable selection markers to identify VHSV-resistant fish. Future work will involve identifying the specific cells that express these microRNAs, as well as the genes that they regulate.

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Expression of microRNAs and interferon-related genes in rainbow trout (Oncorhynchus mykiss Walbaum) infected with Viral hemorrhagic septicemia virus

The fish rhabdovirus Viral hemorrhagic septicemia virus (VHSV) causes severe disease in farmed rainbow trout (Oncorhynchus mykiss). The potential threat from wildlife marine reservoir of VHSV, particularly to sea-farmed fish demands disease protection measures. Identification of biomarkers during infection is important to understand the complex web of interactions involved in the underlying host immune response, which is a requisite to developing effective disease control strategies. MicroRNAs potently mediate post-transcriptional silencing of a broad range of genes by the RNA interference mechanism and have been shown to regulate cellular processes, including immune responses. A microarray experiment in our lab revealed that a number of miRNAs were upregulated in fish liver following VHSV infection. Here, we validated the expression of the miRNAs and analyzed for associated expression of interferon (IFN)-related genes in the liver of VHSV-infected fish. Quantitative RT-PCR verified positive regulation for most of the miRNAs shown to be upregulated by our microarray analysis. Of these, miR-155, miR-462, and miR-731 had the highest expression levels. The increased expression of these miRNAs in infected liver correlated with the upregulation of type I IFN and the type I IFN-inducible Mx and Vig-1 genes, as well as that of IFN-gamma, indicating activation of both general anti-viral IFN response and natural killer cell and/or T cell responses. Further analysis of the expression of the three most highly upregulated miRNAs in fish intramuscularly injected with a type I IFN construct and the general IFN stimulator and TLR-3 agonist, poly I:C showed increased expression of miR-155, miR-462, and miR-731, as well as Mx at the site of injection relative to controls. This suggests that the increased levels of the three miRNAs at the site of administration is associated with type I interferons, which besides their anti-viral effects are known to have major immune regulatory roles in mammals. Besides adding to our understanding of anti-viral immunity, response profiles of miRNAs could serve as molecular signatures of responses to infection. In the future we plan to analyze whether individual variability is associated with differences in disease susceptibility.

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Improved Protection of Rainbow Trout Against Furunculosis by an Autologous Vaccine Under Experimental Conditions

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. To address this issue under experimental conditions, two groups of rainbow trout were vaccinated by i.p. injection with two different oil-adjuvanted vaccines: (1) a commercial vaccine comprising Vibrio anguillarum serotype O1 and O2, and Aeromonas salmonicida subspecies salmonicida bacterins, with all bacteria originating from Atlantic salmon, and (2) an experimental vaccine based on cultures of the same bacterial species originating from rainbow trout reared in Danish sea farms. The experiment also included a third group of non-vaccinated controls. All fish were individually chip-tagged to allow mixing of all groups in three replicate aquaria. After 770 dg (day degrees) or 77 days at 10°C, half of the fish in each group were challenged by i.p. injection of 1x10^5 cells of the A. salmonicida isolate used in the experimental vaccine. The other half was tagged by cutting off the adipose fin (non-injected cohabitants). While the non-vaccinated, i.p.-injected 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 gradually risen to 15-17-20°C, the cohabitants started to die. Some variability was evident between replicate tanks, but the experimental vaccine tended to provide better protection than the commercial counterpart. The results indicate that tailormaking of a vaccine against furunculosis for sea reared rainbow trout in DK is an important approach for optimal protection.

General information
Inter-Species Transmission of Viral Haemorrhagic Septicaemia Virus Between Turbot (Scophthalmus Maximus) and Rainbow Trout (Onchorhynchus Mykiss)

Viral haemorrhagic septicaemia is a serious viral disease of teleost fish with high economic impact on the aquaculture industry. The disease is caused by the viral haemorrhagic septicaemia virus (VHSV), an RNA virus belonging to the family Rhabdoviridae. Compared to other rhabdoviruses infecting fish, VHSV has an exceptional wide host range of more than 70 species across marine and aquatic environments. To establish such a wide host range host-specific adaptation would be disadvantageous, nevertheless, host-specific differences in pathogenicity have been observed for VHSV. The divergence in pathogenicity, however, is not fully resembled in the phylogeny, which indicates a correlation between geographic regions rather than host species. The objective of this study was to identify whether VHSV has the ability to transmit between different host species or whether viral transmission is restricted to one host species through host-specific adaptation. To investigate the existence of inter-species transmission and host-specificity a cohabitation challenge between turbot and rainbow trout was conducted with turbot as donor- and rainbow trout as recipient host species. Turbot were ip challenged with a turbot- or a rainbow trout adapted VHSV isolate and subsequently grouped with naïve rainbow trout. Mortality and viral shed was monitored daily. Both virus isolates showed signs of host-specific adaptation based on differences in replication dynamics, viral production, and virulence. Host-specific adaptation, however, did not result in total restriction of inter-species transmission. Despite of host-specific adaptation, the rainbow trout adapted VHSV isolate was able to cause disease in turbot resulting in subsequent infection of cohabiting rainbow trout, thus indicating the existence of inter-species transmission of VHSV between turbot and rainbow trout.

In vivo screening of modified siRNAs for non-specific antiviral effect in a small fish model: number and localization in the strands are important

Small interfering RNAs (siRNAs) are promising new active compounds in gene medicine but the induction of non-specific immune responses following their delivery continues to be a serious problem. With the purpose of avoiding such effects chemically modified siRNAs are tested in screening assay but often only examining the expression of specific immunologically relevant genes in selected cell populations typically blood cells from treated animals or humans. Assays using a relevant physiological state in biological models as read-out are not common. Here we use a fish model where the innate antiviral effect of siRNAs is functionally monitored as reduced mortality in challenge studies involving an interferon sensitive virus. Modifications with locked nucleic acid (LNA), altritol nucleic acid (ANA) and hexitol nucleic acid (HNA) reduced the antiviral protection in this model indicative of altered immunogenicity. For LNA modified siRNAs, the number and localization of modifications in the single strands was found to be important and a correlation between antiviral
protection and the thermal stability of siRNAs was found. The previously published sisiRNA will in some sequences, but not all, increase the antiviral effect of siRNAs. The applied fish model represents a potent tool for conducting fast but statistically and scientifically relevant evaluations of chemically optimized siRNAs with respect to non-specific antiviral effects in vivo.

Oral transmission as a route of infection for viral haemorrhagic septicaemia virus in rainbow trout, Oncorhynchus mykiss (Walbaum)

Surveys among wild marine fish have revealed occurrence of viral haemorrhagic septicaemia virus (VHSV) infections in a high number of diverse fish species. In marine aquaculture of rainbow trout, preying on invading wild fish might thus be a risk factor for introduction and adaptation of VHSV and subsequent disease outbreaks. Our objective was to determine whether an oral transmission route for VHSV in rainbow trout exists. Juvenile trout were infected through oral, waterborne and cohabitation transmission routes, using a recombinant virus strain harbouring Renilla luciferase as reporter gene. Viral replication in stomach and kidney tissue was detected through bioluminescence activity of luciferase and qRT-PCR. 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 the 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 demonstrate the existence of an oral transmission route for VHSV in rainbow trout. This implies that preying on invading infected wild fish is a risk factor for introduction of VHSV into marine cultures of rainbow trout.
Typing of viral hemorrhagic septicemia virus by monoclonal antibodies

Seven mAbs with specific reaction patterns against each of the four genotypes and eight subtypes of viral hemorrhagic septicemia virus (VHSV) were produced, aiming to establish an immunoassay for typing VHSV isolates according to their genotype. Among the mAbs, VHS-1.24 reacted with all genotypes except genotype Ie, whilst mAb VHS-9.23 reacted with all genotypes except genotype III. mAb VHS-3.80 reacted with genotypes Ib, Ic, Id and II. mAb VHS-7.57 reacted with genotypes II and IVa, and mAb VHS-5.18 with genotype Ib only. Interestingly, mAb VHS-3.75 reacted with all of the genotype III isolates except a rainbow trout-pathogenic isolate from the west coast of Norway, and reacted in addition with the IVb isolate, CA-NB00-01, from the east coast of the USA. Finally, mAb VHS-1.88 reacted with all genotype IVb isolates from the Great Lakes, but not with CA-NB00-01. In conclusion, we can distinguish between all four genotypes and between five of eight subtypes of VHSV by testing isolates in immunoassay using a panel of nine mAbs. By Western blotting and transfection of cell cultures, it was shown that mAb VHS-1.24 recognized an epitope on the viral phosphoprotein (P), whilst all others recognized antigenic determinants on the nucleoprotein (N). From amino acid alignments of the various genotypes and subtypes of VHSV isolates, it was possible to determine the epitope specificity of mAb VHS-1.24 to be aa 32–34 in the P-protein; the specificities of mAbs VHS-3.80, VHS-7.57 and VHS-3.75 were found to be aa 43 and 45–48, aa 117 and 121, and aa 103, 118 and 121 of the N-protein, respectively.

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Assessment of the Epitope Specificity of Monoclonal Antibodies that can Discriminate between the Various Genotypes of VHSV

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Correlation of mRNA Profiles, miRNA Profiles, and Functional Immune Response in Rainbow Trout (Oncorrhyncus Mykiss) Infected With Viral Hemorrhagic Septicemia Virus (VHSV) and in Fish Vaccinated With a DNA Vaccine Against VHSV

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.

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Epizone: Interlaboratory Ring Trial to Compare Dna Transfection Efficiencies
Chemical-based transfection of DNA into cultured cells is routinely used to study for example viral or cellular gene functions involved in virus replication, to analyse cellular defence mechanisms or develop specific strategies to interfere with virus replication. Other applications include rescue of viruses by reverse genetics and/or generation of mutated viruses. A large number of transfection chemicals like calcium phosphate, branched organic compounds, liposomes, cationic polymers etc. are available on the market which are used by different laboratories for different cell lines. To obtain an overview on the efficiencies of varying transfection procedures, an interlaboratory ring trial was initiated within EPIZONE theme 5. A total of 15 participating laboratories from 7 member institutions received RK13 cells, plasmid DNA encoding firefly luciferase under the transcriptional control of the human cytomegalovirus major immediate early promoter, a specially developed lysis buffer and a detailed protocol. Transfected cells were harvested in the laboratories of the participants, frozen and sent to the FLI where both the luciferase activity and protein content of the individual samples were determined to compare transfection efficiency between laboratories with the same protocol and equipment. In addition some laboratories sent samples from cells they are routinely using, transfected with the provided firefly luciferase plasmid, to allow comparison of transfection efficiency between different cell types. About 50 different samples were analysed and the luciferase activity per nanogram total protein (RLU/ng) was determined. The results revealed for RK13 cells a large range of specific luciferase activities between laboratories and, in comparison to RK13 cells, also varying transfection efficacies for other the cell lines. Details will be presented.

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General and family-specific gene expression responses to viral hemorrhagic septicaemia virus infection in rainbow trout (Oncorhynchus mykiss)

The ability of rainbow trout (Oncorhynchus mykiss) to respond successfully to infection by viral hemorrhagic septicaemia virus (VHSV) is expected to involve a large number of biochemical processes. We hypothesized that this would be reflected at the gene expression level in infected fish, and we tested it by examining gene expression levels in the head kidney of trout at a genome-wide scale with a 16K cDNA microarray for salmonids. Expression levels were recorded during 16 days following bath challenge. The challenge experiment included a relatively low susceptibility (32% survival following challenge) and a relatively high susceptibility (18% survival following challenge) trout family that were both split into a group exposed to virus and a non-exposed control group. In total, 939 genes were differentially expressed between infected and non-infected fish (FDR p = 0.05). Five groups of Gene Ontology categories were involved in immune-related processes and over-represented in infected fish: (i) stress and defense response, (ii) NFκB signal transduction, (iii) response to non-self, (iv) antigen processing and presentation, and (v) proteasome complexes. The first four categories were also over-represented among the 642 differentially expressed genes in the low-susceptibility trout family but not among the 556 differentially expressed genes in the high-susceptibility trout family. Expression profiles for most immune genes discussed showed increased transcription from day 3 post-challenge. The results suggest that the innate immune system may play an important role in the successful response to VHSV in rainbow trout. In addition, the results indicate that a superior regulation of the transcription of several key innate immune-related genes contribute to the increased survival in resistant fish. (C) 2011 Elsevier Ltd. All rights reserved.

