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PANDA

Permanent network to strengthen expertise on infectious diseases of aquaculture species and scientific advice to EU policy

Coordination Action, Scientific support to policies

WP4: Report on the current best methods for rapid and accurate detection of the main disease hazards in aquaculture, requirements for improvement, their eventual standardisation and validation, and how to achieve harmonised implementation throughout Europe of the best diagnostic methods

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Cover image: Koi with Koi Herpes Virus Disease: enophthalmia and gill necrosis (M.Engelsma acknowl.)

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Executive summary

Within the PANDA project, the objectives of Work Package 4 (WP4) are to identify the optimal diagnostic methods currently available for the most serious diseases, which were identified by risk analysis (WP2), and to provide recommendations for their standardisation and harmonisation procedures throughout Europe and for any needs to improve their accuracy, rapidity and applicability.

The work package was lead by participant 4, Olga Haenen, who appointed a task force to do the work together. The WP4 task force made tables with current available diagnostic methods, with literature references, and if the test were well established or validated. These tables were put at the panda website to get input from PANDA network members. Additionally, the draft tables were sent to leading experts per pathogen, which were asked for comments. From this latter action many input resulted. During the plenary PANDA workshops in Lelystad (April 2006) and Weymouth (March 2007), the WP4 results were discussed, and plans for making the reports were made. During the whole project time, many lectures were presented to international audiences, and many experts joined the PANDA network.

From the tables and the discussions, this report was made. It was concluded, there are many well established tests for diagnosis of disease and detection of hazardous pathogens of aquaculture species, like defined in the WP2 list. However, many of the diseases or pathogens are not known yet by most laboratories in the EC.

For **fish diseases**, especially for the recently EC-listed aquaculture diseases, acquisition of expertise into the EC, and training in screening and diagnostic techniques on the viruses Epizootic Haematopoietic Necrosis (EHN), Koi Herpes Virus Disease (KHVD), Epizootic Ulcerative Syndrome (EUS) was recommended. The Community Reference Laboratory on Fish Diseases sofar organizes workshops and ring tests for important and current EC listed viruses (Viral Haemorrhagic Septicaemia Virus (VHSV), Infectious Haematopoietic Necrosis Virus (IHNV) and Spring Viraemia of Carp Virus (SVCV)). Extension of the training and ring tests with the fish pathogens EHNV, KHV and EUS is advized, apart from with Infectious Salmon Anaemia Virus (ISAV). For the 3 mentioned fish bacteria, fast and accurate additional tests are needed for confirmation. For the 4 fish parasites, expertise lacks in Europe, to screen for these parasites, and type them. However, as these parasites are not listed yet by EC or OIE, they have a lower priority.

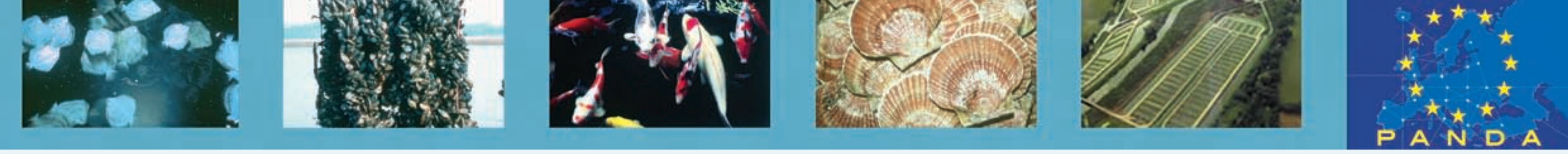
The **mollusc diseases** and pathogens are well covered in expertise and training via the CRL for Mollusc Diseases, which organizes workshops on endemic and exotic important diseases and pathogens for NRL's. Furthermore, they already take the exotic pathogens into account in their ring test.

For **crustacean diagnosis**, appointment of a CRL by the EC is necessary, and training on clinics and diagnosis of Yellowhead disease, White Spot Disease, and Taura syndrome is recommended. The task force furthermore recommended to acquire expertise and testing for the non-WP2 listed Crayfish plague by *Aphanomyces astaci*, as this disease is a threat for crustaceans all over Europe.

The **amphibian diseases**/pathogens RANA virus and *Batrachochytrium dendrobatidis*, a fungus are new to most laboratories. Appointment of a CRL by the EC is necessary, after which certain laboratories should get expertise and skills in testing via training. Many of the internationally available tests are non validated, but for reason of daily use at laboratories well established. However, these tests need validation and ring testing, after they have been implemented into European laboratories.

With the new lists of diseases of the EC and WP2 of PANDA, the tasks to achieve harmonised implementation throughout Europe of the best methods are extended for several responsible bodies: The

European Commission, Community Reference Laboratory, and the National Reference Laboratory with their government will have to put much effort and money, using the PANDA network and world wide experts, to get the expertise into Europe and to the CRL's, NRL's and regional labs. Priorities have to be made in the whole process, and therefore ad hoc expert groups need to be appointed first. In this way, the PANDA network can be further used.



section 1

Introduction

The aquaculture industry is growing, both at European and at world level. More and more globalisation takes place. This includes transport of live aquaculture animals all over the world. These transports carry the risk of transporting aquaculture disease as well, and introducing these diseases into a new region. The EC has good legislation to be able to trade relatively safe, by the current Aquaculture Directive 91/67/EC, and by implementing the EC Directive 2006/88/EC.

Within the PANDA project, work package 2 has identified the most serious aquaculture diseases and hazards, which threaten European aquaculture: exotic, emerging and re-emerging disease hazards of potential risk to Europe, including an assessment of their potential impact on aquaculture and aquatic wildlife in the EU.

The overall objectives of the current work package, WP4 of the PANDA were to identify the optimal diagnostic methods currently available for the most serious diseases, which were identified by risk analysis (WP2), and to provide recommendations for their standardisation and harmonisation procedures throughout Europe and for any needs to improve their accuracy, rapidity and applicability. The work package was lead by participant 4, who appointed a task force to do the work together.

The work approach centred on the following areas:

1.1 Description of work

Task force: A task force of leading European experts in diagnostic methods for aquatic animal diseases (Annex 7.1) was formed by participant 4 in consultation with the Project Steering Group and chaired by participant 4, Olga Haenen. Relevant issues regarding diagnostic aspects of the disease hazards identified in WP2 were identified. A second opinion was asked from selected scientists (Annex 7.2). Members of the developing permanent network were invited to contribute via electronic forum discussions.

Through this network discussion, information was assimilated and collated on: the current status of available diagnostic methods for the diseases identified in WP2 (Table 1.1.a), new developments in methods for disease diagnosis and surveillance, with an assessment of their specificity, sensitivity and speed and their potential applicability to diseases identified in WP2, needs for validation and standardisation of diagnostic methods for the serious aquatic animal diseases, needs for strengthening knowledge and technical skills to achieve harmonised application within the EU for the current best diagnostic methods identified.

Network & Workshops: Workshops of the task force and other experts selected from the network were held to compare and discuss the current diagnostic methods, state of developing new methods, and means to achieve their validation, standardisation and harmonisation. The workshops provided assessment of the state of art and made recommendations for knowledge gap filling and further research and technical skills training needs within the EU and these were passed to WP6 for co-ordination with other training needs identified by WP2 and 3.

Recommendations for guidelines and policy/legislation options with regards to harmonised application of current best practices for rapid diagnosis of the identified diseases in WP2 were developed and finalised under WP7 for submission to the Commission.

Table 1.1.a: PANDA WP2 Disease Hazard List

Animal host group	Disease agent
Fish	Epizootic haematopoietic necrosis virus Red sea bream iridovirus *Streptococcus agalactiae* *Trypanoplasma salmositica* *Ceratomyxa shasta* *Parvicapsula pseudobranchicola* *Neoparamoeba pemaquidensis/perurans* (Amoebic Gill Disease) *Aphanomyces invadans*
Mollusc	*Perkinsus marinus* *Marteilioides* spp. (M. chungmuensis : Marteilioidosis)
Crustacean	Yellowhead Taura Infectious hypodermal and haematopoietic necrosis *Coxiella cheraxi* (crayfish systemic rickettsiosis)
Amphibian	Ranavirus[1]
	Disease agent
Fish	KHV ISAV *Streptococcus iniae* *Lactococcus garvieae* *Gyrodactylus salaris*
Mollusc	*Candidatus Xenohaliotis californiensis* *Nocardia* spp. (Pacific oyster nocardiosis) *Perkinsus olseni/atlanticus*
Crustacean	Whitespot
Amphibian	Ranavirus *Batrachochytrium dendrobatidis* (amphibian chytridiomycosis)

1.2 Deliverables

The work package was planned to have 2 deliverables:

Deliverable 8: Report on the current best methods for rapid and accurate detection of the main disease hazards and requirements for improvements and their eventual standardisation and validation.

This includes newest developments in methods for disease diagnosis and surveillance, and including, if known, their validation status, and their potential applicability to diseases identified in WP2. The needs for validation and standardisation of diagnostic methods for the serious aquatic animal diseases were investigated. During task force discussions it was decided, that 5 other important diseases/pathogens of mollusc or crustaceans should get attention in WP4, apart from the WP2 listed ones, for use of this report by the NRL's a.o. for diagnosis of mollusc and crustacean diseases. These 5 extra diseases/pathogens are treated separately, and are put in an Annexes 7.4 and 7.5.

Finally, the task force summarized training needs within the EU on knowledge gap filling, further research, and technical skills, and these were passed to the WP6 for coordination with other training needs identified by WP2 and 3.

Deliverable 9: Report identifying how to achieve harmonised implementation throughout Europe of the best diagnostic methods for the main disease hazards.

This includes the needs for strengthening knowledge and technical skills to achieve harmonised application within the EU for the current best diagnostic methods identified. Additionally, recommendations for guidelines and policy/legislation options are given, with regards to harmonised application of current best practices for rapid diagnosis. As Directive 2006/88/EC is in place from 2008, this means various new listed diseases/pathogens for aquaculture for Europe. Apart from viruses, bacteria, parasites and fungi are added to the list compared to Directive 91/67/EC. It means more different techniques to be used to cover the diagnosis of these, partly exotic diseases. Inevitably this means an extension of the tasks of the Community Reference Laboratories and National Reference Laboratories in aquaculture diseases. In the last decade, many new member states have accessed the EC. Their tasks will also be extended. Overall, the above facts will result in many training needs as consequence.

In this report both Deliverables are put together.

1.3 Milestones and expected results

M4.1 Completion of the assessment of the scientific literature and unpublished information on current diagnostic methods for the disease hazards identified in WP2. From this, a decision would be made on which are the best methods currently available and these would be described with recommendations made on any research needs to improve their accuracy and rapidity.

M4.2 Completion of the assessment of current status of the validation and standardisation of diagnostic methods for aquatic animal diseases. From this, needs and means for improvement would be identified.

M4.3 Knowledge gaps and skill shortages would be identified. From this, recommendations for training needs would be referred to WP6.

1.4 Structure of the report and how to use it

The data on test methods are grouped for fish (viruses, bacteria, parasites, fungi), followed by mollusc diseases, than the crustacean pathogens, and than the amphibian pathogens. In some of the paragraphs of section 3 some details on the specific tests are given, but for details per test, the specific reference lists are given per disease or pathogen, and those references can be found via the Web of Science, in peer reviewed bulletins. Screening and/or detection methods are kept separate from diagnostic methods, but as expected there is overlap in their lists. Additionally to the WP2 disease/pathogen list, the task force did work on 3 more molluscan and 2 more crustacean important diseases or pathogens, within WP4. The summarized results on these 5 diseases/pathogens can be found in Annexes 7.4 and 7.5. This report identifies the current best methods for rapid and accurate detection of the main disease hazards and requirements for improvements and their eventual standardisation and validation.The gaps and needs identified were translated to recommendations, and those are given section 4.

1.5 General remarks and links with other WPs of PANDA

The WP2 list contains many diseases/pathogens which are exotic to Europe. It means, knowledge on these diseases, and their specific diagnostic techniques are so far often only present at one laboratory or even none within Europe. As a consequence, this WP2 list of hazards, the lists of the new EC Directive, and the list of the Aquatic Animal Health Code of the OIE (2007) are overlapping, Europe starts from scratch with diagnosis of some of these diseases.

The WP4 task force consists of a small group of European multidisciplinary aquatic disease experts, each with their own subjective view on the current plan to achieve harmonisation throughout Europe of the best diagnostic methods for the main disease hazards. This implicates, that views on the harmonisation are subjective and for the present situation. The views may change in time.

The training needs related to WP4 were communicated with WP6. The training needs and recommendations of WP4 can be found both in this report and in the WP6 report of PANDA.





section 2

Materials and methods

2.1 Task force

A task force of leading European experts in diagnostic methods for aquatic animal diseases was formed:

Member	From institution	Country	Task/speciality
Olga Haenen	CIDC-Lelystad, NRL for Fish and Shellfish Diseases, Lelystad	Netherlands	WP4 leader, fish virology, parasitology, fish and amphibian fungi, QA
Inger Dalsgaard	Technical University of Denmark DTU, Danish Institute for Fisheries Research, Copenhagen	Denmark	Fish bacteriology
Niels Olesen	Technical University of Denmark DTU, National Veterinary Institute, CRL for Fish Diseases, Aarhus	Denmark	Fish virology
Jean-Robert Bonami	Pathogens and Immunity,ECOLAG, Université Montpellier	France	Crustacean diseases
Jean-Pierre Joly	IFREMER, CRL for Mollusc Diseases, La Tremblade	France	Mollusc diseases
Isabelle Arzul	IFREMER, CRL for Mollusc Diseases, La Tremblade	France	Mollusc diseases, steering group member

2.2 Network

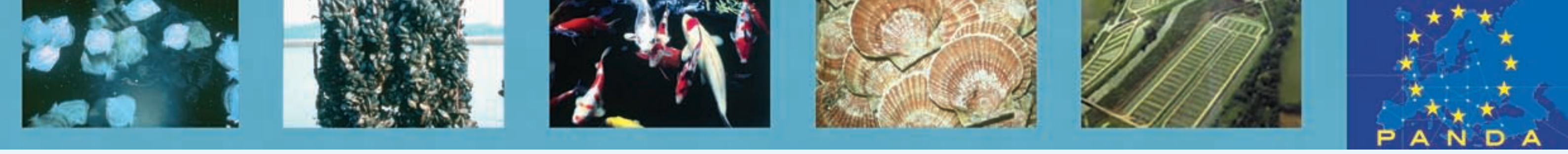
The tables of published screening and diagnostic methods per hazard of the list of WP2 made by the task force were put at the PANDA website during the past 2 years. PANDA members could give comments on the tables via the PANDA forum. In parallel, individual tables were sent to selected external specialists for review (Annex 7.2).

2.3 Workshops and dissemination

PANDA tables were presented and discussed during annual meetings of National Reference Labs for Fish and Shellfish diseases respectively, in 2005 and 2006, in a workshop-like way. At several conferences and courses, PANDA WP4 was presented via oral presentations, abstracts and fliers.

2.4 Analysis of data

After the tables were completed, each task force member wrote parts of the report, and these were collated and intergrated by the WP4 leader (participant 4) to the final deliverables.



2.5 Why harmonization throughout Europe? Background and aim

Related to diagnosis of disease and detection of pathogens in aquaculture, member states should be confident about their test methods: The diagnostic test result of a disease should be the same in one or another member state, so, their tests should have the same Quality Assurance level or validation level. When we take the WP2 list and the lists of 2006/88/EC together, for the exotic diseases, there is expertise on these diseases/pathogens mostly outside Europe, sometimes in the OIE. To be prepared for diagnosis of suspicion of one of these diseases/pathogens, it is necessary to acquire knowledge on their diagnosis in Europe. This means the EC needs to acquire expertise on the exotic diseases, and needs to fund the organization of training on techniques by CRL's for NRL's. This is followed by implementation of tests at NRL level, and their standardization and validation at each individual laboratory, funded by the national government.

2.6 CRL functions

Community Reference Laboratories (CRL) for Fish Diseases (DTU, Århus, Denmark) and Mollusc Diseases (IFREMER, La Tremblade, France) respectively function in educating the National Reference Laboratories (NRL) already for years on the current listed diseases: they organize Annual NRL meetings, and annual or bi-annual ring tests for NRL's. Additionally, the OIE Reference Laboratory for Koi Herpes Virus Disease (CEFAS, Weymouth, UK) organizes ring tests for PCR testing of Koi Herpes Virus.

Related to fish diseases, the education of NRL's by the CRL and CEFAS is specialized to viruses, present in Europe. However, in the new EC Directive 2006/88/EC, a fungus and 2 exotic viruses are added to the lists of fish diseases. Additionally, the mollusc diseases/pathogens list is changed, and various crustacean and amphibian diseases/pathogens are listed for the first time. This means an extension of tasks of all NRL's, and the CRL's for Fish Diseases and Mollusc Diseases, respectively. All labs need to be prepared to diagnose these diseases, or delegate diagnosis to another national laboratory or to the NRL of another member state. According to the EC Directive 2006/88/EC, also a CRL for Crustacean Diseases and a CRL for Amphibian Diseases need to be appointed by the EC.

section 3

Results

3.1 Task force

September 2004, the task force met for the first time, at CIDC-Lelystad, and divided the work. The task force members made tables of published screening and diagnostic methods per hazard of the list of WP2, based on literature searches on the Web of Science a.o., and own experience. The status of, standardis-ation, validation and harmonisation of each test was investigated, as far as possible, and if the tests were best used for screening or for confirmation of disease.

WP4 meetings were held at:

- CIDC-Lelystad, NL: workshop: Sept 2004
- CIDC-Lelystad, NL: workshop: April 2006
- CEFAS, Weymouth, UK: workshop: March 2007

3.2 Network

After draft tables had been made by the task force, individual specialists (Annex 7.2) were invited to assess the tables of published screening and diagnostic methods per hazard of the list of WP2, made by the task force. Their input was included, and put at the PANDA website. The PANDA network could react via the forum, but no input was received via this route.

3.3 Workshops and dissemination

During the NRL meetings on Shellfish and Fish Diseases, the darft tables of published screening and diagnostic methods per hazard of the list of WP2 were discussed, in a workshop-like way. The input was directly integrated into the tables, which were again put at the PANDA website for further possible review. It appeared, that, probably due to the busy agenda of most scientists, only active invitation of review of tables worked out well.

Table 3.3.a: Workshops and dissemination WP4 during the project

Date & venue	Title (lectures unless otherwise mentioned)	Presented at	By
23-24 Sept 2004	WP4 task force start up	CIDC-Lelystad task force meeting	O. Haenen
Jan 2004	Update on WP4: diagnostic methods, standardisation & validation	Barcelona consortium meeting	O. Haenen
July 2004	Update on WP4: diagnostic methods, standardisation & validation	Galway consortium meeting	O. Haenen
Oct 2004	Update on WP4: diagnostic methods, standardisation & validation	Barcelona consortium meeting	O. Haenen
24-25th Feb 2005	Update on WP4: diagnostic methods, standardisation & validation	Paris consortium meeting	O. Haenen

Continued

Table 3.3.a: Workshops and dissemination WP4 during the project (continued)

Date & venue	Title (lectures unless otherwise mentioned)	Presented at	By
15-17 March 2005	Overview of PANDA: aims and objectives of WP4	NRL meeting Shellfish Diseases, La Tremblade, France	CIDC-Lelystad (Olga Haenen) & CRL Shellfish Diseases IFREMER (Jean-Pierre Joly)
May 2005, The Netherlands	Het EU PANDA project (publication)	Published in Aquacultuur, the branche bulletin (in Dutch)	CIDC-Lelystad (Olga Haenen)
May 2005	Update on WP4: diagnostic methods, standardisation & validation	Hydra, Greece consortium meeting	O. Haenen
20-23 June 2005	PANDA : WP4: diagnostic methods, evaluation & validation	NRL meeting Fish Diseases, Aarhus, Denmark	CIDC-Lelystad & CRL DTU, NVI Aarhus (Olga Haenen & Niels Olesen)
Sept 2005	PANDA : WP4 diagnostic methods, evaluation & validation : lecture and flier	EAFP Conference, Copenhagen, Denmark	CIDC-Lelystad (Olga Haenen)
Oct 2005	Update on WP4: diagnostic methods, standardisation & validation	Oslo consortium meeting	O. Haenen
Nov-Dec 2005	Specific WP4 tables per pathogen or host group	Put at the PANDA website, and sent to international specialists for comments	CIDC-Lelystad (Olga Haenen)
22-23 March 2006	Overview of PANDA : aims and objectives of WP4: final discussion on available techniques and gaps	NRL meeting Shellfish Diseases, La Tremblade, France	Jean-Pierre Joly and Isabelle Arzul (Ifremer)
5-8 April 2006	Update on WP4: diagnostic methods, standardisation & validation (and separately discussion in task force)	CIDC-Lelystad PANDA plenary workshop with task forces	O. Haenen
22-24 May 2007	PANDA : Hazards to European fish culture and their diagnosis	Copenhagen, DK	O. Haenen, N.J. Olesen, I. Dalsgaard, I. Arzul
24-25 Nov 2006	Update on WP4: diagnostic methods, standardisation & validation	Copenhagen, DK Consortium meeting	O. Haenen
18-23 March 2007	PANDA : Work package WP4: diagnostic methods, standardisation & validation (and separately discussion in task force)	Weymouth plenary PANDA Workshop	Olga Haenen, Inger Dalsgaard, Jean-Robert Bonami, Jean-Pierre Joly, Niels Olesen, Britt Bang Jensen, Ellen Ariel, Laurence Miossec and Isabelle Arzul
17-23 June 2007	PANDA : Work package WP4: diagnostic methods, standardisation & validationCorfu final	PANDA consortium meeting	Olga Haenen, Inger Dalsgaard, Jean-Robert Bonami, Jean-Pierre Joly, