Report

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Work package 4 report: Broodfish testing for viral infections

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English summary:
This report summarises current scientific information and experience obtained with various methods for testing of salmonid broodfish or spawn for infectious pancreatic necrosis (IPN) virus infection, in order to prevent vertical transmission of the organism to the offspring. The same assessment is being done for nodavirus infections (viral encephalopathy and retinopathy – VER, and viral nervous necrosis –VNN) in marine fish species. Methods for population-level screening to document the absence of these pathogens are relatively well-established, whereas the methods suitable for testing and certification of individual fish are still insufficiently developed and validated to facilitate effective risk management and control. Priority subject for further research in order to improve the management and control of these vertically transmissible fish diseases are suggested.

Norsk sammendrag:
Denne rapporten oppsummerer dagens vitenskapelig informasjon og praktiske erfaring med forskjellige metoder for å teste gytere av laksefisk eller deres kjønnsprodukter for infeksiøs pankreas nekrose virus (IPNV) infeksjon og derigjennom hindre vertikal smitte av viruset til avkommet. En tilsvarende vurdering gjøres for nodavirusinfeksjoner (VER og VNN) hos marine fiskearter. Metodene for sykdomsovervåkning på populasjonsnivå er relativt veletablerte, og kan brukes for å vise at populasjonen er fri for disse organismene, men metoder for å teste og sertifisere enkeltfisk er fortsatt utilstrekkelig dokumentert for å sikre effektiv risikohåndtering og sykdomskontroll. Prioriterte emner for framtidig forskning på området foreslås.

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Introduction

National and international trade in fertilised eggs and gametes for finfish aquaculture is in most parts of the world subject to strict zoo-sanitary regulations and health certification requirements, many of which are built upon rather old and partly scarce scientific data. Aim of this concerted action project is thus to scrutinise and re-assess the scientific basis for current zoo-sanitary control requirements. In the initial part of the project (Work Package 1), we found that there is reasonable evidence for so-called "true" vertical transmission (infection of the developing embryo or transmission inside the fertilised egg) only for a limited number of finfish diseases. These are bacterial kidney disease (BKD), infectious pancreatic necrosis (IPN), salmon rickettsial syndrome caused by *Piscirickettsia salmonis*, and *Flavobacterium psychrophilium* infections. For a number of other infections, there are indications that vertical transmission may occur but in our opinion, more likely as a contamination of the egg surface ("egg-associated transmission"). Infectious haematopoietic necrosis (IHN) and nodavirus infections of marine species (VER/VNN) may serve as examples of this category.

In the second part of the project, we have scrutinised the scientific evidence relating to the ability of the selected infectious agents to survive in the environment or on the egg surface, as well as their susceptibility to various disinfection procedures (Work Package 3). Obviously, these features are largely determining the need for, and the effect of applicable disinfection procedures to inactivate agents that may contaminate the egg surface during incubation. Some agents, especially IPN virus possesses the ability to survive for years even under extreme adverse microenvironments. Whereas the information relating to the rhabdoviruses suggests that commonly applied disinfection procedures are highly effective, there is less data available on ISA or flavobacteria in this respect. No data were found on the susceptibility of *Piscirickettsia salmonis* or several iridoviruses listed in the OIE fish disease code to disinfection procedures applicable to live eggs.

The current work package (WP4) comprises the assessment of sampling procedures and diagnostic methods for testing of parental fish or their sexual products, allowing for broodstock segregation and other zoo-sanitary management precautions, and enabling the certification of gametes or fertilised eggs as being (likely) free from specific disease agents. Two typical but distinct disease management situations are addressed:

1. Testing to document and maintain specific pathogen free (SPF) brood fish populations (in a farm, or in an area), or
2. Testing to eliminate or minimise vertical transfer risks when using parental fish from a population of unknown infection status, or from a covertly infected population.

The first of these situations is of particular relevance to European freshwater rainbow trout farming in several EU member states (Denmark, Sweden, Finland). The second situation is typical for European commercial farming of Atlantic salmon and marine fish species as found in other EU- and EEA states (Norway, Scotland, Ireland, Faeroe Islands, Italy, Greece).

With reference to these two situations, this report gives an overview of strengths, weaknesses and disease control merits of the principal 4 methodologies available for brood fish or gamete testing, namely immunodiagnostic techniques (ELISA, IFAT, immunohistochemistry, etc); nucleic acid detection (RT-PCR, real-time PCR, etc.), cell culture propagation, and serodiagnosis.

The discussion of methods also includes how sensitive the method appears relative to sample conservation, transport and processing, i.e. the robustness of the method for use in an industrial, commercial setting.

The current report deals with the viral fish pathogens that have been found to transmit vertically: infectious pancreatic necrosis virus (IPNV), and the nodaviruses (Viral encephalopathy and retinopathy virus –VER- and viral nervous necrosis virus –VNN). A separate report from this work package dealing with broodfish testing for certain bacterial infections has already been completed.

Materials and methods

Forming the basis for the assessment a workshop with invited experts was conducted in Oslo September 2004, during which summaries of published data, of published and unpublished scientific studies, and of (mostly unpublished) disease control experience was presented and discussed in-depth. A second workshop with invited experts was held in Aarhus March 8-9, 2005 presenting new data from on-going research and continuing the discussion of the experience made with various methods.

Based upon written input from the authors and workshop participants, the first draft report was produced and submitted to contributing experts and the project group, who discussed it during their project meeting in Aarhus on June 24, 2005. Further improvement of the initial version of the report has been made since then. The work has been led by Dr. Niels Jørgen Olesen from the DFVF laboratory in Aarhus, who has been the co-ordinator of this report.

Infectious pancreatic necrosis (IPN)

The screening procedure for infectious pancreatic necrosis (IPN) has for decades been based on IPN virus (IPNV) isolation in cell culture (Agius et al.1982) followed by immunological identification of the agent causing cytopathic effects (CPE), either by serum neutralisation (Hill & Way 1995), enzyme-linked immunosorbent assay (ELISA) (Dixon & Hill 1983), or by molecular methods like RT-PCR (Taksdal et al. 2001). The method is primarily used for documentation and maintenance of freedom of IPNV in fresh water rainbow trout farming. The cell cultivation methods are regarded as very sensitive provided sampling and shipment for laboratory testing is done properly, but the methods require incubation for at least 2 weeks before negative results can be given. The time factor is especially important in brood stock testing of covertly infected populations because of the impracticality and cost of quarantine-incubating single egg batches until the test outcome becomes clear. For testing to document and maintain IPN freedom in brood fish populations in farms, or in an area, the time factor has less importance.

There is a great interest to validate molecular methods for detection of virus directly in fish tissue, that could potentially replace cell culture methods. The major advantage of the molecular methods would be the reduction of time for examination and the possibility for automation of the laboratory procedures. Direct immunological test on fish tissue are generally regarded as being insufficiently sensitive for IPNV screening purposes, whereas they are very reliable and cost efficient for IPN virus identification after propagation in cell cultures.

Broodstock often represent very valuable fish; therefore the development of any method allowing for non-lethal sampling for IPN screening would be of crucial benefit.

Sampling and cell culture techniques

Cell cultivation of IPNV according to the OIE Manual is in general regarded as a very sensitive method for virus detection, but several suggestions for improvement of sensitivity have been published. Agius et al (1982) advocated for a co-cultivation technique on RTG-2 cells using trypsinised fish tissue material to improve sensitivity, as he found only 4 out of 6 and 4 out of 8 samples were positive by the traditional technique, as compared to co-cultivation. This approach was refined by Munro et al. (2004) using macrophage co-cultivation on CHSE-214 cells in a non-lethal testing but recent studies suggest that the technique is not appropriate for use on broodstock fish (Munro, E.S., unpublished. Fisheries Research Services, Aberdeen, Scotland) (see chapter on non-lethal testing). The classical method advice the use of homogenized liver, kidney and spleen, or ovarian fluid from broodfish inoculated onto BF-2, CHSE-214 or RTG-2 cells.

In an inter-laboratory proficiency test, Lorenzen et al (1999) demonstrated significant higher sensitivity of CHSE-214 and BF-2 cells for IPNV infection, compared to RTG-2, FHM and EPC cells. The CHSE-214 came out as the most sensitive but the difference to BF-2 sensitivity was not significant. A broodstock testing series, however, involving 685 Atlantic salmon from the Faeroe Islands and 103 rainbow trout from Danish fish farms revealed 48% (range 20-100 %) positives on BF-2 cells but 90% (range 77-100%) positives on CHSE-214 cells, indicating the superiority in sensitivity of CHSE-214 cells. These data also demonstrated that a combination of the 2 cell lines would increase the sensitivity by 10%, which might be important for broodstock testing where high sensitivity of detection in single fish is crucial (Olesen, DFVF, Århus Denmark – manuscript in prep.).

For each cell line a number of sub lineages exist in different laboratories but a study by McAllister (1997) revealed only little variation in IPN virus susceptibility using 8 CHSE-214 lineages, in contrast to significant differences seen in especially VHSV titres in these lineages. The inter-laboratory test performed by Lorenzen et al. (1999) demonstrated that EPC and RTG-2 did not differ significantly among laboratories, whereas BF-2 and CHSE-214 varied more, and the FHM cells showed the largest variability.

