Report

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Work package 4 report: Broodfish testing for bacterial 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 bacterial kidney disease (BKD - *Renibacterium salmoninarum* infection) in order to prevent vertical transmission of the organism to the offspring. Assessment is also being performed for *Flavobacterium psychrophilum* infections causing rainbow trout fry syndrome (RTFS) or bacterial coldwater disease (CWD), and for *Piscirickettsia salmonis* infection causing salmon rickettsial syndrome (SRS) in salmonid fish species. Methods for screening to document the absence of BKD in fish populations are well established. Some of them have also proven successful for testing individual fish from infected populations in order to avoid vertical transmission of the infectious agent. Several diagnostic methods for flavobacteriosis and piscirickettsiosis have also been established but none of them, as yet, has been validated for use in programmes to prevent vertical transmission of disease. Priority subjects 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 bakteriell nyresyke (*Renibacterium salmoninarum* infeksjon), for derigjennom å hindre vertikal smitte av viruset til avkommet. En tilsvarende faglig vurdering gjøres for *flavobakterium psychrophilum*-infeksjoner og *Pisciricettsia salmonis*-infeksjon hos laksefisk. Metodene for på testing og kontroll av BKD både på populasjonsnivå og på individnivå er veletablerte og gjennomprøvd. Det finnes også en rekke diagnostiske metoder for å avdekke infeksjon med flavobakterier og piscirickettsier, men ingen av dem er validert til bruk i stamfiskkontroll eller som grunnlag for risikohåndtering og sykdomskontroll. Prioriterte emner for framtidig forskning på dette 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 diagnostic methods and procedures 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 in trade and transfer. Focus of the work has been placed on those infections that have been shown or are believed to transmit inside the egg shell, as identified in the WP1 report.

Materials and methods

Forming the basis for the assessment of this work package report, two workshops with invited experts have been conducted, during which summaries of published data, of published and unpublished scientific studies, and of (mostly unpublished) disease control experience has been presented and discussed in-depth. One of the workshops, held in Copenhagen in October 2004, was devoted to the vertically transmissible bacterial infections of fish (BKD, rainbow trout fry syndrome RTFS and Piscirickettsiosis) that are discussed in the current work package report.

Based upon the background knowledge of the workshop participants and on their scientific literature databases, the first chapter drafts were produced by the first and the second author, and submitted to the co-authors and to the contributing experts for supplementation and revision. Further improvement of the initial version of the report has been co-ordinated by INRA Jouy-en-Josas, who has been the co-ordinator of this report.

Results

Bacterial kidney disease (*Renibacterium salmoninarum* infection)

Bacterial kidney disease has been known since the early 1930s, and its economic impact has been perceived serious enough, both in farmed fish and in feral salmonid populations, to give rise to ambitious control programmes. Chemotherapy is of limited use and vaccines are not available, but surveillance and destruction of infected broodfish have appeared most effective for reducing the impact of BKD in progeny fish (Gudmundsdóttir et al., 2000; Pascho et al., 1991). It is likely that no other fish-pathogenic bacterium has been subjected to as extensive efforts in the development of diagnostic techniques as *Renibacterium salmoninarum.* Some pioneer techniques are now of historic interest only, including coagglutination (Kimura and Awakura, 1977; Kimura and Yoshimizu, 1981), immu-

noprecipitation (Kimura et al., 1978) and immunodiffusion (Chen, 1974). Other techniques have proven quite effective (Pascho et al., 2002), such that they may serve as models for application of similar control programmes for other fish pathogens.

Culture

R. salmoninarum is a fastidious growing organism that requires low incubation temperature (usually 15 °C) and sometimes as long as 6-19 weeks to produce characteristic colonies (Benediktsdóttir et al., 1991). Although different formulations were tested after the first successful cultivation report and these allowed assessment of the importance of cysteine and serum enrichment (see Austin and Austin, 1999 for review), most media were considered to produce rather inconsistent results until Evelyn (1977) described the KDM-2 medium. Consisting of peptone 1% (w/v), yeast extract 0.05% (w/v), cysteine hydrochloride 0.1% (w/v), and completed with the addition of 5-10% (v/v) of foetal calf serum and 1% to 1.5% (w/v) agar for solid medium, this medium is still commonly used and has been the basic formulation for subsequent improvements. Daly and Stevenson (1985) suggested replacement of serum with activated charcoal (KDM-C), whereas Austin et al (1983), made the medium more selective (SKDM) by incorporating antimicrobial agents (cycloheximide, D-cycloserine, polymyxin B sulphate, and oxolinic acid) to limit the proliferation of contaminating microorganisms. Despite the use of a selective medium, contamination of cultures can still occur (Gudmundsdóttir et al., 1991; Olsen et al., 1992; Sakai et al., 1987a).

Inconsistency in the quality of different peptone lots may induce important variations in *R. salmoninarum* culture sensitivity (Evelyn and Prosperi-Porta, 1989). The variable performance of different peptone lots may explain in part why comparative studies (Gudmundsdóttir et al., 1991; Olsen et al., 1992; Sakai et al., 1987a) have not always been in agreement or have not discerned clear differences among tested formulations (Starliper et al., 1998). However, the problems with peptone lots can be alleviated by drop-in-oculation of a heavy suspension of *R. salmoninarum* in the centre of culture plates (nurse culture) or by incorporation of filtered or autoclaved supernatant from a previous *R. salmoninarum* broth culture into the medium (Evelyn et al., 1989; 1990). In addition to variable results associated with the peptone component of *R. salmoninarum* media, soluble substances in homogenates of salmonid liver and kidney tissues have been shown to have an inhibitory effect on the growth of *R. salmoninarum* on KDM-2 medium unless the homogenates are washed or diluted (Daly and Stevenson, 1988; Evelyn et al., 1981). In spite of technical demands and the long delays of incubation required for plate reading, culture has been used with some success in several BKD detection programmes incorporating broodstock screening (Jansson et al. 1996).

Immunodiagnostic methods

Microscopic observation

Microscopic observation by use of Gram staining or Lillie's allochrome (Bruno and Munro, 1982) suffered limitations which were soon resolved by the introduction of specific methods for marking *R. salmoninarum* cells. Immunochemistry, based on immunoperoxidase use (Hoffmann et al., 1989; Jansson et al., 1991), proved to be effective, but practical considerations led to a preference for the routine use of immunofluorescence tests. Both direct immunofluorescence tests (Bullock et al., 1980; Cvitanich, 1994; Ochiai et al., 1984) and indirect tests (Bullock and Stuckey, 1975; Laidler, 1980; Lee and Gordon, 1987; Yoshimizu et al., 1988) have been applied, sometimes in combination with avidin/biotin systems (Yoshimizu et al., 1988). Nevertheless, cross-reactions with other fish-associated bacteria have been reported by a number of authors (see review by Pascho et al., 2002). Careful attention is therefore necessary in the selection of antibody and the interpretation of results (Armstrong et al., 1989). An important improvement in the sensitivity of immunofluorescent tests applied to detection of *R. salmoninarum* in coelomic (ovarian) fluid of spawning fish, was achieved when Elliott and Barila (1987) recommended a procedure that concentrated the bacteria on polycarbonate filter membranes prior to immunofluorescence staining. Thus performed, membrane filtration fluorescent antibody tests (MF-FAT) proved more sensitive than immunofluorescence staining of coelomic fluid without prior concentration on membrane filters (Elliott and McKibben, 1997). The MF-FAT is also more sensitive than ELISA procedures for detecting *R. salmoninarum* in coelomic fluid (Pascho et al., 1991; 1998), but examination of numerous individual samples with a fluorescence microscope can be cumbersome.

Enzyme-linked immunosorbent assay (ELISA)

The first application of ELISA to BKD diagnosis was carried out by Pascho and Mulcahy (1987). The double antibody sandwich method was used, and plates were coated with antibodies directed to the soluble and thermostable p57 antigen of *R. salmoninarum,* known to be released in colonized tissues during the course of infection. Sakai et al. (1987a) described almost simultaneously an ELISA procedure performed on ester cellulose membranes which required more technical investment but allowed detection of 103 bacterial cells per ml, proving much more sensitive than IFAT and immunodiffusion. Monoclonal antibodies prepared against the p57 protein were soon tested in order to reduce the risk of cross-reactions (Hsu et al., 1991; Rockey et al., 1991). Although the ability of all strains to produce p57 was questioned by Bandín et al. (1992; 1993) the method was quickly adopted, and commercial kits were approved for BKD diagnosis. In a subsequent study, Bandín et al. (1996) attested to the practical effectiveness of ELISA tests, noting only a single cross-reaction, with *Stenotrophomonas maltophilia.*

In large-scale detection studies, however, polyclonal sera are generally preferred over monoclonal antibodies. Their broad specificity and higher sensitivity (Jansson et al., 1996) compensate for the apparent limited occurrence of cross-reactions. Laboratory ELISAs, in which samples are inoculated into microtiter plates and results are analysed with a spectrophotometer, are also more sensitive than ELISA field kits, in which samples are placed in test tubes and results are read by visual comparison of test samples to standards (Pascho et al., 2002; Reddington, 1993). The laboratory ELISA is one of the few detection methods that allow one to quantify the degree of infection in fish tissues, similar to direct enumeration or plate counting. This feature can be of great use for broodfish segregation or culling procedures.

The ELISAs that use antibodies prepared against the p57 protein and other soluble antigens of *R. salmoninarum* can detect infections in tissues remote from the one sampled (Elliott and Pascho, 2001; Pascho and Mulcahy, 1987), because these antigens circulate throughout the body (Rockey et al., 1991; Turaga et al., 1987). ELISA procedures cannot distinguish live from dead *R. salmoninarum,* however, and the persistence of *R. salmoninarum* antigens (Pascho et al., 1997) can therefore cause problems in the interpretation of ELISA results when management practices such as antibiotic chemotherapy, vaccination or disinfection are being evaluated (Pascho et al., 2002). Although an ELISA can be very sensitive for detection of *R. salmoninarum* antigen in tissue samples and in blood, research has indicated that some polyclonal antibody ELISAs (Pascho et al., 1991; 1998) and monoclonal antibody ELISAs (Griffiths et al., 1996) lack sensitivity for detecting the bacterium in coelomic fluid of spawning salmonids.