Gene regulatory mechanisms in infected fish

This talk will highlight the regulatory mechanisms of gene expression especially the programmed form of mRNA decay which is known as RNA interference (RNAi) and how this and other mechanisms contribute to the regulation of genes involved in immunity. In the RNAi mechanism small double stranded RNA molecules produced by the eukaryotic cell is...
used to program the RNA Induced Silencing Complex (RISC) for cleavage of specific mRNA transcripts and/or translational repression in the cytoplasm or even chromatin methylation in the nucleus. All processes leading to silencing of the target gene. MicroRNAs (or miRNAs) are one class of such small RNAs which are expressed from the genome. The RISC system allows for non-perfect base pairing of miRNAs to their target genes why one small RNA can in theory silence large groups of genes at the same time. It is therefore anticipated that they are able to depress whole pathways for the fine-tuning of physiological states like immunological reaction. But miRNAs are themselves under control of regulatory sequences for their timed expression. We will give an example of the finding of two rainbow trout microRNAs, which are up-regulated in the liver during infection with viral hemorrhagic septicemia virus (VHSV), and a genomic upstream sequence which we believe contains their promoter. Particular transcription factor binding motifs inside this potential promoter area point to its use in dsRNA induced antiviral defence. Other sites point to a role in leukocyte differentiation. Thus the expression of these miRNAs might be steered by different mechanisms in different cell types and have different roles in terms of the genes they target in different cell types. Thus gene regulation and function is better looked upon as a web of interactions. Data from zebrafish studies seem to show that these microRNAs are only expressed above a certain stage in the development of the fish.

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**Inhibition of Reporter Genes by Small Interfering RNAs in Cell Culture and Living Fish**
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 septicemia 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. Various types of fish including albino trouts and transparent fish were used as animal models to get better visualization of reporter gene expression. High variability of reporter gene expression was found between individual fish but it seems that in glass catfish, siRNAs are able to reduce reporter gene expression in the muscle showing that it is possible to use siRNA as technology to target genes locally in living fish. In parallel experiments, which will not be reported here, we examine the delivery of siRNAs using pharmacological formulations in order to achieve systemic delivery and knock down effect.

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**Inhibition of Reporter Genes by Small Interfering RNAs in Cell Culture and Living Fish**
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 trouts 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.

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microRNA regulation in rainbow trout infected with a fish pathogenic rhabdovirus
Rainbow trout is a major worldwide aquaculture species and viral disease has a high cost to fish farmers every year why effective treatment and a deeper understanding of immune components involved in the coexistence between fish and virus is of big concern to our field. We present here a study of microRNA regulation in rainbow trout during infection with the fish pathogenic rhabdovirus viral haemorrhagic septicaemia virus (VHSV). Infected fish as well as infected and immune stimulated cell cultures have been tested for microRNA regulation by microarray using a ‘all species’ approach followed by qPCR. Two regulated rainbow trout microRNAs have been cloned, sequenced and upstream promoter areas characterized and tested for functionality upon immune stimulation.

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Rainbow trout surviving infections of viral haemorrhagic septicemia virus (VHSV) show lasting antibodies to recombinant G protein fragments
Rainbow trout antibodies (Abs) binding to recombinant fragments (frgs) derived from the protein G of the viral haemorrhagic septicemia virus (VHSV)-07.71 strain, could be detected by ELISA (frg-ELISA) in sera from trout surviving laboratory-controlled infections. Abs were detected not only by using sera from trout infected with the homologous VHSV isolate but also with the VHSV-DK-201433 heterologous isolate, which had 13 amino acid changes. Sera from healthy trout and/or from trout surviving infectious haematopoietic necrosis virus (IHNV) infection, were used to calculate cut-off
absorbances to differentiate negative from positive sera. Specific anti-VHSV Abs could then be detected by using any of the following frgs: frg11 (56–110), frg15 (65–250), frg16 (252–450) or G21-465. While high correlations were found among the ELISA values obtained with the different frgs, no correlations between any frg-ELISA and complement-dependent 50% plaque neutralization test (PNT) titres could be demonstrated. Between 4 and 10 weeks after VHSV infection, more trout sera were detected as positives by using heterologous frg-ELISA rather than homologous PNT. Furthermore, the percentage of positive sera detected by frg11-ELISA increased with time after infection to reach 100%, while those detected by complement-dependent PNT decreased to 29.4%, thus confirming that the lack of neutralizing Abs does not mean the lack of any anti-VHSV Abs in survivor trout sera. Preliminary results with sera from field samples suggest that further refinements of the frg-ELISA could allow detection of anti-VHSV trout Abs in natural outbreaks caused by different heterologous VHSV isolates. The homologous frg-ELISA method could be useful to follow G immunization attempts during vaccine development and/or to best understand the fish Ab response during VHSV infections. The viral frgs approach might also be used with other fish species and/or viruses.

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Recombinant hybrid infectious hematopoietic necrosis virus (IHNV) carrying viral haemorrhagic septicaemia virus (VHSV) G or NV genes show different virulence properties
Viral haemorrhagic septicaemia virus (VHSV) is the economically most important viral disease in European rainbow trout farming. The virus was introduced to fresh water farms in the 1950ies from a reservoir of VHSV in the marine environment. Isolates from wild marine fish and fresh water farms are difficult to distinguish serologically but they show different virulence profiles: marine isolates typically cause little or no mortality in rainbow trout fry following experimental waterborne challenge, while freshwater isolates often kill the majority of the fish. Genetic analysis reveal that the change in host range (to include rainbow trout) likely have occurred several times. Virus from the marine environment therefore continues to represent a threat to the expanding trout aquaculture industry in the marine environment. Identification of potential virulence markers are therefore of great importance. By a reverse genetics approach using the related novirhabdovirus infectious hematopoietic necrosis virus (IHNV) as basis, four hybrid IHNV-VHSV variants were generated. These chimeric variants included substitution of the IHNV glyco(G) or nonstrutrual (Nv) protein with the corresponding G or Nv-protein from either a freshwater or a marine VHSV strain. Following rescue of the hybrid viruses, comparative challenge experiments in rainbow trout fingerlings have been performed. The pathogenicity of the recombinant IHNV-VHSV hybrid viruses were similar, regardless of whether the G or Nv originate from marine or fresh water VHSV. Recombinant IHNV gained higher virulence following substitution of the homologous G gene with the VHSV G gene, while the opposite was the case following substitution of the Nv gene. These findings suggest that higher virulence of VHSV compared to IHNV might be related to the G protein, while the VHSV Nv may not efficiently support in vivo propagation of IHNV.

General information
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Organisations: Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, National Institute for Agronomic Research
Search for genetic virulence markers in viral haemorrhagic septicaemia virus (VHSV) using a reverse genetics approach

VHSV is a negative strand RNA virus causing serious disease in farmed rainbow trout. Although VHSV has been eradicated by stamping out procedures in several fresh water bodies, recently including all streams in Denmark, the wildlife marine reservoir still represents a threat against rainbow trout farming. Particularly in Scandinavia, outbreaks of VHS in sea reared rainbow trout have demonstrated that although marine variants of VHSV are considered to be avirulent to rainbow trout, the virus is potentially able to adapt to this host and cause disease. Limited knowledge about the genetic background for virulence to rainbow trout makes it difficult to differentiate between dangerous and harmless VHSV variants. With the aim of identification of genetic virulence markers, we have implemented reverse genetics technology for generation of hybrid virus variants. By substituting different regions in the genome of a virulent VHSV variant with the homologous regions from the genome of an avirulent variant, a set of chimeric viral genomes were generated. Following rescue of the hybrid viruses, the plan is to do comparative challenge experiments in rainbow trout fingerlings in order to assess which substitutions that affect the pathogenicity of the virus.

Small regulatory RNAs of the RNA interference (RNAi) pathway as a prophylactic treatment against fish pathogenic viruses

Small RNAs acting in the recently discovered gene regulatory mechanism called RNA interference has a potential as diagnostic signatures of disease and immunological state and when produced synthetically as prophylactic treatment of such diseases. In the RNAi mechanism the cell produces different small RNAs which inhibit gene expression through more or less specific interaction with messenger RNAs resulting in repression of translation to protein. In this way cells can turn of genes of specific pathways thereby leading to altered physiological stages of tissues and possibly of whole organisms. The mechanism can be programmed with several types of small double stranded RNAs - the type of which defines the destiny of the target. One such class of regulatory RNAs called microRNAs are upregulated due to various physiological responses of the cell and they suppress many genes simultaneously believed to be connected through common or related pathways. Another class of small RNAs, the so called small interfering RNAs (siRNAs) has received attention due their high degree of target specificity. Because synthetic siRNAs can be designed to target specific disease causing genes such as viral genes or oncogenes they hold promise in the treatment against cellular diseases in veterinary as well as human medicine This presentation will give an overview of the RNAi mechanism, and examples from our studies of microRNA regulation in rainbow trout during infection with the fish pathogenic rhabdovirus viral hemorrhagic septicemia virus (VHSV) and examples of some of our results on delivery and effect of siRNAs designed to target viral genes of VHSV. The VHS disease causes high mortalities in salmonid fish aquacultures why intervention strategies are highly in demand.
Species specific inhibition of viral replication using dicer substrate siRNAs (DsiRNAs) targeting the viral nucleoprotein of the fish pathogenic rhabdovirus viral hemorrhagic septicemia virus (VHSV)

Gene knock down by the use of small interfering RNAs (siRNAs) is widely used as a method for reducing the expression of specific genes in eukaryotic cells via the RNA interference pathway. But, the effectivity of siRNA induced gene knock down in cells from fish has in several studies been questioned and the specificity seems to be a general problem in cells originating from both lower and higher vertebrates. Here we show that we are able to reduce the level of viral gene expression and replication specifically in fish cells in vitro. We do so by using 27/25-mer DsiRNAs acting as substrates for dicer for the generation of siRNAs targeting the nucleoprotein N gene of viral hemorrhagic septicemia virus (VHSV). This rhabdovirus infects salmonid fish and is responsible for large yearly losses in aquaculture production. Specificity of the DsiRNA is assured in two ways: first, by using the conventional method of testing a control DsiRNA which should not target the gene of interest. Second, by assuring that replication of a heterologous virus of the same genus as the target virus was not inhibited by the DsiRNA. Target controls are, as we have previously highlighted, essential for verification of the specificity of siRNA-induced interference with virus multiplication, but they are still not in general use.

Temperature influences the expression profiling of immune response genes in rainbow trout following DNA vaccination and VHS virus infection
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 15°C). 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 (15°C); 7 days pv (10°C); 23 days pv (5°C), 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 (5°C), 64 (10°C) and 72 (15°C), 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 synchronously with expression of the vaccine G gene. These findings encompass that for example in fish kept at 5°C, putative CD3, CD4, CD9, CD28, 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 10°C and lowest in fish at 5°C. 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.
Viral haemorrhagic septicaemia virus (VHSV) in rainbow trout: virulence variability within genotype Ib isolates

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Cellular and molecular immune responses of the sea bass (Dicentrarchus labrax) experimentally infected with betanodavirus

Naïve sea bass juveniles (38.4 ± 4.5 g) were intramuscularly infected with a sublethal dose of betanodavirus isolate 378/03, followed after 43 days by a similar boosting. This infection resulted in an overall mortality of 7.6%. At various intervals, sampling of fish tissues was performed to investigate: i) B and T lymphocyte content in organs and tissues; ii), proliferation of leucocytes re-stimulated in vitro with inactivated virus; iii) presence of serum antibody specific for betanodavirus; iv) expression of genes coding for the following immunoregulatory molecules involved in innate and acquired responses: type I IFN, Mx, IL-1, Cox-2, IL-10, TGF-β, TCRβ, CD4, CD8α, IgM, by using a quantitative PCR array system developed for sea bass. The obtained results showed a detectable increase of T cells and B cells in PBL during betanodavirus infection. Furthermore, leucocytes obtained from blood, head kidney, and gills showed a detectable “in vitro” increase in viability upon addition of inactivated viral particles, as determined by measuring intracellular ATP concentration. ELISA analysis of sera showed that exposure to nodavirus induced a low, but specific antibody titer measured 43 days after infection, despite the presence of measurable levels of natural antibody. Finally, a strong upregulation of genes coding for type I IFN, Mx, and IgM was identified after both infection and boosting. Interestingly, an upregulation of Cox-2 until boosting, and of TGF-β and IL-10 after boosting was also observed, while the other tested genes did not show any significant variations with respect to mock-treated fish. Overall, our work represents a first comprehensive analysis of cellular and molecular immune parameters in a fish species exposed to a pathogenic virus.