In a comparative study by Bovo et al. (1985) the pike gonad (PG) cell line was recommended for isolating IPNV, showing higher titres and more positive samples than BF-2, RTG-2 and EPC cells, respectively. Unfortunately CHSE-214 was not included in the study and PG cells never became very disseminated among fish diseases laboratories. A survey conducted by Rodriguez et al. (1993) revealed

that CHSE-214 and RTG-2 were equally and highly susceptible to field IPNV isolates, yielding more positive samples than were detected by inoculation onto FHM and EPC cells.

The processing of organ samples consists of a homogenization step that can be carried out by mortar and pestle, stomacher, Omnitron or Polytron (sonicating). Hedrick et al (1986) demonstrated that all 4 apparatuses were equally suitable for recovering IPNV from trout tissues. Smail et al. (2003) however demonstrated that sonication of cell pellets after homogenisation yielded a higher number of isolations.

Pooling of samples (equal amounts of organ material from up to 5 or 10 individual fish to produce one homogenate) has generally been accepted in population screening programmes to document freedom from infection. There are, however, divergent views as to which degree factors that may be present in single fish (such as for example neutralising antibodies) may affect the test applied to the entire pool. A clarification on this potential source of bias especially for individual fish testing and certification is urgently needed.

In conclusion, we regard the most important steps for ensuring high sensitivity in the examination of fish for IPNV by cell cultivation are as follows:

1. Transport and storage

The transport and storage of tissue samples for examination by cell cultivation must be performed in a manner to prevent inactivation of virus. Fish tissue sampled for virus isolation must be transported in medium for maintaining virus infectivity, and shall not be frozen. Storage of tissue material in transport medium with 50% glycerol added gives an optimal protection of IPNV, unfortunately this storage method will inactivate a number of membrane viruses such as the rhabdoviruses and orthomyxoviruses, and samples in glycerol will thus only be suitable for the detection of IPNV. Long-time storage of IPNV isolates should be done in glycerol at 4°C to -20°C.

2. Sample material

As described in the IPN chapter of the OIE manual (anonymous, 2003) are organs (liver, kidney, spleen) and ovarian fluid from broodfish at spawning time all deemed suitable for virological examination of asymptomatic fish for IPNV. We consider kidney material to be the organ of choice, which should be included if the fish can be sacrificed (see chapter on non-lethal testing).

3. Isolation of virus

After homogenization of tissue by any suitable apparatus, inoculation onto CHSE-214 monolayers should be recommended as first priority, BF-2 as second, and both for obtaining maximum sensitivity.

4. Virus identification

This step is not crucial for test sensitivity. ELISA is reliable, specific, rapid and easy but demand high virus titres in the cell culture and cannot reveal double infections. Neutralisation tests are easy to perform, and will detect double infection with other viruses replicating in the cell cultures used, but is more time consuming before identification (2-4 days of incubation required). IFAT is rather work-intensive and the reading is non-standardised but useable. RT-PCR is regarded sensitive, but the specificity is only assessed for a few of the published protocols. It is also quite costly in terms of equipment and labour compared to ELISA and neutralisation.

Molecular techniques

No published reports are available concerning comparison of molecular detection and cell culture methods for IPNV in carrier broodfish. In a pilot study at the National Veterinary Institute (NVI) Oslo it was shown that a considerably higher number of broodfish kidney samples proved to be IPNV-positive by RT-PCR than by isolation in cell culture (Dannevig, unpublished results). NVI also participated in an informal ring-test for detection of IPNV in kidney tissue from brood fish by RT-PCR or cell culture performed by a Norwegian salmon breeding company. Again, RT-PCR proved to produce the highest number of positive samples, though there was remarkable inconsistency among the laboratories involved (A. Storset AquaGen AS, pers. comm.). Comparison of PCR assays, flow cytometry and serological methods revealed that PCR and flow cytometry where the most sensitive methods for detection of IPNV in salmonid sperm (Rodriguez Saint-Jean et al. 2002).

Even in sexually immature salmonids, only few studies comparing RT-PCR and cell culture have been reported. Blake et al. (1995) reported that PCR assays may be a reliable alternative to the cell culture method for detecting aquatic birnaviruses. However, this study included only twelve 5-fish pools of carrier yearlings of brook trout *(Salvelinus fontinalis)* and in one of the spleen pools, the PCR was negative despite a positive cell culture assay. In another study, 8 Atlantic salmon parr surviving experimental infection with IPNV tested kidney-positive for IPNV by RT-PCR, whereas none were positive by the cell culture method (Taksdal et al. 2001). The same outcome was found in another 25 parr from the same source population of survivors that had been injected with cortison prior to testing. In yet another study, 13 organ pools from rainbow trout that were identified IPNV-positive by cell culture were subjected to RT-PCR by Barli-Maganja et al. (2002), all proving RT-PCR positive.

We regard the most i important steps for ensuring high sensitivity in the examination of brood fish samples by RT-PCR are as follows:

1. Transport and storage

The transport and storage of tissue samples for examination by molecular techniques must be performed in a manner to prevent degradation of RNA. Fish tissue transported in medium for maintaining virus infectivity is not optimal for preservation of RNA.We rather recommend that the specimen should be rapidly deep-frozen (-70°C) and transported to the laboratory on dry ice. Alternatively, the samples may be transported and stored in medium/solutions with special properties that conserve RNA (RNALater®).

2. Sample material

Several studies have reported that IPNV in carrier fish is associated with adherent leukocytes. Kidney tissue is generally chosen as test material since leukocytes are present in high numbers in this tissue. RNA is easily isolated from kidney tissue in high yields. Ovarian fluids, milt and eggs are also suitable test material regarding successful isolation of RNA, though isolation of RNA from eggs is more complicated than from other tissues. The isolation and enrichment of the target cells may greatly improve the sensitivity of the test as described by Munro and co-workers (2004).

3. Isolation of RNA

Several methods for RNA extractions are available, many of them based on commercial kits. The methods based on silica-membrane columns have the advantages that they do not involve use of hazardous chemicals compared to the classical phenol-chloroform method. For isolation of viral RNA from body fluids or from cell culture supernatants containing little cellular RNA, special kits are now available. They contain extra carrier RNA to improve the extraction of viral RNA, and consequently the amount of RNA extracted from the sample cannot be quantified. Validation studies comparing the performance of various kits or reagents are still lacking and the use of internal controls are therefore recommended.

4. Selection of primers

The selected primers must be from conserved regions of the virus genome to ensure detection of possible virus variants. The possibility that carrier fish may harbour other virus variants than those detected in disease outbreaks should be considered.

5. Procedure for RT-PCR

Many laboratories use a two-step RT-PCR, whereby the RT and PCR steps are run in separate tubes. The introduction of one-step procedures where the two reactions are run in a single tube must be welcomed. However, in this case, no cDNA is left for use in additional amplifications, but the risk for contamination between samples may be greatly reduced. At the current state of documentation and validation, the use of internal controls to verify the detailed RT-PCR procedure is recommended.

Non-Lethal Testing Methods

Methods available for non-lethal broodstock testing include analysis of serum for virus-specific antibodies, virus isolation or detection from mucus swabs or blood, and the examination of seminal or ovarian fluids for the presence of the virus or its genome.

Serology

Taking samples of blood to test for specific serum antibodies to IPNV has long been an attractive proposition for non-lethal screening of broodstock populations and even of individual broodfish, since it may yield evidence of previous exposure to the virus and therefore likely of the donor (population) being carrier of the virus. However, despite several studies using different antibody detection methods, no reliable correlation between absence or presence of antibody, and the absence or presence of virus has been achieved.

Smail et al (1985) compared antibody neutralising titre against virus titre from two farm populations of persistently infected Atlantic salmon post-smolts and found no significant positive or negative correlation between the two, which was in consistent with the results of Reno (1976) and Reno et al. (1978). All fish were found to be IPNV positive, some at levels exceeding 10^3-10^4 $TCID_{50}$/gram of kidney tissue, compared to only 39.7% and 43.8% antibody positive fish, respectively, on the two farms. A more recent investigation into experimentally infected and farmed populations of rainbow trout revealed that 30% of fish were virus positive by culture in contrast to 71% antibody-positive by ELISA or serum neutralization (Dixon & deGroot, 1996). All the virus positive fish were also antibody positive by ELISA and/or neutralisation, but not consistently with either one method. The authors concluded that although the antibody ELISA did not detect antibody in all virus positive individuals, it has potential for identifying rainbow trout populations previously exposed to IPNV, that therefore are likely to contain carriers.

The research findings to date suggest that analysis of serum for virus-specific antibodies would not accurately determine the current virus status of individual salmonids. Whilst some methods, such as the antibody ELISA, have been shown capable of detecting the presence of carriers in a population, they are not suited for confirming absence of IPNV in individual spawners. The use of serology to document the long-term specific pathogen free (SPS) status of broodstock sites and operations should, however, be encouraged in order to benefit from larger sample numbers.

IPNV antigen detection in non-lethal samples

Mucus

Munro et al. (2004) compared the isolation rate of IPNV from mucus samples (gill, skin, and rectum) versus kidney tissue from Atlantic salmon growers. Culture of the mucus samples detected IPNV from 9 out of 25 fish compared to 14 out of 25 from kidney tissue, suggesting that the mucus was a sub-optimal tissue.

Whole blood

Unpublished data from a Chilean study comparing blood and kidney tissue from Atlantic salmon broodfish using cell culture, RT-PCR and PCR-ELISA led to the conclusion that whole blood was an unsuitable sample material for broodstock screening (Muñoz, Fundacion Chile & BiosChile, Puerto Montt, Chile and Joel Leal, Aquatic Health Diagnostic and Research Services, Puerto Varas, Chile, pers. comm.).

Blood leukocytes

Yu et al. (1982) documented that IPNV could be isolated from purified blood leucocytes obtained from both rainbow trout and brook trout using a co-cultivation method, and Rodriguez et al. (2001) developed a rapid and sensitive method for the detection of IPNV in asymptomatic adult (post-mature) rainbow trout using purified blood leucocytes. Johansen et al. (1995) confirmed that IPNV would multiply in adherent blood leucocytes from Atlantic salmon and in 2004 Munro et al. developed a sensitive non-destructive method for detecting IPNV from carrier grower Atlantic salmon by culture of virus from adherent blood leucocytes. However, attempts to document the suitability of this method for detection of IPNV carrier broodfish have proved unsuccessful (Eann Munro, pers. observation).

Ovarian fluid components

Smail & Munro (1985) compared virus isolation from kidney and ovarian fluid of 40 Atlantic salmon broodfish at a commercial site with a history of IPNV. In this study, 15 fish were positive on both tissues, whereas 11 fish were positive only in kidney and 6 fish were positive only in the ovarian fluid. 8 fish in this study proved IPNV negative. Enhanced cell culture isolation of IPNV from the pelleted cell component of ovarian fluid from brook trout was described by McAllister et al. (1987). As mentioned above did RT-PCR of 13 organ pools from rainbow trout confirm the presence of IPNV-positive found by by cell culture (Barliç-Manganja et al. 2002).

In a Chilean cell culture screening including 150 Atlantic salmon broodfish of each sex it was found that the IPNV prevalence in females was 2% in kidney samples vs. zero in ovarian fluid samples. In male fish, the prevalence was 2.8% in kidney samples vs. 0.7% in the seminal fluid samples (P. Bustos, ADL Diagnostic Chile LTD., Castro, Chile, pers. comm..). Unpublished data from Scotland (Eann Munro, pers. obs.) using the cell culture method supported this finding (All 153 samples were IPNV-negative on ovarian fluid cell pellet, whereas 88 of these fish were kidney positive for IPNV by cell culture). Unfortunately, none of these studies have as of yet been published, nor have studies to demonstrate which virus titer is required in either kidney or sexual fluids to associate with IPNV infection of the resultant offspring.

Milt

A quick and simple indirect immunofluorescence and cell sorting assay (FACS) for detection of IPNV in whole semen has been described in rainbow trout (Rodríguez Saint-Jean et al. 1992), where 5 milt samples produced IFA positive results and all were verified IPNV positive by cell culture isolation. This technique gave the added advantage of rapid acquisition of results, and may warrant a revisit and a feasibility study.

Conclusions and further research needs

For population-level broodstock testing, and where lethal sampling is possible and acceptable, kidney material should be the tissue of choice. Kidney tissue should be examined on cell cultures (CHSE-214 and eventually BF-2) according to the method described by the OIE. Samples must be collected and placed cold (1-4°C) in suitable transport medium (as Eagles MEM or L-15 medium) until analysis. If the samples cannot be analysed within 48 hours, and transport medium should be supplemented with 50% glycerol for virus protection, in the case of which storage can be done at 0 to -20°C, still avoiding freezing. Pooling of equal amount of kidney tissue from up to 5 individual fish is considered acceptable for population-level screening to document freedom from IPNV infection. We also believe that serology for IPNV antibodies, and IPNV antigen ELISA on kidney samples, both applied to a large number of individual fish and over time, may provide valuable data to document the absence of IPNV from broodstock sites and –populations. These methods, are, however not recommended by the OIE and must therefore still be regarded experimental for this purpose.

Cell culture isolation from kidney samples using the above described methods is also recommended to determine the IPNV infection status of individual fish of from carrier populations or from populations with unknown infection status. Whereas pooling of samples is acceptable for population-level screening, the feasibility of organ pools for certification of individual fish as being IPNV-free is still undetermined.

Where non-lethal examination is required, the OIE recommendations include the use of ovarian fluid for cell culture, whereas no recommendation is given for analysis of non-lethal samples from male fish. To which degree the sensitivity of ovarian fluid testing is

influenced by centrifugation, and which fraction (cell pellet or fluid) should be tested remains to be firmly established. RT-PCR and other molecular methods for non-lethal testing of individual broodfish must, due to the current lack of standardisation, documentation and validation still be regarded at the experimental stage. We believe, however, that RT-PCR performed on ovarian and seminal fluids may yield valuable information to assist zoo-sanitary broodstock management, and to reduce the risks of vertical transmission of IPNV via embryonated salmonid eggs.

The advantage of cell cultivation is good sensitivity and a very high specificity (no false positives unless by contamination), thus securing the zoo-sanitary relevance of any positive finding. When finding genomic material from the IPN virus via RT-PCR it is still questionable if virus is actually present in an infective form that may represent a real risk for vertical transmission. Until this aspect has been clarified, there is an obvious risk that rejection of breeders based upon RT-PCR results may go beyond what is biologically necessary.

Recommendations for future research

1. The key to rapid progress in further validation of diagnostic methods for management of vertical transfer risks with regards to IPNV are the quantitative aspects, namely the association between titre of IPNV in kidney or sexual fluids, and the vertical transmission rate. Once this relationship is established, various testing methods and control schemes satisfying the required analytical sensitivity can be validated and confirmed through experimental and field studies. We believe that this may provide a strong stimulus for the aquaculture industry to implement the corresponding control methods on a broader scale than today.

2. While awaiting these results, there is an urgent need for documentation and standardisation of methods for individual-fish testing and certification:
   - Large amounts of historical data from various countries and regions, derived from routine testing and disease management operations should be scrutinised in order to reveal evidence of disease control success or failure.
   - Methods applicable to non-lethal sampling tissues from sexually mature salmonids, above all on ovarian and seminal fluids and whole sperm should be documented in greater detail using spiked samples and preferably experimentally infected fish.
   - There is a need to evaluate methods allowing for predictive testing of individual fish such as real-time PCR on whole blood or blood leucocytes derived from fish approaching sexual maturity, in order to stimulate improved on-farm health management of broodstock.

3. The effect of pooling tissue samples (both the pooling of organs from the same broodfish, and the pooling of the same organ from up to 5 broodfish) on the sensitivity of the cell culture method should be investigated.

4. A feasibility study for the establishment and maintenance of SPF populations of Atlantic salmon broodstock should be carried out.

5. Methods to improve the cost-efficacy of population-level IPNV screening and the certification of freedom from IPNV should be documented and validated. This goes in particular for the suitability of serological screening in Atlantic salmon (similar to what is done in rainbow trout by Dixon and de Groot (1996).

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Viral encephalopathy and retinopathy (VER)/ Viral nervous necrosis (VNN)

Viral encephalopathy and retinopathy (VER) is usually observed in larvae and juveniles even in hatcheries supplied with UV treated water, furthermore the viral antigen has been frequently detected in gonads and their products (Arimoto et al., 1992; Mushiake et al., 1994; Nishizawa et al., 1996; Nguyen et al., 1997; Watanabe et al., 1998; Mori et al., 1998 Dalla Valle et al., 2000; Watanabe et al., 2000). More recently the virus has been detected in eggs and larvae from spawners experimentally infected by IM injection (Breuil et al., 2002). These studies suggest that infected parents may transmit the infection to their offspring but it is still to be proven if the virus is transmitted inside the egg or on the egg surface.

From a practical point of view the difference between "true vertical transmission" and "egg-associated transmission" have not much sense, since it is very difficult to disinfect embryonated eggs of the susceptible species. Any effective control program should therefore first and foremost be based on the selection of virus free brood-stock, in order to avoid the presence of carrier fish in the spawner population. The identification of the carrier status represents the most critical step and a highly sensitive and non-destructive method, permitting the detection of sub-clinical infection in parental fish, is required.

The first detection of noda-virus in gonads dates back to 1992 when it was described by Arimoto et al., applying an antigen ELISA method in striped jack *(Pseudocaranx dentex)*, which represents the most investigated species. In table 1 the species, the techniques applied for broodfish testing and the material tested in the most relevant papers on broodfish screening are summarized.

Table 1: Relevant papers published on broodfish screening for nodavirus infection.

SPECIES	TECHNIQUE USED	SAMPLE MATERIAL	REFERENCES
Striped jack	ELISA	Gonads (ovary and testis)	Arimoto et al. 1992
Striped jack	ELISA	Blood (serum)	Mushiacke et al. 1992
Striped jack	ELISA	Blood (serum)	Mushiacke et al. 1993
Striped jack	PCR	Gonads	Mushiacke et al. 1994
	ELISA	Gonads	
	ELISA	Blood (serum)	
Striped jack	PCR	Gonadal fluids	Nishizawa et al. 1996
Striped jack	PCR	Gonads	Nguyen et al. 1997
	IFAT	Gonads	
Barfin flounder	ELISA	Blood (serum)	Watanabe et al. 1998
	PCR	Eggs/sperm	
Striped jack	ELISA	Blood (serum)	Mori et al. 1998
	PCR	Gonads	
Barfin flounder	ELISA	Blood (serum)	Yoshimitzu 1998
Sea bass	ELISA	Blood (serum)	Breuil et al. 1999
Sea bass	NESTED PCR	blood, sperm, ovary biopsy	Dalla Valle et al. 2000
Striped jack	ELISA	Blood (serum)	Watanabe et al. 2000
	PCR	Sperm and ovarian fluid	

Abundance of nodaviruses in various tissues

High prevalence of nodaviruses has been found by nested PCR in blood, seminal fluid, ovaries and different tissues other than brain, which is considered the target organ during clinical outbreaks. In table 2, some results obtained by Dalla Valle et al. (2000) are summarized.

Table 2: Nodavirus positive results observed in different tissues (after Dalla Valle et al. 2000).

SPECIES	TISSUE	N°	METHOD	% POSITIVE FISH
Sea bass	Blood	79	NESTED PCR	59,4
Sea bream	Blood	91	NESTED PCR	34,6
Sea bass	Seminal fluid	17	NESTED PCR	47,1
Shi drum	Seminal fluid	20	NESTED PCR	65
Sea bass	Ovary biopsy	17	NESTED PCR	35,3
Shi drum	Ovary biopsy	15	NESTED PCR	33

According to Arimoto et al. (1992), virus seems to be more prevalent in gonads than in brain in mature fish surviving the infection; in fact while 65 % positive fish ovaries were detected by ELISA, no positive fish brains were found. In the same experiment no virus was detected in testis, suggesting that females would play a more important role than males when it comes to vertical transmission of VER. These results confirm the possibility to obtain sufficient information on the health status of each single spawner through non-lethal sampling.

In a Norwegian material, 76% of the brains from spawners of halibut *(Hippoglossus hippoglossus)* exposed to natural infection eight years before were positive by one or more of the applied methods (real-time PCR, RT-PCR or immunohistochemistry; Hogne Bleie, National Veterinary Insitute Bergen, Norway, pers. comm.). The same author (pers. comm.) obtained significant different results between RT-PCR and real-time PCR when processing the same tissues in parallel.

The discrepancies between the findings in striped jack and halibut might either be due to the application of different diagnostic methods and protocols, or to different pathogenetic mechanisms of nodaviruses in the two species.

Molecular diagnostic techniques

Results similar to those reported above were obtained by Breuil and co-workers (2002) when testing ovarian biopsies, unfertilized eggs, fertilized eggs and larvae obtained from experimentally infected broodfish with ELISA, RT-PCR or nested RT-PCR (table 3).

Table 3 : ELISA, RT-PCR and nested RT-PCR detection of VER/VNN virus in biopsies and spawning products from 7 experimentally infected sea bass females (after Breuil et al. 2002).

ORGANS Postitive #	ELISA Postitive # (%)	RT-PCR Postitive # (%)	Nested RT-PCR Postitive # (%)
Ovarian biopsies (n=7)	0	0	6 (85,7%)
Unfertilized eggs (n=7 spawns)	3 (48,2%)	5 (71,4%)	7 (100%)
Fertilized eggs (n=7 spawns)	3 (42,8%)	7 (100%)	7 (100%)
Larvae (n=7 batches)	7 (100%)	7 (100%)	7 (100%)

On the other hand, highly different prevalences were reported by Dalla Valle et al. (2000) when examining the same samples by RT-PCR and nested PCR, respectevely (table 4).

Table 4: Results obtained with RT-PCR or nested PCR applied to the same tissues (after Dalla Valle et al. 2000).

TISSUE	N°	NEGATIVE	POSITIVE	
			RT-PCR	nested PCR
Sea bass				
• brain	59	15	19 (32,2%)	25 (42,4%)
• blood	79	32	0	47 (59,5%)
• sperm	17	9	0	8 (47,1%)
• ovarian biopsy	17	11	0	6 (35,3%)
• Larvae	18	5	0	13 (72,2%)
Sea bream				
• brain	18	11	2 (11,1%)	5 (27,7%)
• blood	91	60	0	31 (34,1%)
• larvae	15	12	0	3 (20%)
Shi drum				
• sperm	20	7	0	13 (65%)
• ovarian biopsy	15	10	0	5 (33,3%)
Red sea bream				
• brain	8	5	3 (37,5%)	0

The most significant differences were detected in blood, sperm, and ovarian samples in which virus should be harbored, particularly in carrier fish, at very low titers. So it seems to be enough clear that efficient screening of broodfish could be obtained only if very sensitive methods are applied. With regard to this hypothesis Nishizawa et al. (1996), reported the failure of RT-PCR in detecting all positive broodfish of a reared striped jack *(Pseudocaranx dentex)* population and concluded that the detection limit of the applied method (100 fg of viral genome) is insufficient for an effective screening.

Antibody detection by ELISA

Most published attempts to screen broodstock for nodaviruses use molecular techniques and not ELISA, obviously due to lower sensitivity of the latter method and despite its higher applicability. Nevertheless several papers have been published concerning the application of ELISA to detect the presence of antibodies in spawners.

According to Watanabe (table 5), antibody ELISA titres ≥ 1:40 are indicative of infection while values ≤ 1: 10 are indicative of a viral negative condition.

Table 5: Comparison between PCR performed on broodfish tissues samples, ELISA titers in blood from the same fish, and disease outbreaks in larvae (after Watanabe et al. 2000).

ELISA Ab titer	N° of fish		PCR	Outbreaks of VER/VNN in larval production
≤1:10	23	31	0/54	0/6
1:20	8	8	0/16	No larvae
1:40	19	13	0/32	1/7
≥1:40	81	14	11/18	1/2

More recently it has been shown by Breuil et al. (2002) that detection of circulating antibodies by ELISA could be affected by the specificity of viral strains. According to the results obtained the Sb2 strain seems to possess a very low immunogenicity, particularly when infection occurs at low temperatures. Similar results have been confirmed in another laboratory by serum neutralization test, suggesting the need to perform the monitoring control during the summer period.

Conclusions and further research needs

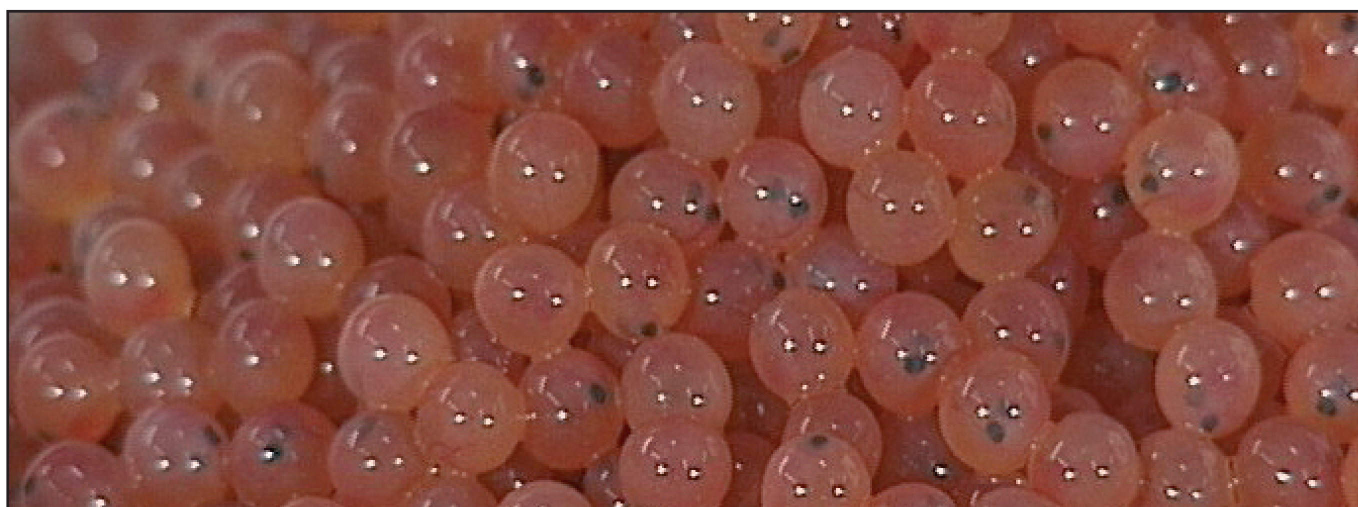
The lack of knowledge with respect to the epidemiology and pathogenic mechanisms of VER/VNN make control measures very difficult; for this reasons a complex approach based on broodfish monitoring is recommended. The selection of free broodstock should include both ELISA to detect specific antibodies and PCR techniques on blood, sperm and ovarian fluid. Due to the reported failure of PCR to detect nodaviruses in some carrier fish, the development and validation of nested PCR or real-time PCR protocols is recommended.

Further research is urgently necessary for a better understanding of the pathogenic mechanisms involved in vertical transmission and the following topics should be investigated:

1. The distribution of antigen in ovaries, sperm and biological fluids of carrier fish.

2. Sensitivity comparisons between RT-PCR, nested PCR and real-time PCR to detect nodavirus antigen in blood, sperm and ovariau fluid, respectively.

3. Comparison between PCR protocols and antibody ELISA to identify carrier fish.



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