Additional useful immunological methods have been described in efforts to improve the specificity of *Renibacterium* detection. Many of them, however, require special equipment that limits their use to research purposes. One such method is Western blot or immunoblot (Griffiths et al., 1989; 1991), which separates antigens by molecular mass as well as by reactivity with specific antibody.

Molecular methods

Three different papers issued in 1994 provided the first information on the application of the polymerase chain reaction (PCR) to BKD detection. Brown et al (1994) and León et al. (1994) used direct PCR. Brown et al. (1994) amplified a sequence of the p57 protein gene for detecting the causative bacterium in eggs, whereas León et al. (1994) detected it in tissues. Turgut et al. (1999) later confirmed the validity of the method proposed by León et al (1994). Magnússon et al (1994) developed a nested reverse transcription PCR for the demonstration of bacterial cells in coelomic fluids, but the technique may prove too difficult to use for routine control programs. Various PCRs have been frequently employed for many sample types in subsequent studies (see review by Pascho et al., 2002). Whereas PCRs for DNA or rRNA cannot distinguish live from dead organisms, Cook and Lynch (1999) developed a nested reverse transcription PCR for mRNA; this procedure detects viable (or recently killed) *R. salmoninarum* cells. Although most PCRs cannot be used to quantify *R. salmoninarum* infection levels, Elliott and Pascho (2004) reported preliminary development of a real-time quantitative PCR for quantification of *R. salmoninarum* in fish tissues.

A noteworthy advantage of PCR is avoidance of the cross-reactions known to occur in immunological tests (Brown et al., 1995; reviewed by Pascho et al., 2002). Nevertheless, cross-reactivity with other bacterial species has occasionally been reported (Magnússon et al., 1994). The PCR, particularly nested PCR, can also be a very sensitive procedure. Using nested PCR, Chase and Pascho (1998) and Cook and Lynch (1999) could detect as few as 10 bacterial cells, increasing PCR sensitivity about 100 times compared with the performance of direct tests.

Comparative studies

The choice of a reliable method for routine examination of fish populations may depend on several considerations, among which a balance between sensitivity, specificity and practical constraints is generally decisive. Culture, FAT, ELISA and PCR presently appear to be the most popular methods. It is more difficult to state the relative advantages of these different methods, as many of the comparative studies carried out to provide objective assessments have resulted in conflicting conclusions (Table 1). This may be due in part to differences in experimental protocols, but it seems that local factors, including geographic context, differences among tested fish populations, or the prevalence of infection, may also introduce some degree of variation. Eventually, the experience and training of the BKD control teams may be of equal or greater significance than the selected method itself for the reliability and effectiveness of BKD testing. Nevertheless, some general criteria can be used to evaluate advantages and disadvantages of diagnostic tests for a particular situation (Table 2). In critical circumstances, it may be necessary to use more than one diagnostic method. In such cases a method suitable for large-scale testing (ELISA, for example) can be used for initial screening, and a second method based on a different diagnostic principle (PCR, for example) should be used for confirmation of positive results.

Another point of significance is the number of fish to be tested to maximize the chances of detecting infected animals. This is a problem of statistics, and available guidelines such as the OIE Manual of Diagnostic Tests for Aquatic Animals (anonymous, 2003) provide tables establishing these numbers according to the prevalence of the disease. A limitation often arises, however, from the high numbers needed when prevalence is low. In such cases, sampling may

become unrealistic with regard to the technical work required to achieve the proper level of sampling. It is clear that any method that is easy to perform with a minimum of technical investment has advantages in such conditions, and this may be of prime importance in the selection of the method to apply. Additionally, for some fish populations, regular monitoring and testing of clinically diseased fish may be more successful than a single large sample of apparently healthy fish for detecting infected animals.

Table 1. Comparison studies of different methods used in BKD detection

KDM2 culture ≥ IFAT > Gram > immunodiffusion	Evelyn, 1978; Evelyn et al., 1981
IFAT > KDM2 culture	Mitchum et al., 1979
IFAT > culture, direct enumeration	Paterson et al., 1979
MF-FAT > IFAT (coelomic fluid)	Elliott and McKibben, 1997
ELISA > SKDM culture	Gudmundsdóttir et al., 1993
	Olea et al., 1993
ELISA > FAT	Meyers et al., 1993
ELISA > DFAT > CIE > immunodiffusion	Pascho et al., 1987
blot- ELISA > IFAT, coagglutination > immunodiffusion	Sakai et al., 1987a, b; 1989
Western blot = culture > DFAT	Griffiths et al., 1991
	Turaga et al., 1987
Western blot > culture, DFAT	Olivier et al., 1992
culture > IFAT (coelomic fluid)	Armstrong et al., 1989
culture > IFAT > ELISA and Western blot	Griffiths et al., 1996
Mab-ELISA > FAT	Hsu et al., 1991
ELISA = MAb-ELISA > culture	Jansson et al., 1996
PCR > culture	Miriam et al., 1997
PCR > MF-FAT > ELISA (coelomic fluid)	Pascho et al., 1998
PCR > ELISA	Chase and Pascho, 1998
culture > Western blot (in carrier detection)	McIntosh and Austin, 1996
CIE > coagglutination > culture > DFAT > Gram > immunodiffusion	Cipriano et al., 1985

Abbreviations: DFAT: direct immunofluorescence; IFAT indirect immunofluorescence; MF-FAT: membrane filtration immunofluorescence; ELISA: enzyme-linked immunosorbent assay (Mab- ELISA: monoclonal antibody ELISA); CIE: counter-immunoelectrophoresis; PCR: polymerase chain reaction; KMD2: kidney disease medium; SKDM: selective kidney disease medium.

Conclusions and research needs

Vertical transmission of *R.salmoninarum* can be avoided by using broodstocks free of the bacterium. In endemic situations, culling or segregation of broodstocks has proven to be an effective way of limiting the impact of BKD (Elliott et al., 1995; Gudmundsdóttir et al., 2000; Maule et al., 1996; Pascho et al., 1991), and extensive experience has been acquired about the detection methods specially adapted to this purpose. If maximum sensitivity is needed, it seems that PCR, namely nested PCR, should be the reference method. Successive amplification steps, however, make PCR more susceptible to contamination with foreign DNAs, such that strict quality control procedures are required to avoid serious problems. Furthermore, standard PCR does not permit the diagnostician to differentiate active infection from the residual traces of an infection that has been overcome, it cannot be calibrated to provide quantitative information on the intensity of tissue or sample infection as can ELISA and FAT, and it is still less suited to the detection of live bacteria than cultivation. Amplification of mRNA rather than DNA, through RT-PCR and a real-time application, would help to alleviate such difficulties. Developed for experimental research, these methods still appear cumbersome and difficult to validate for routine processing of large numbers of samples. It is clear that, while improvements in control strategies will be dependent on advances in epidemiological knowledge, the adaptation of modern molecular procedures to the special constraints of large scale detection would represent a valuable progression in BKD control effectiveness.

monly used diagnostic tests for *Renibacterium salmoninarum* (modified from Pascho et al., 2002).

	Diagnostic Test					
Criterion	**Culture**	**Smear FAT[a]**	**MF-FAT[a]**	**Field ELISA[a]**	**Laboratory ELISA[a]**	**PCR[a]**
Specificity	No	Yes	Yes	Yes	Yes	Yes
Sensitivity	Low/high[b]	Moderate	High	Low	Moderate/high[c]	High
Quantitative	No/yes[d]	Semi-	Yes	No	Semi-	No/yes[e]
Detects live bacteria only	Yes	No	No	No	No	No/yes[f]
Detects remote infections	No	No	No	Yes[g]	Yes[g]	No
Non-lethal sample	Yes[h]	No	Yes[h]	No	Yes[i]	Yes[h,i]
Rapid test (=2 days)	No	Yes	Yes	Yes	Yes	Yes
Time/cost savings for multiple samples	No	No	No	No	Yes	No
Specialized equipment	No	Yes[j]	Yes[j]	No	Yes[k]	Yes[l]
Technical expertise required	Low	Moderate	Moderate	Low	High	High
Commercial reagents	Yes	Yes	Yes	Yes	Yes	Custom

[a]**Abbreviations:** FAT: immunofluorescence (fluorescent antibody test, used for tissue smears); MF-FAT: membrane filtration immunofluorescence (used for coelomic fluid); ELISA: enzyme-linked immunosorbent assay; PCR: polymerase chain reaction. [b]The presence of other organisms in samples can reduce the detection of *R. salmoninarum.* [c]Limited experimentation has shown a higher sensitivity for a polyclonal antibody ELISA than for a monoclonal antibody ELISA. [d]Spread plate or drop-inoculated cultures can be quantitative. [e]Preliminary development of a real-time quantitative PCR procedure for *R. salmoninarum* has been completed; other PCR procedures for this bacterium are not quantitative. [f]Only PCR procedures designed to detect mRNA detect live (or recently killed) *R. salmoninarum* only. [g]An ELISA that uses antibody directed against soluble antigen(s) of *R. salmoninarum* can detect infections in tissues remote from those sampled, provided that the concentration of antigen released by the bacterium into the blood and tissues exceeds the minimum detection limits of the assay. [h]Coelomic (ovarian) fluid can be used as a non-lethal sample. [i]Blood can be used as a non-lethal sample. [j]A fluorescence microscope is required. [k]Specialized equipment includes a microtitre plate reader (spectrophotometer, often attached to a computer). A reagent dispenser and microtirte plate washer are essential for ELISA analyses involving large numbers of samples. [l]Specialized equipment for basic PCR includes a thermal cycler, gel electrophoresis system including power supply, and a UV gel viewer (if ethidium bromide staining is used) and gel documentation system. For quantitative PCR, an automated sequence detector is required, and a 96-well centrifuge for nucleic acid extractions and a 96-well spectrophotometer are desirable.

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Flavobacterium psychrophilum infection (Rainbow trout fry syndrome)

The case of *Flavobacterium psychrophilum* infection is quite different from that of renibacteriosis. Worldwide spreading did not occur before the mid-eighties. As long as the disease was confined to salmonid populations of North-Western America little attention had been paid to health control programmes, limiting to routine egg disinfection recommendations (see WP 3). As soon as the suspicion of true vertical transmision was substantiated, however (see WP 1), extensive studies aiming at developing detection methods adapted to broodstock control started to multiply. Although this late interest is reflected by a clear preference for molecular techniques, is seems evident that the experience accumulated with BKD has served as a reference in most of the relevant works. So far, however, no detection method has been applied routinely to large scale *F. psychrophilum* detection programmes.

Culture

Difficulties in *F. psychrophilum* culture stem from slow growth (up to 4-5 days), from low temperature preference (15-18 °C), from possible confusions with morphologically resembling organisms (*Chryseobacterium* spp., but also undetermined flavobacteria commonly called "psychrophilum-like"), and from special cultural requirements. Fish pathogenic *Flavobacterium* species growth does not generally occur on usual nutrient media. A noticeable progress was achieved by Anacker and Ordal (1955), with the culture of *F. columnare* on Cytophaga agar (AOA) which contained reduced amounts of peptone (0.05 %), yeast extract 0.05 %, beef extract 0.02 % and sodium acetate 0.02 %. *Flavobacterium psychrophilum,* however, prefers higher rates of nutrients, and several ways of enrichment, such as increasing tryptone to 0.5 % (Bernardet and Kerouault, 1989) or incorporating serum (Obach and Baudin-Laurencin, 1991) proved more suitable, as did TYES, another formula including calcium and magnesium salts (Holt et al., 1993) sometimes completed with skimmed milk 20 %.

In spite of all these improvements some inconstency was still observed, namely in the numbers of viable or culturable bacterial cells obtained from cultures performed in similar conditions. Among further suggestions, including special attention paid to the beef extract brand (Lorenzen, 1993), those which seemed to result in the most interesting performances were the incorporation of serum and mineral elements traces (Michel et al., 1999) or carbon hydrates (Daskalov et al., 1999) to AO medium. An extensive study recently reported by Cepeda et al. (2004) reached the same conclusions. The objective of all these works was mainly to improve the mass production of viable bacterial cultures, but it is clear that isolation would gain to be performed on as suitable media as possible. Isolation may often be hampered by the presence of contaminant bacteria in biological materials. To avoid this problem, several authors (Kumagai et al., 2004; Madsen et al., 2005) attempted at incorporating different antimicrobial products into culture media with some success, although a slight decrease in *F. psychrophilum* viability was sometimes noticed. Before performing isolation, a pre-incubation step of eggs in liquid medium, with or without antibiotics, was also used by both groups (Dalsgaard & Madsen, 2002; Kumagai et al., 2004; Madsen et al., 2005). When using selective procedures or media, it is careful to include standard procedures or media for comparison.

Because *F. psychrophilum* is frequently involved in septicemic infections, control of broodfish may be performed from internal organs (kidney and spleen) as well as from sexual products (eggs, sperm and ovarian fluid), so minimizing the risk of contamination usually associated with skin or mucus sampling. Direct culture was successfully applied to *F. psychrophilum* detection in fish and sexual products by Rangdale et al. (1996), Izumi and Wakabayashi (1997), Ekman et al. (1999), Dalsgaard and Madsen (2002) and Madsen et al. (2005).

Immunodiagnostic methods

Lorenzen and Karas (1992) were the first workers to propose the use of IFAT in diagnostic of RTFS. Although high antibody titres are sometimes difficult to obtain through conventional rabbit immunization, polyclonal antibody proved convenient for IFAT applications. Cross absorption with bacterial cells belonging to different *F. psychrophilum* serotypes even permit to prepare type-specific antisera. Rangdale et al. (1996), Izumi and Wakabayashi (1997) and Amita et al. (2000) used IFAT in experimental or field studies, concurrently with other methods. A more elaborate fluorescent technique, relying on membrane filtration followed with in situ hybridization, was used by Vatsos et al. (2002) for the detection of culturable and non-

culturable forms of the bacterium in water samples, but no attempt was done for adapting the process to fish disease control.

Enzyme-linked immunosorbent assay (ELISA) was tested by Rangdale and Way (1995) and Lorenzen and Olesen (1997, before Mata and Santos (2001) introduced further improvements with the combination of the biotin-avidin system. Use as a detection procedure has been limited, however, by the development of molecular approaches, which up to now seemed to be preferred by most of the authors interested in *F. psychrophilum* detection. Actually, the stake of the relevant studies was generally to demonstrate definitively the presence of the bacterium inside fertilized eggs. This explains why the most sensitive methods were chosen at first, although both IFAT and ELISA should probably be quite adaptable to the control of large numbers of fish.

Molecular methods

First application of PCR to coldwater disease diagnosis was made possible with the description of primers (PSY1 and PSY2) designed by Toyama et al. (1994) from the 16S rDNA gene sequence. Although the original procedure was adopted in some subsequent studies (Vatsos et al., 1999), the agreement on the specificity of PSY1 and PSY2 primers was not complete, and other combinations were tested (Urdaci et al., 1998), with limited success, however. In fact, tissue inhibitors apparently occurred in some biological products and the variability of the flavobacteria 16S rDNA sequence did not appear to be very important, which made difficult to identify highly specific primers and lowered the test sensitivity. This led Izumi and Wakabayashi (2000) to consider another target gene, gyrB, for obtaining more reliable primers.

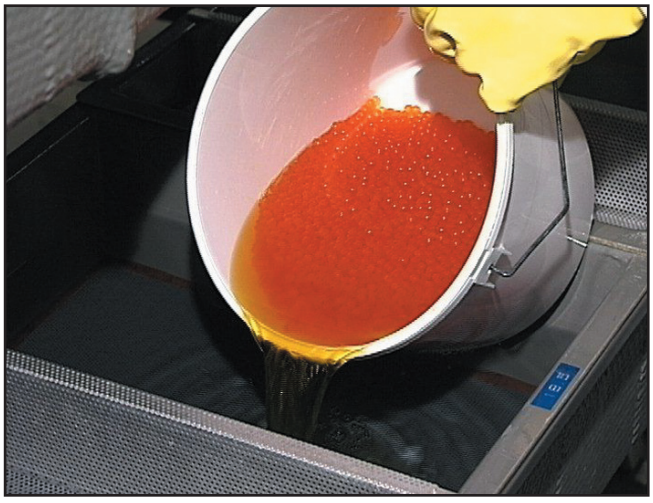
Attempts at improving the specificity and sensitivity of 16S rDNA amplification tests were made by several authors. Although hybridization of amplified products with specific probes was suggested (Urdaci et al., 1998), a majority did address nested PCR. Izumi and Wakabayashi (1997) used universal primers 20F and 1500R (Weisburg et al., 1991) and Toyama's primers PSY1 and PSY2 to adapt a two-step procedure which was tested in field surveys, in comparison with culture and IFAT. Different studies conducted in Japan (Amita et al., 2000), in Europe (Wiklund et al., 2000; Dalsgaard and Madsen, 2002) and in the USA (Taylor and Winton, 2002) with the same system confirmed its usefulness for *F. psychrophilum* detection, although its suitability for egg content screening was more debated. In their work, aiming at optimising nested PCR for several fish pathogens including *F. psychrophilum,* Taylor and Winton (2002) just changed the universal primers used in the first reaction step. The resulting schedule was lately employed to produce new data supporting true vertical transmission of the agent (Taylor, 2004). In the meantime, Baliarda et al. (2002) extended the 1st step amplified sequence to the interspace region (ISR) of the rDNA gene, still adding some degree of specificity.

A different application was explored by del Cerro (2002) with the Taq-Man based PCR method, in which products resulting from DNA cleavage by Taq-polymerase are fluorescent. As the resulting signal is proportional to the quantity of target DNA, quantification of the PCR reaction appears feasible and could prove of great interest in broodstock control. The problem of tissue inhibitors has still to be solved, yet.

Conclusions and research needs

It remains much to learn about *F. psychrophilum,* its prevalence, its behaviour and its ability to survive and evolve in water as well as in host fish, and it is likely that more advanced epidemiological and physiological knowledges would greatly help in the choice and application of diagnostic and surveillance methods. Presently, culture remains the golden standard, but other methods adapted to individual broodfish or large population samples, including IFAT and ELISA, should be developed and validated.

Molecular approaches have been more extensively applied. Detection of the bacteria in sexual products (milt and eggs) may depend on the intensity of the infection, the sensitivity of the technique, and the presence of inhibitors. This means that several aspects should be considered for optimising detection procedures. Other sites of sampling (for instance testicular tissues) could be investigated, the methods of DNA extraction could be adjusted, and above all, comparative studies should be planned. Although several sets of primers and different target genes have been proposed, comparisons performed on similar samples have never been carried out, and very few authors reported parallel results obtained from molecular and non-molecular techniques. Yet, it may be expected that according to the context, quantitative results obtained from ELISA, Taq-Man PCR, and perhaps RT-PCR (if correctly adapted to practical needs) will be of prime importance in establishing a solid scientific basis for *F. psychrophilum* detection and control.



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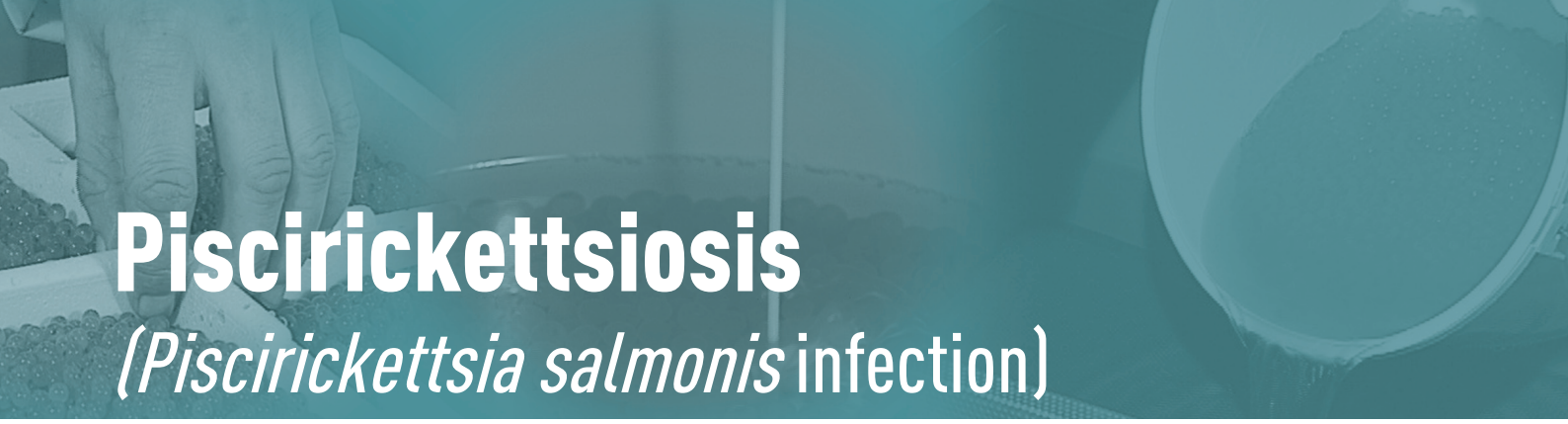

Piscirickettsiosis
(*Piscirickettsia salmonis* infection)

The testing of salmonid broodfish for *Piscirickettsia salmonis* first began in Chile in the late 1990ies, and the predilected tissue used for serological testing by IFAT or ELISA was kidney, following the BKD testing model already being commonly carried out (P. Bustos, pers. comm.). Given the later publications suggesting that *P. salmonis* may transmit vertically by attaching to and penetrating salmonid eggs (Larenas et al. 2003), testing of broodfish populations or of individual broodfish is believed useful in order to exclude egg or sperm batches from populations or individual spawners with detectable infection levels. Since 1999-2000, significant parts of the Chilean salmon farming industry has practiced such broodstock testing. However, most of the Chilean salmon industry have little certainty about the benefits of these procedures. There are serious doubts about the cost-effectiveness of these measures, also because the piscirickettsiosis outbreaks in seawater have remained without evident changes, the marine fish farming area is obviously endemic with the pathogen, and there is an absence of outbreak or clinical cases in freshwater farming. In addition, the Chilean fish health authorities have not included compulsatory measures to prevent vertical transmission of piscirickettsiosis in broodstock, quite unlike what is being required for control of IPNV and BKD (P. Bustos, pers. comm.).

So far, all methods for detection and identification of *Piscirickettsia salmonis* have been developed for use in clinically diseased fish and there are no scientific publications to document their use or performance when applied to broodstock testing or – segregation. Moreover and except for the PCR, there is a nearly complete lack of published information to show the analytical or clinical sensitivity of any of the diagnostic procedures. Consequently, the methods recommended by the OIE (anonymous, 2003) focus on monitoring methods and diagnostic procedures to detect clinically overt disease, or techniques to confirm *P. salmonis* infection. No method is recommended for screening latent carriers of the disease, or for specifically testing broodfish or their sexual products.

For population diagnosis, individual fish showing aberrant swimming behaviour or any moribund or newly dead fish showing gross necropsy signs as described for the disease (white necrotic patches or mottles, or haemorrhages on internal organs; peritonitis; haemorrhages) should be given preference when selecting fish for testing. However, the clinical expression of this disease in broodfish is very scarce in Atlantic salmon and rainbow trout, being more pronounced in coho salmon. Therefore, discharge of visually affected fish is believed useful only in the first weeks of the spawning season (P. Bustos, pers. comm.).

For all methods, kidney, liver and blood are the recommended tissues for testing (anonymous, 2003). The demonstration of *P. salmonis* in ovarian fluid and sperm from infected salmonids has been reported by Larenas et al. (2003) suggesting that these fluids may have a specific potential for future screening of spawners. Recent information suggests that the location of the causative agent inside reproductive tissues could be a very important factor to take into account (P. Bustos, pers. comm.).

Direct demonstration of *P. salmonis* in tissues

Giemsa or acridine-orange (Lannan & Fryer 1991) staining of tissue impressions or smears is easy to perform and enhances the microscopic detection of rickettsia-like organisms inside or outside cells. When testing for population diagnosis, the identity of suspicious organisms should be confirmed by indirect fluorescent antibody technique (IFAT) or nucleic acid amplification as described below.

Isolation and propagation of *P. salmonis* in cell culture

Tissues should be prepared aseptically, if necessary stored between 0-4^{0}C without freezing, inoculated onto CHSE-214 or EPC cell cultures grown without antibiotic supplementation, and maintained at 15-18^{0}C for up to 28 days. Cultures not exhibiting cytopathogenic effects should be passaged once and observed for another 14 days (Lannan & Fryer 1991). Due to the lack of antibiotic supplement in the cell culture media, this method is prone to bacterial contamination and the time necessary to confirm a negative finding is extremely long. The cell culture method is therefore best suited for routine monitoring and screening of presumably *P. salmonis*- negative populations.

Indirect demonstration of antigen in tissues

The detection of *P. salmonis* by use of IFAT on tissue smears, imprints and blood serum was described by Lannan et al. (1991) and Lannan & Fryer (1991), and this method is recommended for confirmatory diagnosis of piscirickettsiosis by the OIE (anonymous, 2003). IFAT has also successfully been employed for demonstrating *P. salmonis* contamination of ovarian fluid and milt (Larenas et al. 2003). Anti-*P. salmonis* antibodies (monoclonal or polyclonal) for IFAT are currently available from several suppliers of diagnostic reagents, and one Chilean supplier offers both IFAT and direct FAT test kits commercially. Chilean laboratory experience suggests increased sensitivity of the IFAT when including imprints from both kidney and liver tissue, as compared to kidney imprints alone (P. Bustos, pers. comm.) An ELISA protocol using some of these antibodies has been reported to yield good agreement with the corresponding IFAT (Aguayo et al. 2002) but further publications on this method are lacking. Immunohistochemistry (IHC) for use on fixed tissue sections have been described by Alday-Sanz et al. (1994). Whereas IFAT or FAT are suitable for testing of spawners, the IHC may be useful in population monitoring but technically less advantageous for testing during the spawning season.

Demonstration of *P. salmonis* genomic material by PCR

The detection of nucleotide sequences from *P. salmonis* by polymerase chain reaction (PCR) was described by Mauel et al (1996), who reported an analytical sensitivity of their single-step PCR corresponding to 60 $TCID_{50}$ per sample. However, only 50% of infected tissue samples were positive by this procedure. Using a nested PCR with non-specific amplification of 16sRNA in the first step, the sensitivity corresponded to one $TCID_{50}$ per analysis, and all the infected tissue samples were positive. This procedure is currently among the OIE recommended methods for diagnosis of piscirickettsiosis (anonymous, 2003). Another PCR protocol for *P. salmonis* based on different primers has been published by Marshall et al.(1998). Heath et al. (2000) described a competitive PCR assay apparently able to amplify between 1-10 *P. salmonis* genome equivalents against a background of > 99.9% salmonid DNA. There is no publication on the application of any of these PCR procedures on fish reproductive tissues. PCR testing of broodfish samples for *P. salmonis* as well as for BKD and IPN are, however, being offered to the Chilean aquaculture industry by commercial diagnostic laboratories (J. Leal, pers. comm.). PCR techniques may have an interesting potential both in population testing and in the testing of individual spawners from infected populations or areas.

Conclusion and research needs

The lack of scientific validation of applicable diagnostic procedures for piscirickettsiosis is dramatic, leaving both authorities and the international aquaculture industry with a lack of factual background for policy development. This situation questions testing and certification requirements imposed upon international trade, and hampers industrial initiatives to implement cost-effective measures to prevent vertical transmission. To remedy this situation we suggest that the following research should have priority:

1) Studies on the (quantitative) abundance of *P. salmonis* infection of various tissues necessary to induce vertical transmission of the infection.

2) Evaluation of the diagnostic characteristics (sensitivity, specificity, repeatability, reproducibility) of rapid methods to detect relevant levels of tissue infection with *P. salmonis*.

3) The comparative sensitivity of various diagnostic methods (cell culture, IFAT, ELISA, PCR) for blood, ovarian fluid and seminal fluids to predict the infection status of individual fish.

4) Clinical sensitivity and predictive value of relevant methods when applied specifically to samples from infected but healthy broodfish populations.

5) Reports from current broodstock testing and –segregation efforts, and studies on the disease control experiences, costs and benefits associated with these activities.

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Conclusion on bacterial diseases

The history of *Renibacterium salmoninarum* research has shown that developing and improving diagnostic methods for the detection of bacteria inside eggs and sexual products was a long-lasting endeavour, that required both a subtle choice between the specificity and sensitivity of the used techniques and minimal knowledge about the agent epidemiology and biological properties. The experience accumulated with BKD can however, without too much faltering and with shorter delay of time, help us achieve suitable methods to the detection of *F. psychrophilum* and *P. salmonis*. As pointed out for each of these agents, critical questions are still to be solved. Yet, the noticeable advances of the last past years in immunological and molecular technologies, together with a facilitated access to marketed reagents and to regularly up-dated information will reinforce the chances of rapidly finding solutions to these problems. Providing the necessary support and manpower will be supplied, significant progress should be quickly expected and will result in well-performing control systems able effectively to prevent vertically transmissible bacterial diseases.

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