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Organisations: Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, Università degli Studi della Tuscia, Andalusian Institute of Agricultural and Fisheries Research and Training, Instituto
Experimental vaccination of small turbot against bacterial and viral pathogens

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Contributors: Lorenzen, E., Rasmussen, J. S., Kjær, T. E., Einer-Jensen, K., Engell-Sørensen, K., Dalsgaard, I., Nylén, J., Buchmann, K., Lorenzen, N.
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Expression Profiling of Immune Response Genes in Rainbow Trout Following DNA Vaccination and VHS Virus Infection

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Contributors: Rasmussen, J. S., Christensen, M. B., Einer-Jensen, K., Lorenzen, E., Lorenzen, N.
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Identification of Genetic Virulence Markers in VHS Virus

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Contributors: Stegmann, A., Lorenzen, N., Bremont, M., Einer-Jensen, K.
Immersion exposure of rainbow trout (Oncorhynchus mykiss) fry to wildtype Flavobacterium psychrophilum induces no mortality, but protects against later intraperitoneal challenge

Flavobacterium psychrophilum, the causative agent of RTFS or rainbow trout fry syndrome, causes high mortality among hatchery reared rainbow trout (Oncorhynchus mykiss) fry in Europe and the USA. Despite several attempts, no efficient vaccines have yet been developed, the main obstacle being that the fry have to be vaccinated very early, i.e. around 0.2–0.5 g, where RTFS usually starts to give problems in the fish farms. Consequently, only oral or bath vaccines are relevant. Immersion of fry in inactivated or attenuated bacteria has resulted in RPS values of less than 50%. However, the results are biased by the fact that the fish have been challenged by intraperitoneal (ip) or subcutaneous (sc) injection against which an immersion/oral vaccine may not protect. Therefore, the present study was undertaken in order to investigate whether the presumably most potent immersion immunization, i.e. bathing in high titres of non-attenuated isolates of F. psychrophilum, was able to induce immunity to a subsequent ip challenge. Immersion in live bacteria for 30 or 50 min caused no mortality and protected a major fraction of the fry against challenges 26 and 47 days later with RPS values of 88.2 and 60.3%, respectively. Increased specific antibody titres suggested that adaptive immune mechanisms were involved in the protection.

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In vivo screening of backbone modified siRNAs for their ability to induce interferon based off-target effects

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Contributors: Schyth, B. D., Bramsen, J. B., Kjems, J., Wengel, J., Lorenzen, N.
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N-Linked Glycans on the Viral Glycoprotein are not Required for Induction of Protective Immunity to VHSV when Delivered as a DNA Vaccine

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Protection Against Viral Haemorrhagic Septicemia Virus (VHSV) in Rainbow Trout Using a DNA Vaccine with MX1 Promotor Controlled Expression of the Viral G Protein

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Studies on herd-immunity and primary versus secondary infection of VHSV in challenge and vaccination trials with rainbow trout

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Temperature effects on vaccine induced immunity to viruses in fish
Abstract In poikilothermic vertebrates such as teleost fishes, temperature affects all physiological processes including host-pathogen interactions like immune response and propagation of infection. Whether an infection with a pathogenic virus in fish results in development of clinical disease often depends on the balance between virus multiplication and anti viral immune reactions in the host. Water temperature is one of the most important factors influencing the balance between the fish and its environment. Usually, an optimal immune response of a particular fish species is obtained at its normal summer temperature whereas low temperatures may be immunosuppressive. Although innate and adaptive immune response mechanisms should be considered as integrated parts of the immunedefence, low temperatures appears to affect (inhibit) adaptive mechanisms more than innate mechanisms. This might represent a problem in terms of inducing a protective immune response by vaccination in aquaculture, since it is often desirable to vaccinate fish during autumn, winter, or spring. In experimental vaccination trials with rainbow trout (Oncorhynchus mykiss) using a DNA-vaccine encoding the viral glycoprotein of viral haemorrhagic septicamaea virus (VHSV), non-specific as well as specific immune mechanisms seemed to be delayed at low temperature. At five weeks post vaccination fish kept at 5C had no detectable response of neutralising antibodies while two thirds of the fish kept at 15C had sero-converted. While protective immunity was still established at both temperatures, specificity analysis suggested that protection at the lower temperature was mainly due to non-specific innate antiviral mechanisms, which appeared to last longer at low temperature. This was presumably related to a prolonged persistence of the vaccine. In DNA vaccination trials with spring viremia of carp (SVC)
in common carp (Cyprinus carpio), protection at low temperature (10°C) appeared to require considerably longer time to develop compared to at 19°C, stressing that determination of optimal vaccination strategies in terms of temperature related effects need to be based on experimental evidence with the actual host and pathogen species rather than general principles.

Using small interfering RNAs (siRNAs) to combat a fish pathogenic virus

Viral diseases of fish and a possible role for small regulatory RNAs in their antiviral defence

Adaptive versus innate immune mechanisms in trout responding to rhabdovirus antigens.
Distinction of genotypes of viral haemorrhagic septicaemia virus (VHSV) by monoclonal antibodies

VHSV isolates can be divided into 4 major genotypes and a number of subtypes with an almost distinct geographical distribution. Host range and pathogenicity appear to some extent to be linked with genotypes. Once new genotypes of VHSV will be introduced into new areas, they can cause severe outbreaks of VHS among susceptible fishes. According to the OIE Aquatic Animal Health Code, even if the same disease agent is present in both the import and the export country, the importing country can demand health certificate of the exporting country for imports when the pathogenicity or host range of the strain in the exporting country is significantly higher or larger than that in the importing country. In order to prevent introduction to or spreading in a country of new genotypes of VHSV and to facilitate the responsibilities of exporting and importing countries, such as issuing health certificates and carry out quarantine and disease control programs, a quick and simple detection method for discriminating between each the genotypes of VHSV is strongly desired. Monoclonal antibodies (MAbs) VHS-10 and VHS-5.18 specifically recognizing VHSV genotypes IVa and Ib respectively, as well as MAb IPSB11 recognizing all known VHSV isolates, were prepared earlier. In the present study, more new genotype specific monoclonal antibodies against VHSV were produced, aiming at establishing a complete immunoassay for typing of VHSV according to genotype. BALb-c mice were immunized with purified preparations of 7 different genotypes of VHSV (I, Ia, Ib, II, III, IVa and IVb). Six MAbs from these hybridoma clones were selected and their MAbs reactivity in IFAT and ELISA tested against a large panel of 79 VHSV isolates. The isolates representing all known geno- and subgenotypes of VHSV. Among the new MAbs, VHS-1.24, reacted with all types except genotype Ie (the Black Sea variant of VHSV), while MAb VHS-9.23 reacted with all genotypes except genotype III. MAb VHS-3.80 reacted with genotypes Ib, Ic, Id and II, only. MAb VHS-7.57 reacted with genotype II and IVa. Interestingly, MAb VHS-3.75 reacted with all genotype III isolates except the rainbow trout pathogenic isolate from Norway (NO-2007-50-385) (Dale et al. in press), but did react with the New Brunswick VHSV IVb isolate (Oliver 2002, Gagné et al. 2007). Another MAb (VHS-1.88) reacted with genotype IVb only, except with the New Brunswick isolate. The present findings support a phenotypic difference between NO-2007-50-385 and the other virus representatives in genotype III, and genotype IVb may eventually be split up in two subgroups (the Great Lakes isolates and New Brunswick isolate). In conclusion, we can now distinguish between all genotypes and some of subtypes of VHSV by testing isolates in IFAT or ELISA with 9 MAbs (Table 1).
Dual DNA vaccination of rainbow trout (Oncorhynchus mykiss) against two different rhabdoviruses, VHSV and IHNV, induces specific divalent protection

DNA vaccines encoding the glycoprotein genes of the salmonid rhabdoviruses VHSV and IHNV are very efficient in eliciting protective immune responses against their respective diseases in rainbow trout (Oncorhynchus mykiss). The early anti-viral response (EAVR) provides protection by 4 days post vaccination and is non-specific and transient while the specific anti-viral response (SAVR) is long lasting and highly specific. Since both VHSV and IHNV are endemic in rainbow trout in several geographical regions of Europe and Atlantic salmon (Salmo salar) on the Pacific coast of North America, co-vaccination against the two diseases would be a preferable option. In the present study we demonstrated that a single injection of mixed DNA vaccines induced long-lasting protection against both individual and a simultaneous virus challenge 80 days post vaccination. Transfected muscle cells at the injection site expressed both G proteins. This study confirms the applied potential of using a combined DNA vaccination for protection of fish against two different rhabdoviral diseases.

Functional demonstration of adaptive immunity in zebrafish using DNA vaccination.

Due to the well characterized genome, overall highly synteny with the human genome and its suitability for functional genomics studies, the zebrafish is considered to be an ideal animal model for basic studies of mechanisms of diseases and immunity in vertebrates including humans. While several studies have documented existence of a classical innate immune response, there is mainly indirect evidence of functional adaptive immunity. To address this aspect, groups of zebrafish were vaccinated with DNA-vaccines against the rhabdoviruses VHSV, IHNV and SVCV. Seven weeks later, the fish were challenged with SVCV by immersion. Despite some variability between replicate aquaria, there was a protective effect of the homologous vaccine and no effect of the heterologous vaccines. The results therefore confirm the existence of not only a well developed but also a fully functional adaptive immune system in zebrafish.
Genetic and serological typing of European infectious haematopoietic necrosis virus (IHNV) isolates

Infectious haematopoietic necrosis virus (IHNV) causes the lethal disease infectious haematopoietic necrosis (IHN) in juvenile salmon and trout. The nucleocapsid (N) protein gene and partial glycoprotein (G) gene (nucleotides 457 to 1061) of the European isolates IT-217A, FR-32/87, DE-DF 13/98 11621, DE-DF 4/99-8/99, AU-9695338 and RU-FR1 were sequenced and compared with IHNV isolates from the North American genogroups U, M and L. In phylogenetic studies the N gene of the Italian, French, German and Austrian isolates clustered in the M genogroup, though in a different subgroup than the isolates from the USA. Analyses of the partial G gene of these European isolates clustered them in the M genogroup close to the root while the Russian isolate clustered in the U genogroup. The European isolates together with US-WRAC and US-Col-80 were also tested in an enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies (MAbs) against the N protein. MAbs 136-1 and 136-3 reacted equally at all concentrations with the isolates tested, indicating that these antibodies identify a common epitope. MAb 34D3 separated the M and L genogroup isolates from the U genogroup isolate. MAb 1DW14D divided the European isolates into 2 groups. MAb 1DW14D reacted more strongly with DE-DF 13/98 11621 and RU-FR1 than with IT-217A, FR-32/87, DE-DF 4/99-8/99 and AU-9695338. In the phylogenetic studies, the Italian, French, German and Austrian isolates clustered in the M genogroup, whereas in the serological studies using MAbs, the European M genogroup isolates could not be placed in the same specific group. These results indicate that genotypic and serotypic classification do not correlate.

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Interference of an ERM-vaccine with a VHS-DNA vaccine in rainbow trout

Simultaneous vaccination of fish against several diseases is often desirable in order to minimise cost and handling of the fish. Intramuscular DNA-vaccination of rainbow trout against viral haemorrhagic septicaemia virus (VHSV) has proved to provide very good protection. However, preliminary results showed that intraperitoneal injection of a commercial vaccine against Enteric Redmouth Disease (ERM) based on formalin-killed bacteria in oil adjuvant immediately followed by intramuscular injection of an experimental DNA-vaccine against VHSV, decreased the protective effect of the DNA-vaccine against challenge with VHSV 11 weeks post vaccination (pv). This experiment was performed with rainbow trout of 30 g injected with 0.5 g VHS-DNA vaccine. The experiment was later repeated with smaller fish (2.5g) and using two different doses of DNA-vaccine, 1 g and 0.05 g. Both doses provided good protection in the control groups not given the ERM vaccine. But among fish given both vaccines, those vaccinated with the lower DNA dose had significantly higher mortality when challenged with VHSV 9 weeks pv. When challenged with VHSV 8 days pv, not even the 1 µg DNA dose protected such fish. A plasmid dose of 0.05 g VHSV DNA vaccine would normally induce good protection in small fish (2-3 g). To ensure complete protection in larger fish, higher doses are needed. This could explain the negative effect of ERM vaccination observed in the 30 g fish described above. It thus appears, that if the fish are vaccinated with a VHS-DNA vaccine dose according to their size, a simultaneous intraperitoneal vaccination against ERM can compromise the protective effect of the DNA-vaccine. The negative effect appears to be strongest in the early phase following vaccination. The immune mechanisms behind this interference will be discussed.

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Organisations: Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, Marine Scotland, Norwegian School of Veterinary Medicine
In Vivo Screening of Chemically Modified RNA duplexes for their Ability to Induce Innate Immune Responses

Due to their sequence specific gene silencing activity siRNAs are regarded as promising active compounds in gene medicine. But one serious problem with delivering siRNAs as treatment is the now well-established non-specific activities of some RNA duplexes. Cellular reactions towards double stranded RNAs include the 2′-5′ oligoadenylate synthetase system, the protein kinase R, RIG-I and Toll-like receptor activated pathways all resulting in antiviral defence mechanism. We have previously shown that antiviral innate immune reactions against double stranded RNAs could be detected in vivo as partial protection against a fish pathogenic virus. This protection corresponded with an interferon response in the fish. Here we use this fish model to screen siRNAs containing various chemical modifications of the RNA backbone for their antiviral activity, the overall aim being identification of an siRNA form with minimal immunostimulatory effects.

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IN VIVO SCREENING OF CHEMICAL MODIFICATIONS OF siRNAs FOR EFFECT ON THE INNATE IMMUNE RESPONSE IN FISH

Abstract Due to their sequence specific gene silencing activity siRNAs are regarded as promising new active compounds in gene medicine and functional studies. But one serious problem with delivering siRNAs as treatment is the now well-established non-specific activities of some RNA duplexes. Cellular reactions towards double stranded RNAs include the 2′-5′ oligoadenylate synthetase system, the protein kinase R, RIG-I and Toll-like receptor activated pathways all resulting in antiviral defence mechanism. We have previously shown that antiviral innate immune reactions against injected siRNAs could be detected in vivo as reduced susceptibility to a fish pathogenic virus. This protection corresponded with an interferon response. Here we use this fish model to screen siRNAs containing various chemical modifications of the RNA backbone and find that it is possible to differentiate between the antiviral activities of these duplexes. We conclude that the fish in vivo model is a potent tool for gaining insight into the overall triggering of antiviral reactions by siRNAs in vertebrates. The perspective is to learn how to avoid triggering of non-specific antiviral responses and still allow uptake of siRNAs into RISC for specific gene silencing.

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Organisations: Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, Aarhus University, University of Southern Denmark
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MicroRNA Expression during Viral Infection or PolyI:C Stimulation in a Fish Model

Fish are important as small vertebrate models for studying various aspects of development and disease. MicroRNA regulation in fish has so far received attention especially in studies of their expression and function during embryonic development. In the studies carried out at the National Veterinary Institute in Århus we aim at using fish models for studying microRNA regulation during viral infection. In the studies presented here we make use of a qPCR method to detect miRNAs in fish cells. We present results regarding the expression of the immunologically relevant microRNAs, miR-155, miR-146a and miR-146b in fish cells during infection with the fish pathogenic virus viral hemorrhagic septicemia virus (VHSV) and during immune stimulation with double stranded RNA (polyI:C). We highlight the need of finding stable normalization genes for microRNA detection.

New tools to study RNA interference to fish viruses: Fish cell lines permanently expressing siRNAs targeting the viral polymerase of viral hemorrhagic septicemia virus

Previous studies have indicated that low transfection efficiency can be a major problem when gene inhibition by the use of small interfering RNAs (siRNAs) is attempted in fish cells. This may especially be true when targeting genes of viruses which are fast replicating and which can still infect cells that have not been transfected with the antiviral siRNAs. To increase the amount of antiviral siRNAs per cell a different strategy than transfection was taken here. Thus, we describe carp epiboly papulosum cyprinid (EPC) cell clones expressing siRNAs designed to target the L polymerase gene of the viral hemorrhagic septicemia virus (VHSV), a rhabdovirus affecting fish. Eight siRNA sequences were first designed, synthesized and screened for inhibition of in vitro VHSV infectivity. Small hairpin (sh) DNAs corresponding to three selected siRNAs were then cloned into pRNA-CMV3.1/puro plasmids, transfected into EPC cells and transformed clones were obtained by puromycin selection. Sequence-specific interference with VHSV could only be observed with EPC clones transformed with a mixture of the three shDNAs, rather than with those clones obtained with individual sh DNAs. However, interference was not specific for VHSV as infection with an heterologous fish rhabdovirus, was also reduced to a similar extent. It was shown that this reduction was not due to an Mx response in the transformed cell clones. Here, we discuss some of the possible reasons for such data and future work directions. EPC clones stably expressing rhabdoviral specific siRNA sequences could be a strategy to further investigate the use of RNA interference for targeting costly fish pathogenic viruses.
Screening of Modified RNA duplexes

Because of sequence specific gene targeting activity siRNAs are regarded as promising active compounds in gene medicine. But one serious problem with delivering siRNAs as treatment is the now well-established non-specific activities of some RNA duplexes. Cellular reactions towards double stranded RNAs include the 2'-5' oligoadenylate synthetase system, the protein kinase R, RIG-I and Toll-like receptor activated pathways all resulting in antiviral defence mechanism. We have previously shown that antiviral innate immune reactions against double stranded RNAs could be detected in vivo as partial protection against a fish pathogenic virus. This protection corresponded with an interferon response in the fish. Here we use this fish model to screen siRNAs containing various chemical modifications of the RNA backbone for their antiviral activity, the overall aim being identification of an siRNA form with minimal immunostimulatory effects.

O-114: Distinction between genotypes of viral haemorrhagic septicaemia virus (VHSV) using monoclonal antibodies

Studies on herd-immunity and primary versus secondary infection of VHSV in challenge and vaccination trials with rainbow trout
Abstract for Scofda meeting 4-5.11.09:

Studies on herd-immunity effect and primary versus secondary infection of VHSV by Ellen Lorenzen, Torben Eigil Kjær & Niels Lorenzen, National Veterinary Laboratory, Århus

The phenomenon of "herd-immunity" is one of the basal principles behind vaccination as well as selective breeding, i.e. the more non-susceptible individuals in a population, the lower the risk of disease among susceptible individuals. Thus as part of a recent field trial with a VHS-DNA-vaccine vaccinated as well as naïve fish from a Danish fish farm were brought to the laboratory at a size of 24g to be subjected to an experimental challenge with VHSV. The setup included 7 aquaria with 100 fish in each: 2 aquaria with 100 vaccinated fish (+VHS-challenge), 2 aquaria with 100 naïve fish (+VHS-challenge), 2 aquaria with 50 vaccinated + 50 naïve fish (+VHS-challenge), and 1 aquarium with non-challenged control fish (vaccinated + naïve). Mortality in the aquaria with only vaccinated fish was 2-3 %. Mortality in the aquaria with only naïve fish was 60-70 %. However, mortality among naïve fish in the mixed aquaria was only 6-18 %, the mortality among vaccinated fish being 0-6 %, and we interpreted this as an effect of herd-immunity, where the vaccinated fish protected the naïve fish, probably by secreting less virus compared to the naïve fish. We tried to demonstrate this phenomenon in 3 later experiments, but without success, probably due to a too high challenge load in relation to the susceptibility of the fish included in these studies, i.e. it was shown that the challenged vaccinated fish secreted large amounts of virus, although still less than challenged naïve fish. However, these results led to the question if the fish die due to the challenge virus or due to the virus secreted from fish in the same aquarium that become diseased at an early time point. This question was addressed in 3 subsequent parallel challenge experiments (3 different virus doses) including only one fish in 24 aquaria and 24 fish in 3 aquaria. The study showed, that at high challenge doses, mortality was comparable in the single-fish group (24 aquaria) and the group with 24 fish in each of 3 aquaria. At lower challenge doses, however, the survival rate increased in the single fish group the lower the virus titer during challenge. i.e. at lower challenge doses, secondary infections seem more pronounced. These results will be presented and discussed.

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Contributors: Lorenzen, E, Kjær, T. E., Lorenzen, N.
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The protective mechanisms induced by a fish rhabdovirus DNA vaccine depend on temperature

DNA vaccines encoding the viral glycoproteins of viral haemorrhagic septicaemia virus (VHSV) and infectious haematopoietic necrosis Virus (IHNV) have proved highly efficient in rainbow trout (Oncorhynchus mykiss) under experimental conditions. Non-specific as well as specific immune mechanisms seem to be activated. Temperature is an important external parameter affecting the immune response in fish. The present study aimed at determining the effectiveness of a DNA vaccine against VHSV at different temperatures. Rainbow trout fingerlings acclimated at 5 degrees C, 10 degrees C or 15 degrees C, were given an intramuscular injection of 1 µg purified plasmid DNA and challenged with virulent VHSV 8 or 36-40 days later. The vaccine protected the fish well at all three temperatures, but the involvement of innate and adaptive mechanisms differed: at low temperature. non-specific protection lasted longer and at 36 dpv fish kept at 5 degrees C had no detectable response of neutralizing antibodies while 67% of the fish kept at 15 degrees C had seroconverted. Induction of Mx as measured in liver samples was delayed at 5 degrees C with no detectable response 7 dpv whereas fish maintained at 10 C had significantly elevated levels of Mx3-transcripts at that time point. Immunohistochemical studies of the injection site of vaccinated fish also showed a clear effect of temperature: in fish maintained at 15 degrees C the vhsG-protein appeared earlier on the surface of transfected myocytes and the inflammatory response clearing away these myocytes arose earlier Compared to fish kept at the lower temperatures of 5 and 10 degrees C.

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THE PROTECTIVE MECHANISMS INDUCED BY A FISH RHABDOVIRUS DNA-VACCINE DEPENDS ON TEMPERATURE

DNA-vaccines encoding the viral glycoproteins of viral haemorrhagic septicemia virus (VHSV) and infectious haematopoietic necrosis virus (IHNV) have proved highly efficient in rainbow trout (Oncorhynchus mykiss) under experimental conditions. In the early phase following vaccination, innate cross-protective mechanisms are dominating but the protection becomes highly specific within 3-4 weeks at 12-15°C. Temperature is known as an important external parameter affecting the immune response in fish and the present study aimed at characterizing temperature effects on the immune response to a VHS DNA vaccine. Rainbow trout fingerlings acclimated at 5°C, 10°C or 15°C, were given an intramuscular injection of 1g purified plasmid DNA and challenged with virulent VHSV 9 or 36-40 days later. The vaccine protected the fish well at all three temperatures, however the non-specific mechanisms lasted for a longer period of time at lower temperatures, hereby apparently compensating for a delayed adaptive response. At 36 dpv fish kept at 5°C thus had no detectable response of neutralising antibodies while 67% of the fish kept at 15°C had seroconverted. Immunohistochemical time-course studies of the injection site of vaccinated fish also showed a clear effect of temperature: in fish maintained at 15°C the vhsG-protein occurred earlier on the surface of transfected myocytes than in fish kept at the lower temperatures and the inflammatory response clearing away these myocytes similarly arose earlier at high temperature. Long persistence of transfected myocytes expressing the vaccine antigen might explain the prolonged stimulation of innate protective mechanisms at low temperature. From a practical point of view the results suggest that DNA vaccination against rhabdoviruses might be applied as a prophylactic measure within a broad temperature range.

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Cell-mediated immune responses in rainbow trout after DNA immunization against the viral hemorrhagic septicemia virus
To identify viral proteins that induce cell-mediated cytotoxicity (CMC) against viral hemorrhagic septicemia virus (VHSV)-infected cells, rainbow trout were immunized with DNA vectors encoding the glycoprotein G or the nucleocapsid protein N of VHSV. The G protein was a more potent trigger of cytotoxic cells than the N protein. Peripheral blood leukocytes (PBL) isolated from trout immunized against the G protein killed both VHSV-infected MHC class I matched (RTG-2) and VHSV-infected xenogeneic (EPC) target cells, suggesting the involvement of both cytotoxic T lymphocytes (CTL) and NK cells, respectively. In contrast, PBL from trout that were immunized against the N protein only killed VHSV-infected RTG-2 cells, indicating that this protein only elicits a CTL response. Further, a significant killing capacity of these PBL was only observed during summer months. PBL from fish that were immunized against the VHSV G protein significantly killed VHSV-infected but not infectious hematopoietic necrosis virus (IHNV)-infected targets indicating antigen specificity. Thus, this is the first report on cytotoxic immune responses after DNA vaccination in fish. Furthermore, cells isolated from the inflamed site of DNA injection were stained and transferred to isogeneic DNA-vaccinated recipients. Most of the stained donor leukocytes accumulated at the recipients' DNA injection site showing, for the first time, leukocyte homing in fish. Transferred donor leukocytes mainly migrated to the homologous vaccine injection site rather than to injection sites of heterologous vaccines, suggesting the antigen specificity of homing. By demonstrating CMC responses to distinct viral proteins and homing in rainbow trout, these results substantially contribute to the understanding of the teleost immune system.
A High Throughput In Vivo Model for Testing Delivery and Antiviral Effects of siRNAs in Vertebrates

Despite the promise of small interfering RNAs (siRNAs) in antiviral therapy, few in vivo studies of them as inhibitors of viral replication and disease have been published, a lack that is most probably due to problems with obtaining successful delivery. Here we introduce a novel in vivomodel composed of small juvenile rainbow trout and a fish pathogenic virus to analyze the delivery and antiviral effects of formulated siRNAs. Intrapitoneally (IP) injected siRNAs formulated in polyctonic liposomes, and to a lesser degree naked siRNAs, primarily entered free IP cells, including macrophage-like cells. Uptake in these cells correlated with antiviral activity, seen as reduced mortality of virus-challenged fish. However, protection at the disease level was not dependent upon which of three tested siRNAs was used, and protection correlated with up-regulation of an interferon (IFN)-related gene in the liver, indicating a systemic IFN response. The results emphasize the compromise in using transfection reagents for improved uptake of siRNAs, where these reagents also increase the risk of the siRNAs ending up in a cellular compartment in which stimulation of non-specific anti-viral defence mechanisms will be initiated.

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Cell-mediated cytotoxicity in rainbow trout, Oncorhynchus mykiss, infected with viral haemorrhagic septicaemia virus
Mammalian cytotoxic T cells as part of the adaptive immune system recognize virus-infected target cells by binding of their T-cell receptors (TCR) to classical MHC class I molecules loaded with viral peptides. Our previous studies have shown that the allele of the single dominant polymorphic classical MHC class I locus Onmy-UBA is identical in the rainbow trout clone C25 and in the permanent rainbow trout cell line RTG-2. This enabled us to develop an assay to measure antiviral cytotoxicity in rainbow trout using a system of MHC class I-matched effector and target cells. Peripheral blood leucocytes (PBL) isolated from low dose viral haemorrhagic septicaemia virus (VHSV)-infected rainbow trout killed MHC class I-matched and later also xenogeneic MHC class I-mismatched VHSV-infected cells. When compared to PBL from uninfected control fish PBL from infected fish showed a higher transcriptional level of the CD8 alpha gene which is a typical marker for mammalian cytotoxic T cells. Concurrently, the expression of the natural killer cell enhancement factor (NKEF)-like gene was enhanced as measured by real-time RT-PCR. Taken together, these results suggest that both innate and adaptive cell-mediated immune responses represented by NK and cytotoxic T cells, respectively, are triggered after VHSV infection. PBL that were able to kill VHSV-infected MHC class I-mismatched xenogeneic cells were generated later during infection than PBL capable of lysing VHSV-infected MHC class I-matched targets. This is contradictory to the generally accepted rule that innate immune mechanisms represent the first line of defence after viral infections.

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Contributors: Utke, K., Bergmann, S., Lorenzen, N., Kollner, B., Ototake, M., Fischer, U.
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Classification of viral haemorrhagic septicaemia virus (VHSV) and how do we define the disease VHS?

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Contributors: Olesen, N. J., Madsen, S., Einer-Jensen, K., Skall, H. F., Lorenzen, N.
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Antiviral activity of Small interfering RNAs: Specificity testing using heterologous virus reveals interferon-related effects overlooked by conventional mismatch controls

RNA interference by small interfering RNAs (siRNAs) is considered to be a highly specific method for knockdown of gene expression in eukaryotic cells via degradation of target mRNA. Mutated siRNA molecules with 1–4 mismatching nucleotides compared to the target mRNA are regularly used as specificity controls. Using siRNAs for inhibition of a fish-pathogenic rhabdovirus, we report that inclusion of a heterologous virus, as target control is essential for verification of the specificity of siRNA-induced interference with virus multiplication. Transfection with three different siRNAs specific to the viral glycoprotein gene of the target-virus efficiently inhibited viral multiplication in infected cell cultures, while two of three corresponding mismatched siRNAs did not have this effect. This suggested specific interference, but similar results were obtained when the same siRNAs were tested against a heterologous virus. Further analyses revealed that the siRNAs induced a non-target-specific anti-viral effect correlating with upregulation of the interferon induced Mx gene.

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Expression of the glycoprotein of viral haemorrhagic septicaemia virus (VHSV) on the surface of the fish cell line RTG-P1 induces type 1 interferon expression in neighbouring cells

In the present study using a luciferase/Mx promoter reporter system, it was shown that the rainbow trout gonad cell line (RTG-P1), a fibroblastic cell line, produces IFN when transfected with a plasmid encoding the glycoprotein of VHSV but not with plasmid vector alone. Only a small percentage of the cells expressed the G protein on the surface membrane as indicated by immunostaining of transfected cells. When transfection was performed in the presence of monoclonal antibodies (Mab) to the glycoprotein, the production of interferon mRNA transcripts was reduced by over 50%. This indicates that the surface expression of G protein was the major mechanism of interferon induction and that most of the interferon was being expressed by cells neighbouring the transfected cells. Crown

Genetic stability of the VHSV consensus sequence of G-gene in diagnostic samples from an acute outbreak

The negative stranded RNA virus viral haemorrhagic septicaemia virus (VHSV) is an important disease-causing agent in aquacultured fish and internationally harmonized diagnostic procedures are continuously under development. The present study concerns the suitability of genotyping by sequencing of RT-PCR products for epidemiological analysis. Focus was put on a specific case story involving an acute outbreak of VHS in a Danish rainbow trout farm which otherwise had been free of VHSV during the previous 5 years. Tissue materials from individual fish were collected during routine inspection and the initial diagnosis was based on isolation of the virus by cell cultivation and subsequent identification by ELISA. Additional tissue samples were collected 25 days after the initial sampling. RT-PCR amplification and sequencing of the entire glycoprotein-gene (1524 nt) was performed on RNA purified from collected tissue material as well as from inoculated cell culture. No nucleotide substitutions where identified when aligning the obtained sequence data for the two sample types. The presented data indicate that the overall consensus sequence of the virus outbreak was stable during the survey, and that initial passage of the virus on BF-2 cells did not result in changes within the G-gene at a detectable level. The results suggest that genotyping of VHSV isolates based on RT-PCR products amplified from infected primary tissues material is a reliable tool for epidemiological studies.
Monitoring of the immune system in fish and shellfish

Deoxyribonucleic acid (DNA) vaccination is based on the administration of the gene encoding the vaccine antigen, rather than the antigen itself. Subsequent expression of the antigen by cells in the vaccinated hosts triggers the host immune system. Among the many experimental DNA vaccines tested in various animal species as well as in humans, the vaccines against rhabdovirus diseases in fish have given some of the most promising results. A single intramuscular (IM) injection of microgram amounts of DNA induces rapid and long-lasting protection in farmed salmonids against economically important viruses such as infectious haematopoietic necrosis virus (IHNV) and viral haemorrhagic septicaemia virus (VHSV). DNA vaccines against other types of fish pathogens, however, have so far had limited success. The most efficient delivery route at present is IM injection, and suitable delivery strategies for mass vaccination of small fish have yet to be developed. In terms of safety, no adverse effects in the vaccinated fish have been observed to date. As DNA vaccination is a relatively new technology, various theoretical and long-term safety issues related to the environment and the consumer remain to be fully addressed, although inherently the risks should not be any greater than with the commercial fish vaccines that are currently used. Present classification systems lack clarity in distinguishing DNA-vaccinated animals from genetically modified organisms (GMOs), which could raise issues in terms of licensing and public acceptance of the technology. The potential benefits of DNA vaccines for farmed fish include improved animal welfare, reduced environmental impacts of aquaculture activities, increased food quality and quantity, and more sustainable production. Testing under commercial production conditions has recently been initiated in Canada and Denmark.
Genotyping of the fish rhabdovirus, viral haemorrhagic septicaemia virus, by restriction fragment length polymorphisms

The aim of this study was to develop a standardized molecular assay that used limited resources and equipment for routine genotyping of isolates of the fish rhabdovirus, viral haemorrhagic septicaemia virus (VHSV). Computer generated restriction maps, based on 62 unique full-length (1524 nt) sequences of the VHSV glycoprotein (G) gene, were used to predict restriction fragment length polymorphism (RFLP) patterns that were subsequently grouped and compared with a phylogenetic analysis of the G-gene sequences of the same set of isolates. Digestion of PCR amplicons from the full-length G-gene by a set of three restriction enzymes was predicted to accurately enable the assignment of the VHSV isolates into the four major genotypes discovered to date. Further sub-typing of the isolates into the recently described sub-lineages of genotype I was possible by applying three additional enzymes. Experimental evaluation of the method consisted of three steps: (i) RT-PCR amplification of the G-gene of VHSV isolates using purified viral RNA as template, (ii) digestion of the PCR products with a panel of restriction endonucleases and (iii) interpretation of the resulting RFLP profiles. The RFLP analysis was shown to approximate the level of genetic discrimination obtained by other, more labour-intensive, molecular techniques such as the ribonuclease protection assay or sequence analysis. In addition, 37 previously uncharacterised isolates from diverse sources were assigned to specific genotypes. While the assay was able to distinguish between marine and continental isolates of VHSV, the differences did not correlate with the pathogenicity of the isolates.

Kinetics of Mx expression in rainbow trout (Oncorhynchus mykiss) and Atlantic salmon (Salmo salar L.) parr in response to VHS-DNA vaccination

The duration of the Mx mRNA response to an intramuscular injection of the viral haemorrhagic septicaemia virus (VHSV) glycoprotein (G) gene DNA vaccine as well as to the control plasmid was determined in rainbow trout at 14 degreesC over a period of 11 weeks. The Mx response was detectable on day 7, peaked on day 14 and returned to pretreatment levels on day 21 and thereafter. No increase in Mx expression was detectable to the control plasmid. In further experiments, the kinetics of the Mx response were compared in rainbow trout and Atlantic salmon parr kept at 10 degreesC and injected with the DNA vaccine or the synthetic double-stranded RNA, poly LC. In both species there was a rapid response to poly LC detectable from day 1, reaching maximum from days 3 to 9 and decreasing to background level by day 12. The peak
level and return to background was reached slightly later in salmon. In both species the response to the VHS/DNA vaccine was slower to begin, not being detectable on days 1 and 3, but elevated levels were found on day 6. However, in the salmon part, the peak level was on day 6 and the signal disappeared by day 12, while in the rainbow trout, the response peaked at day 12 and lasted until day 21. The kinetics of the Mx response to the VHS/DNA vaccine in rainbow trout correlate with the early non-specific protection against VHS in this species following vaccination. It is speculated that the more transient Mx response in Atlantic salmon parr to the DNA vaccine may be related to the innate resistance of salmon to VHS.

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Contributors: Acosta, F., Petrie, A., Lockhart, K., Lorenzen, N., Ellis, A.
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Parallel phylogenetic analyses using the N, G or Nv gene from a fixed group of VHSV isolates reveal the same overall genetic typing
Different genetic regions representing the viral phospho-(P), nucleocapsid-(N) or glyco-protein (G) gene have been used for phylogenetic studies of viral haemorrhagic septicaemia virus (VHSV). Since these analyses were performed on different virus isolates using various genomic regions, it has been difficult to evaluate how the choice of target region affects the output of the analyses. To address this, we sequenced and performed parallel phylogenetic analysis of an N gene fragment, the entire Nv (non-structural protein) and G genes, and 4 different fragments of the G gene from a fixed virus panel. The overall genotyping of the selected isolates was identical for the 7 target regions, but separation of Genotype I sub-lineages was best when the analysis was performed on the full length G gene (1524 nucleotides, nt). Good resolution was furthermore obtained using smaller sequencing windows represented by a G gene fragment (nt 360 to 720) or the Nv gene (366 nt), although these regions had different characteristics with respect to resolution of Genotype I sublineages and resolution within Sub-lineage Ia. Phylogenetic analysis based on the deduced amino acid sequences was also performed. The phylogenetic relationship between the nucleotide and amino acid sequences of the isolates corresponded best in the case of the N gene/protein. For the 6 other genomic regions, genetically distant isolates occasionally grouped together when compared at protein levels. No clear relationship between the G gene genotyping and serotyping with neutralising (G protein specific) antibodies was observed, stressing that epidemiological analysis based on phenotypic characteristics such as serotype could be misleading.

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Contributors: Einer-Jensen, K., Ahrens, P., Lorenzen, N.
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Time course study of in situ expression of antigens following DNA-vaccination against VHS in rainbow trout (Oncorhynchus mykiss Walbaum) fry

The present study was performed as a time course study of fish vaccinated with 20 μg plasmid DNA vaccine encoding either the VHSV G-protein or the VHSV N-protein. Samples of the injection site were collected sequentially over a 7-week period. The study revealed an intense positive staining by immunohistochemistry for the viral G-protein mainly in the membrane of intact myocytes, most prominent by days 10-27, and with concomitant infiltration of inflammatory cells by days 13-38 that subsequently lead to a marked reduction in the number of myocytes expressing the G-protein. By immunofluorescence, infiltrating cells positive for MHC II, IgM, and C3 were demonstrated. By contrast, in fish vaccinated with the VHSV-N construct, fewer, diffusely positive myocytes were found, most prominent by days 13-38, these having a positive reaction for the N-protein mainly in the cytoplasm and variably in the membrane. N-protein positive myocytes did not attract infiltrating cells to the same degree. Positive reaction for the N-protein almost ceased by day 48 post-vaccination.

Evolution of the fish rhabdovirus viral haemorrhagic septicaemia virus

Viral haemorrhagic septicaemia (VHS) caused by the rhabdovirus VHSV is economically the most important viral disease in European rainbow trout farming. Until 1989, this virus was mainly isolated from freshwater salmonids but in the last decade, it has also been isolated from an increasing number of free-living marine fish species. To study the genetic evolution of VHSV, the entire G gene from 74 isolates was analysed. VHSV from wild marine species caught in the Baltic Sea, Skagerrak, Kattegat, North Sea, and English Channel and European freshwater isolates, appeared to share a recent common ancestor. Based on the estimated nucleotide substitution rate, the ancestor of the European fresh water isolates was dated some 50 years ago. This finding fits with the initial reports in the 1950s on clinical observations of VHS in Danish freshwater rainbow trout farms. The study also indicates that European marine VHSV and the North American marine line separated approx. 500 years ago. The codon substitution rate among the freshwater VHSV isolates was found to be 2-5 times faster than among marine isolates. The data support the hypothesis of the marine environment being the original reservoir of VHSV and that the change in host range (to include rainbow trout) may have occurred several times. Virus from the marine environment will therefore continue to represent a threat to the trout aquaculture industry.
Genotyping of viral haemorrhagic septicaemia virus from worldwide using the non-virion gene

Use of plasmid DNA for induction of protective immunity

A DNA vaccine directed against a rainbow trout rhabdovirus induces early protection against a nodavirus challenge in turbot
were completely protected against a nodavirus challenge performed 8 days post vaccination, while the cumulative mortality in the control group reached 54%. A DNA vaccine carrying the gene encoding the capsid protein of AHNV revealed no protective properties against the nodavirus challenge. Histological examination of muscle tissue sections from the vaccine injection site showed that the DNA vaccine against VHSV triggered a pronounced inflammatory response in turbot similar to what has earlier been observed in rainbow trout.

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DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: size, dose, route of injection and duration of protection-early protection correlates with Mx expression
Rainbow trout of different sizes (10 and 100 g) were injected intramuscularly (i.m.) or intraperitoneally (i.p.) with different doses (range 10ng-10mug) of a viral haemorrhagic septicaemia (VHS)-DNA vaccine (pcDNA3vhsG). As controls, fish were injected with the pcDNA3 plasmid alone, or with inactivated VHS virus. Fish were challenged at different times post-vaccination (p.v.) to assess protection. At certain times p.v., serum samples were analysed for neutralising antibody and liver tissue was analysed for Mx mRNA expression. A DNA dose of 0.5 mug injected by the i.m. route induced protection in fish of all sizes in challenges performed either I or 4 weeks p.v. This dose also conferred effective protection up to 9 months p.v. in fish >100 g. With lower doses of DNA (0.1 and 0.01 mug) and challenge at 4 weeks p.v., 10 g fish were partially protected but protection was not observed in 100 g fish. Vaccination by the i.p. route induced no or lower levels of protection compared with the i.m. route. Fish vaccinated with 0.5 mug DNA i.m. had no detectable serum neutralising antibody (NAb) at 4 weeks p.v. (with the exception of a single 10 g fish) but antibody was detected at 8 weeks and 6 months p.v. but not at 9 months p.v. However, cohorts of these fish showed effective protection at all timepoints. Lack of detectable levels of NAb (at 9 weeks p.v.) despite partial protection in challenge at 4 weeks p.v. was also observed with 0.01 mug doses of DNA i.m. NAb was detected in sera of fish at 8 weeks after vaccination with 0.1 mug i.m. but not in fish vaccinated with doses of 0.01-0.5 mug i.p. Early protection (1 week p.v.) correlated with elevated Mx gene expression.

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Immunity induced shortly after DNA vaccination of rainbow trout against rhabdoviruses protects against heterologous virus but not against bacterial pathogens

It was recently reported that DNA vaccination of rainbow trout fingerlings against viral hemorrhagic septicaemia virus (VHSV) induced protection within 8 days after intramuscular injection of plasmid DNA. In order to analyse the specificity of this early immunity, fish were vaccinated with plasmid DNA encoding the VHSV or the infectious haematopoietic necrosis virus (IHNV) glycoprotein genes and later challenged with homologous or heterologous pathogens. Challenge experiments revealed that immunity established shortly after vaccination was cross-protective between the two viral pathogens whereas no increased survival was found upon challenge with bacterial pathogens. Within two months after vaccination, the cross-protection disappeared while the specific immunity to homologous virus remained high. The early immunity induced by the DNA vaccines thus appeared to involve short-lived non-specific anti-viral defence mechanisms.

Immunity to viral haemorrhagic septicaemia (VHS) following DNA vaccination of rainbow trout at an early life-stage

Rainbow trout fry of average weight 0.5 g were vaccinated against viral haemorrhagic septicaemia (VHS) by intramuscular injection of 1 mug of plasmid DNA encoding the VHS virus glycoprotein gene. Challenge with a lethal dose of virus at two different time points, 9 and 71 days post-vaccination respectively, revealed that a highly protective and lasting immunity was established shortly after vaccination, in accordance with earlier experiments with larger fish. The defence mechanisms activated by the DNA vaccine are thus functional at an early life-stage in rainbow trout.
Neutralisation and binding of VHS virus by monovalent antibody fragments
We have previously reported the cloning and characterisation of the heavy and light chain variable domain genes encoding three monoclonal antibodies (Mabs) that bind viral haemorrhagic septicaemia virus (VHSV). Two of these antibodies, 3F1H10 and 3F1A2 both neutralised the virus though 3F1A2 appeared to recognise a broader range of virus isolates. The variable domains of these two antibodies differ by only four residues (Lorenzen et al., 2000a. Fish Shellfish Immunol. 10, 129-142). To further study the mechanism of neutralisation, Fab fragments as well as a series of recombinant bacterial single chain antibody (scAb) fragments were generated from the three anti-VHSV Mabs and their variable domain genes, respectively. Fabs and scAbs derived from the neutralising Mabs were both able to neutralise the VHSV type 1 isolate DK-F1. In addition, a series of scAb fragments were produced using the 3F1H10 variable heavy (VH) chain and variable light (V kappa) chain domains but containing, either alone or in dual combination, each of the four different residues present in 3F1A2. The dissociation constants of Mabs 3F1H10 and 3F1A2 and their respective Fab and scAb fragments were measured by BIAcore analysis and found to correlate with the capacity of each molecule to neutralise DK-F1. These investigations, together with computer assisted molecular analysis of the theoretical influence of each mutation on antigen binding, led to the identification of a single mutation at position 35a in the VH domain as having the most marked impact on viral neutralisation.

Protection of rainbow trout against infectious hematopoietic necrosis virus four days after specific or semi-specific DNA vaccination
A DNA vaccine against a fish rhabdovirus, infectious hematopoietic necrosis virus (IHNV), was shown to provide significant protection as soon as 4 d after intramuscular vaccination in 2 g rainbow trout (Oncorhynchus mykiss) held at 15 degreesC. Nearly complete protection was also observed at later time points (7, 14, and 28 d) using a standardized waterborne challenge model. In a test of the specificity of this early protection, immunization of rainbow trout with a DNA vaccine against another fish rhabdovirus, viral hemorrhagic septicemia virus, provided a significant level of cross-protection against IHNV challenge for a transient period of time, whereas a rabies virus DNA vaccine was not protective. This indication of distinct early and late protective mechanisms was not dependent on DNA vaccine doses from 0.1 to 2.5 mug.
Rainbow trout offspring with different resistance to viral haemorrhagic septicaemia

To study immunological and immunogenetical parameters related to resistance against viral haemorrhagic septicaemia (VHS), attempts to make gynogenetic strains of rainbow trout selected for high and low resistance to VHS were initiated in 1988. The first gynogenetic generation of inbreeding resulted in the more resistant offspring E8 and the low resistance offspring K3; the K3 offspring having the same high mortality as the susceptible reference strain of outbred trout in infection trials. A second gynogenetic generation derived from the E8 strain resulted in some low resistance offspring, and two gynogenetic families in which all, or nearly all, fish survived challenge with VHS virus. In this study, an attempt to associate the distribution of different MHC class II genotypes with low and high resistance gynogenetic offspring was performed. Two different MHC haplotypes could be distinguished, and in both low and high resistance families all three genotypes were found, which could be explained by the fact that the mother fish carried the heterozygous genotype. Although no significant differences in MHC IE genotypes were found between the high and low resistance offspring, a significantly different distribution of haplotypes in the low resistance offspring was observed, that could not be explained by a one- or two-locus model.
**DNA vaccination of rainbow trout against viral hemorrhagic septicemia virus: A dose-response and time-course study**

Viral hemorrhagic septicemia (VHS) in rainbow trout Oncorhynchus mykiss is caused by VHS virus (VHSV), which belongs to the rhabdovirus family. Among the different strategies for immunizing fish with a recombinant vaccine, genetic immunization has recently proven to be highly effective. To further investigate the potential for protecting fish against VHS by DNA vaccination, experiments were conducted to determine the amount of plasmid DNA needed for induction of protective immunity. The time to onset of immunity and the duration of protection following administration of a protective vaccine dose were also analyzed. The dose-response analysis revealed that significant protection of rainbow trout fingerlings was obtained following intramuscular injection of only 0.01 mug of plasmid DNA encoding the VHSV glycoprotein gene. In addition, higher doses of DNA induced immunity to a virus isolate serologically different from the isolate used for vaccine development. Following administration of 1 mug of a DNA vaccine, significant protection against VHS was observed in the fish as early as 8 d postvaccination. At 168 d postvaccination, the fish had increased in size by a factor of 10 and protection against a lethal dose of VHSV was still evident. The results confirm the great potential for DNA vaccination in inducing efficient immunoprophylaxis against viral diseases in aquacultured fish.

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**Immunoprophylaxis in fish by injection of mouse antibody genes**

Antibodies are a crucial part of the body's specific defense against infectious diseases and have considerable potential as therapeutic and prophylactic agents in humans and animals. The development of recombinant single-chain antibodies allows a genetic application strategy for prevention of infectious diseases. To test this in a fish model, a gene construct encoding a neutralizing single-chain antibody to the fish-pathogenic rhabdovirus VHSV (viral hemorrhagic septicemia virus) was administered to rainbow trout by intramuscular injection of plasmid DNA. Circulating recombinant antibodies could later be detected in the fish, and protective immunity to the viral disease was established.

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Three monoclonal antibodies to the VHS virus glycoprotein: comparison of reactivity in relation to differences in immunoglobulin variable domain gene sequences

Three monoclonal antibodies (MAbs) to the VHSV G protein were compared in different immunoassays and the variable domain cDNA sequences from the respective immunoglobulin (Ig) genes were determined. One MAb (IP1H3) was non-neutralising and recognised different virus isolates equally well in ELISA. The other two were neutralising and recognised the same or closely related epitopes. One of these two MAbs (3F1H10) was more restricted in its ability to neutralise heterologous VHSV isolates than the other (3F1A2). A semi-quantitative relationship between binding of the two neutralising MAbs in ELISA and their neutralising activity was evident. Binding kinetic analyses by plasmon resonance identified differences in the dissociation rate constant (kd) as a possible explanation for the different reactivity levels of the MAbs. The Ig variable heavy (VH) and light (V kappa) domain gene sequences of the three hybridomas were compared.

The inferred amino acid sequence of the two neutralising antibody VH domains differed by three amino acid residues (97% identity) and only one residue difference was evident in the Vκ domains. In contrast, IP1H3 shared only 38 and 39% identity with the 3F1A2 and 3F1H10 VH domains respectively and 49 and 50% identity with the 3F1A2 and 3F1H10 Vκ domains respectively. The neutralising antibodies were produced by hybridomas originating from the same fusion and the high nucleotide sequence homology of the variable Ig gene regions indicated that the plasma cell partners of the hybridomas originated from the same virgin B lymphocyte. The few differences observed in the VH and V kappa amino acid sequences were probably due to somatic mutations arising during affinity maturation and might explain the observed reactivity differences between the two MAbs.

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Immunity to rhabdoviruses in rainbow trout: the antibody response

Interactions between host and pathogen, as in the case of fish pathogenic viruses, represent interesting models for analyses of the relationships between structure and function of the teleost immune system. Two salmonid rhabdoviruses, IHNV and VHSV, have received special attention due to their occasional detrimental effect on rainbow trout farming. Research efforts have been focused on understanding the mechanisms involved in protective immunity. Several specific and nonspecific cellular and humoral parameters are believed to be involved, but only the antibody response has been characterised in detail so far. Analysis of the specificity of anti-virus trout antibodies has been complicated by a generally insufficient ability of the antibodies to bind the viral proteins in assays such as immunoblotting. However, other assays, specifically designed for detection of fish anti IHNV/VHSV antibodies, have demonstrated that rainbow trout can produce specific and highly functional antibodies that are able to neutralise virus pathogenicity in vitro as well as in vivo. The apparently more restricted antibody response to IHNV and VHSV antigens in fish compared to mammals could possibly be explained by different kinds of epitopes being differently immunogenic in fish and in mammals. Also, it may be assumed that the requirements for the assay-antigens in terms of antigenicity, may differ for mammals and fish. The present text includes an initial presentation of the pathogens, followed by basic and applied aspects of antibody response and antibody reactivity with IHNV and VHSV antigens.
Immunity to VHS virus in rainbow trout

Viral hemorrhagic septicemia virus (VHSV) is the rhabdovirus that causes most disease problems in farmed rainbow trout in Europe. Survivors of infection are usually immune to reinfection but as with other fish viruses, development of a modern recombinant vaccine has been complicated by the limited knowledge of the immune mechanisms and antigens involved in induction of immunity. Neutralizing and protective monoclonal antibodies recognize the envelope glycoprotein (G protein) which is the only viral protein known to be present on the surface of the virus particle. Immunoblotting analyses with monoclonal antibodies as well as with sera from immunized trout have indicated that protein conformation plays an important role in neutralization epitopes. The virus neutralizing activity often found in sera from convalescent trout is highly dependent on a poorly defined complementing activity in normal trout serum. Attempts to demonstrate involvement of the complement component C3 were not successful, but inhibition experiments indicated that the classical pathway for complement activation was needed. Being the target of neutralizing antibodies, the G protein is an obvious candidate for a recombinant vaccine. However, recombinant forms of the G protein expressed in Escherichia coli have been poorly immunogenic in fish, presumably due to incorrect protein conformation. Expression in insect cells has resulted in more potent products but, more recently, considerably higher levels of protection were found following vaccination with naked DNA encoding the G protein under the control of a CMV promoter. Genetic resistance to VHS would be a desirable alternative to vaccination but the time required to obtain this makes it a long-time goal. Results from breeding programs in France and Denmark nevertheless indicate that such a strategy may provide considerable improvement in resistance.
**Isolation of viral haemorrhagic septicaemia virus (VHSV) from wild marine fish species in the Baltic Sea, Kattegat, Skagerrak and the North Sea**

In order to analyse the occurrence of viral haemorrhagic septicaemia virus (VHSV) in the marine environment surrounding Denmark, fish tissue samples were collected on four cruises with the research vessel H/S Dana in 1996 and 1997. The sampling comprised 923 samples totalling 7344 fish representing 29 different species. VHSV was isolated from 24 fish samples from the Baltic Sea, four samples from Skagerrak and three samples from the North Sea. The virus-positive host species included herring Clupea harengus (11 isolates), sprat Sprattus sprattus (eight isolates), cod Gadus morhua (six isolates), rockling Rhinonemus cimbrius (one isolate), Norway pout Trisopterus esmarkii (one isolate), blue whiting Micromesistius poutassou (one isolate), whiting Merlangius merlangus (two isolates) and lesser argentine Argentina sphyraena (one isolate). VHSV has previously been reported from cod and herring, but not from the other five species. A virus belonging to serogroup II of the aquatic birnaviruses was isolated from three samples of flounder Platichthys flesus and three samples of dab Limanda limanda and a virus preliminary identified as iridovirus (lymphocystis virus) was isolated from seven samples of long rough dab Hippoglossoides platessoides. (C) 1999 Elsevier Science B.V. All rights reserved.

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**Production of neutralizing antisera against viral hemorrhagic septicemia (VHS) virus by intravenous injections of rabbits**

Rabbit antisera against viral hemorrhagic septicemia virus (VHSV) produced by two immunization procedures were compared for neutralization and immunochemical properties against homologous and heterologous strains. The VHSV isolate used as the immunogen was a member of a serogroup not neutralized by previously available antisera. The results from this study suggested that frequent intravenous (IV) injections of rabbits with viral antigens were superior to adjuvant-mediated, combined subcutaneous and intraperitoneal (SC/IP) injections for the production of neutralizing antisera. All IV injected rabbits produced high neutralization titers against the homologous VHSV isolate but not against an isolate from a different serogroup. The SC/IP injected rabbits had no significant neutralization titers against either the homologous VHSV strain or two isolates of a heterologous VHSV strain. Sera from all injected rabbits reacted in indirect immunofluorescence (IF) assays with either strain; however, the SC/IP injected rabbits produced higher titers against the heterologous VHSV strain by ELISA (enzyme-linked immunosorbent assay). By Western blotting, neutralizing antisera primarily stained the viral glycoprotein (G) whereas the nonneutralizing sera stained all the viral structural proteins equally well. Our results demonstrate that immunization procedures to produce antisera against VHSV in rabbits determine whether the resultant antibodies will have primarily neutralizing or binding capabilities.

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Recombinant vaccines: experimental and applied aspects
Development of vaccines for aquaculture fish represent an important applied functional aspect of fish immunology research. Particularly in the case of recombinant vaccines, where a single antigen is usually expected to induce immunity to a specific pathogen, knowledge of mechanisms involved in induction of a protective immune response may become vital. The few recombinant vaccines licensed so far, despite much research during the last decade, illustrate that this is not a straightforward matter. However, as vaccine technology as well as our knowledge of the fish immune system is steadily improved, these fields will open up a number of interesting research objectives of mutual benefit. Recent aspects of recombinant protein vaccines, live recombinant vaccines and DNA vaccines are discussed.

Rhabdovirusinfektioner
Characterization of intramolecular disulfide bonds and secondary modifications of the glycoprotein from viral hemorrhagic septicemia virus, a fish rhabdovirus
Viral hemorrhagic septicemia virus (VHSV) infections cause high losses in cultured rainbow trout in Europe. Attempts to produce a recombinant vaccine based on the transmembrane glycoprotein (G protein) have indicated that proper folding is
important for the antigenicity and immunogenicity of the protein. The present study was initiated to identify the disulfide bonds and other structural aspects relevant to vaccine design. The N-terminal amino acid residue was identified as being a pyroglutamic acid, corresponding to Gln21 of the primary transcript. Peptides from endoproteinase-degraded G protein were analyzed by mass spectrometry before and after chemical reduction, and six disulfide bonds were identified: Cys29-Cys339, Cys44-Cys295, Cys90-Cys132, Cys172-Cys177, Cys195-Cys265, and Cys231-Cys236. Mass spectrometric analysis in combination with glycosidases allowed characterization of the glycan structure of the G protein. Three of four predicted N-linked oligosaccharides were found to be predominantly bi-antennary complex-type structures. Furthermore, an O-linked glycan near the N terminus was identified. Alignment of the VHSV G protein with five other rhabdovirus G proteins indicates that eight cysteine residues are situated at conserved positions. This finding suggests that there might be some common disulfide bonding pattern among the six rhabdoviruses.

Mapping of linear antibody epitopes of the glycoprotein of VHSV, a salmonid rhabdovirus

Antibody Linear epitopes of the glycoprotein G (gpG) of the viral haemorrhagic septicaemia virus (VHSV), a rhabdovirus of salmonids, were mapped by pepscan using overlapping 15-mer peptides covering the entire gpG sequence and ELISA with polyclonal and monoclonal murine and polyclonal trout antibodies. Among the regions recognized in the pepscan by the polyclonal antibodies (PAbs) were the previously identified phosphatidylserine binding heptad-repeats (Estepa & Coll 1996; Virology 216:60-70) and leucocyte stimulating peptides (Lorenzo et al. 1995; Virology 212:348-355). Among 17 monoclonal antibodies (MAbs), only 2 non-neutralizing MAbs, I10 (aa 139-153) and IP1H3 (aa 399-413), could be mapped to specific peptides in the pepscan of the gpG. Mapping of these MAbs was confirmed by immunoblotting with recombinant proteins and/or other synthetic peptides covering those sequences. None of the neutralizing MAbs tested reacted with any of the gpG peptides. Previously mapped MAb resistant mutants in the gpG did not coincide with any of the Linear epitopes defined by the pepscan strategy, suggesting the complementarity of the 2 methods for the identification of antibody recognition sites.

Protective immunity to VHS in rainbow trout (Oncorhynchus mykiss, Walbaum) following DNA vaccination

Rainbow trout fingerlings were immunized by intramuscular injection of a plasmid DNA vector encoding the viral haemorrhagic septicaemia virus (VHSV) glycoprotein (G) or nucleocapsid protein (N) genes under the control of a cytomegalovirus promoter. Challenge with VHSV 52 days later demonstrated that both viral genes, and the G gene in
particular, were able to induce protective immunity against VHS. In contrast to sera taken from fish injected with the N gene, neutralizing antibody activity could be detected both before and after challenge in the sera of a major proportion of the fish injected with the G gene.

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**Isolation of VHSV from the marine environment**

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**MHC Polymorphism in rainbow trout families with different resistance to VHS**

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**Vaccination of rainbow trout against VHS using live attenuated vaccines: Danish field trials from 1978 to 1983.**

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Differentiation of VHS virus isolates by use of monoclonal antibodies

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Multiplication of VHS virus in insect cells

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Contributors: Lorenzen, N., Olesen, N. J.
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Simultaneous demonstration of Flexibacter psycrophilus and IPN virus in formaline fixed paraffin embedded rainbow trout fry

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Use of polymerase chain reaction (PCR) for differentiation of serological similar VHS virus isolates from Europe and America.

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Antibody response in rainbow trout vaccinated against viral haemorrhagic septicaemia (VHS) with inactivated virus
Expression of the VHS virus glycoprotein in insect cells

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Infectious hematopoietic necrosis virus

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The role of complement in antibody mediated neutralization of a fish rhabdovirus

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Publication status: Published
Organisations: Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute
Contributors: Lorenzen, N., Olesen, N. J., Jørgensen, P., Jensen, L., Koch, C.
Publication date: 1993
Peer-reviewed: No
Event: Abstract from The Nordic Symposium on Fish Immunology, Lysekil, Sweden.
Detection of rainbow trout antibody to Egtved virus by enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF), and plaque neutralization tests (50 %PNT)

General information
Publication status: Published
Organisations: Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, Technical University of Denmark
Contributors: Olesen, N. J., Lorenzen, N., Jørgensen, P.
Pages: 31-38
Publication date: 1991
Peer-reviewed: Yes

Publication information
Journal: Diseases of Aquatic Organisms
Volume: 10
Issue number: 1
ISSN (Print): 0177-5103
Original language: English
Source: orbit
Source ID: 241238

Infectious Hematopoietic Necrosis (IHN) and Viral Hemorrhagic Septicemia (VHS): Detection of Trout Antibodies to the Causative Viruses by Means of Plaque Neutralization, Immunofluorescence, and Enzyme-Linked Immunosorbent Assay

General information
Publication status: Published
Organisations: Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, Technical University of Denmark
Contributors: Jørgensen, P., Olesen, N. J., Lorenzen, N., Winton, J., Ristow, S.
Pages: 100–108
Publication date: 1991
Peer-reviewed: Yes

Publication information
Journal: Journal of Aquatic Animal Health
Volume: 3
Issue number: 2
ISSN (Print): 0899-7659
Original language: English
Source: orbit
Source ID: 241236

Molecular analysis of a viral glycoprotein with a view to vaccine development

General information
Publication status: Published
Organisations: Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, Technical University of Denmark
Contributors: Lorenzen, N., Olesen, N. J., Jørgensen, P., Etzerodt, M., Holtet, T., Thøgersen, H.
Publication date: 1991
Peer-reviewed: No
Event: Abstract from Biokemisk forenings årsmøde, .
Source: orbit
Source ID: 241576

Research output: Contribution to journal › Journal article – Annual report year: 1991 › Research › peer-review

Research output: Contribution to conference › Conference abstract for conference – Annual report year: 1993 › Research
Paternal Association of Increased Susceptibility to Viral Haemorrhagic Septicaemia (VHS) in Rainbow Trout (Oncorhynchus mykiss)

Farming of rainbow trout (Oncorhynchus mykiss) in Europe is hampered by unacceptably heavy losses due to the severe infectious disease viral haemorrhagic septicaemia (VHS). Strain-dependent variation of VHS resistance exists. A long-term breeding programme to increase VHS resistance in rainbow trout has been started in Denmark. This programme will be based on experimental VHS challenge of the parental fish (n = 84) as well as their normal and gynogenetic offspring (16 fullsib F1 groups). We found a paternal influence on the average VHS resistance of the offspring; partial regression coefficients for sire-offspring were estimated at 0.30 ± 0.09 and for dam-offspring at −0.1 ± 0.12.

General information
Publication status: Published
Organisations: National Veterinary Institute, Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, Technical University of Denmark
Pages: 1188–1191
Publication date: 1991
Peer-reviewed: Yes

Publication information
Journal: Canadian Journal of Fisheries and Aquatic Sciences
Volume: 48
Issue number: 7
ISSN (Print): 0706-652X
Original language: English
DOIs: 10.1139/f91-143
Source: orbit
Source ID: 241237
Research output: Contribution to journal › Journal article – Annual report year: 1991 › Research › peer-review

Sero logical differentiation of Egtved virus (VHSV) using neutralizing monoclonal and polyclonal antibodies

General information
Publication status: Published
Organisations: Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, Technical University of Denmark
Contributors: Olesen, N. J., Lorenzen, N., Jørgensen, P.
Publication date: 1991
Peer-reviewed: No
Event: Abstract from 5th International Conference on Diseases of fish and shellfish, Budapest, Hungary.
Source: orbit
Source ID: 241577
Research output: Contribution to conference › Conference abstract for conference – Annual report year: 1991 › Research

Antibody response to VHS virus glycoprotein in rainbow trout

General information
Publication status: Published
Organisations: Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, Technical University of Denmark
Contributors: Lorenzen, N., Olesen, N. J., Jørgensen, P.
Publication date: 1990
Peer-reviewed: No
Event: Abstract from 1st Nordic Symposium on Fish Immunology, Tromsø, Norway.
Source: orbit
Source ID: 241575
Research output: Contribution to conference › Conference abstract for conference – Annual report year: 1990 › Research

Neutralization of Egtved virus pathogenicity to cell cultures and fish by monoclonal antibodies to the viral G protein

General information
Publication status: Published
Organisations: Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, Technical University of Denmark
**Immunization of rainbow trout with affinity purified Egtved virus proteins, preliminary results.**

**General information**
Publication status: Published
Organisations: Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, Technical University of Denmark
Contributors: Lorenzen, N., Olesen, N. J., Jørgensen, P.
Publication date: 1989
Peer-reviewed: No
Event: Abstract from 4th International Conference of the European Association of Fish Pathologists, Santiago de Compostela, Spain.
Source: orbit
Source ID: 241574
Research output: Contribution to conference › Conference abstract for conference – Annual report year: 1989 › Research

**Monoclonal antibodies against Egtved virus glycoprotein: Application in development of a subunit vaccine**

**General information**
Publication status: Published
Organisations: Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, Technical University of Denmark
Contributors: Lorenzen, N., Olesen, N. J., Jørgensen, P.
Publication date: 1989
Peer-reviewed: No
Event: Poster session presented at Annual Meeting of Scandinavian Society for Immunology, Copenhagen, Denmark.
Source: orbit
Source ID: 241573
Research output: Contribution to conference › Poster – Annual report year: 1989 › Research

**Monoclonal antibodies against Egtved virus structural proteins: Application in diagnosis and vaccine development**

**General information**
Publication status: Published
Organisations: Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, Technical University of Denmark
Contributors: Lorenzen, N., Olesen, N. J., Jørgensen, P.
Publication date: 1989
Peer-reviewed: No
Event: Abstract from 6th International Conference on Comparative and Applied Virology, Banff, Canada.
Source: orbit
Source ID: 241571
Research output: Contribution to conference › Conference abstract for conference – Annual report year: 1989 › Research

**Monoclonal antibodies used in the development of a genetically engineered vaccine against a fish virus**

**General information**
Production and Characterization of Monoclonal Antibodies to Four Egtved Virus Structural Proteins

General information
Publication status: Published
Organisations: Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, Technical University of Denmark
Contributors: Lorenzen, N., Olesen, N. J., Jørgensen, P.
Publication date: 1988
Peer-reviewed: Yes

Publication information
Journal: Diseases of Aquatic Organisms
Issue number: 4
ISSN (Print): 0177-5103
Original language: English
Source: orbit
Source ID: 241233
Research output: Contribution to journal › Journal article – Annual report year: 1988 › Research › peer-review

Detection of Egtved virus and rainbow trout antibody to Egtved virus by enzyme-linked immunosorbent assay (ELISA).

General information
Publication status: Published
Organisations: Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, Technical University of Denmark
Contributors: Olesen, N. J., Lorenzen, N., Jørgensen, P.
Publication date: 1987
Peer-reviewed: No
Event: Abstract from 3rd International Conference of the European Association of Fish Pathologists, Bergen, Norway.
Source: orbit
Source ID: 241570
Research output: Contribution to conference › Conference abstract for conference – Annual report year: 1987 › Research

Passive protection of rainbow trout (Salmo gairdneri) against Egtved virus with monoclonal antibodies.

General information
Publication status: Published
Organisations: Section of Fish Diseases, Division of Poultry, Fish and Fur Animals, National Veterinary Institute, Technical University of Denmark
Contributors: Lorenzen, N., Jørgensen, P., Olesen, N. J.
Publication date: 1987
Peer-reviewed: No
Event: Abstract from International Meeting on Fish Immunology, Plymouth, England.
Source: orbit
Source ID: 241569
Research output: Contribution to conference › Conference abstract for conference – Annual report year: 1987 › Research

Projects:
Vaccination of Seabass against a lethal viral disease and characterization of protective immunity
Barsøe, S., PhD Student, National Institute of Aquatic Resources
Olesen, N. J., Main Supervisor
Lorenzen, N., Supervisor
Technical University of Denmark
01/12/2017 → 03/09/2021
Award relations: Vaccination of Seabass against a lethal viral disease and characterization of protective immunity
Project: PhD

Piscine orthoreovirus in salmonids: geographic distribution, molecular characterization, pathogenesis under experimental conditions
Vendramin, N., PhD Student, National Institute of Aquatic Resources
Olesen, N. J., Main Supervisor
Rimstad, E., Supervisor
Lorenzen, N., Examiner
Evensen, Ø., Examiner
Garver, K. A., Examiner
Technical University of Denmark
01/12/2016 → 12/03/2019
Award relations: Piscine orthoreovirus in salmonids: geographic distribution, molecular characterization, pathogenesis under experimental conditions
Project: PhD

Delivery of small interfering RNAs (soRNAs) for treatment of viral disease in fish aquaculture
Larashati, S., PhD Student, National Veterinary Institute
Lorenzen, N., Main Supervisor
Rasmussen, J. S., Supervisor
Schyth, B. D., Supervisor
Heegaard, P. M. H., Examiner
Collet, B., Examiner
Stipendie fra udlandet
01/11/2009 → 29/01/2014
Award relations: Delivery of small interfering RNAs (soRNAs) for treatment of viral disease in fish aquaculture
Project: PhD

Flavobacterium psychrophilum, forebyggelse og immunforsvar
Henriksen, M. M. M., PhD Student, National Veterinary Institute
Dalsgaard, I., Main Supervisor
Kania, P., Supervisor
Lorenzen, N., Supervisor
Olesen, N. J., Examiner
Aasted, B., Examiner
Wiklund, T. C. O., Examiner
Buchmann, K., Supervisor
Technical University of Denmark
15/06/2010 → 26/02/2014
Award relations: Flavobacterium psychrophilum, forebyggelse og immunforsvar
Project: PhD

Expression of rhabdovirus-induced fish-specific microribonucleic acids in rainbow trout (Oncorhynchus mykiss)
Bela-Ong, D., PhD Student, National Veterinary Institute
Lorenzen, N., Main Supervisor
Schyth, B. D., Supervisor
Skovgaard, K., Examiner
Giehm Mikkelsen, J., Examiner
Wiegertjes, G., Examiner
Technical University of Denmark
01/02/2011 → 26/09/2014
Award relations: Expression of rhabdovirus-induced fish-specific microribonucleic acids in rainbow trout (Oncorhynchus mykiss)
Project: PhD
Non-coding RNA mediated gene regulation during influenza infection
Brogaard, L., PhD Student, National Veterinary Institute
Skovgaard, K., Main Supervisor
Larsen, L. E., Supervisor
Lorenzen, N., Examiner
Tchilian, E. Z., Examiner
Salicio, S. C., Examiner
Technical University of Denmark
15/07/2013 → 31/01/2018
Award relations: Non-coding RNA mediated gene regulation during influenza infection
Project: PhD

DAFINET: Danish Fish Immunology Research Network
Buchmann, K., Project Manager, University of Copenhagen
Pedersen, K., Project Participant, University of Copenhagen
Jørgensen, T. R., Project Participant, University of Copenhagen
VIuff, B. M., Project Participant, University of Copenhagen
Salomonsen, J., Project Participant, University of Copenhagen
Aasted, B., Project Participant, University of Copenhagen
Mazaheri, S., Project Participant, University of Copenhagen
Lorenzen, N., Project Manager, National Veterinary Institute, Division of Poultry, Fish and Fur Animals
Einer-Jensen, K., Project Participant, National Veterinary Institute, Division of Poultry, Fish and Fur Animals
Rasmussen, J. S., Project Participant, National Veterinary Institute, Division of Poultry, Fish and Fur Animals
Kjær, T., Project Participant

Improved vaccination strategies in marine aquaculture
Lorenzen, N., Project Manager, National Veterinary Institute, Division of Poultry, Fish and Fur Animals, Section of Fish Diseases
Rasmussen, J. S., Project Participant, National Veterinary Institute, Division of Poultry, Fish and Fur Animals, Section of Fish Diseases
Olesen, N. J., Project Participant, National Veterinary Institute, Division of Poultry, Fish and Fur Animals, Section of Fish Diseases
Dalsgaard, I., Project Manager, National Institute of Aquatic Resources
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Buchmann, K., Project Participant, University of Copenhagen
Juel Hansen, P., Project Manager, University of Copenhagen
Henriksen, N. H., Project Manager, Danish Aquaculture Association
Hørløck, V., Project Manager, Aller Aqua A/S
Engell-Sørensen, K., Project Manager, Fishlab
Nielsen, T., Project Manager, Aquasearch Ova Aps
Madsen, S. B., Project Participant, Aquasearch Ova Aps
Nylén, J., Project Manager, Schering-Plough A/S
Melingen, G. O., Project Participant, Schering-Plough A/S

Project ID: 22452
Forskningsprojekter - Andre ministerier og styrelser: DKK1,444,780.00
01/04/2008 → 30/09/2012

Collaborators: Schering-Plough A/S, Aller Aqua A/S, University of Copenhagen, Aquasearch Ova Aps, Fishlab, Danish Aquaculture Association
Award relations: Improved vaccination strategies in marine aquaculture

Project: Research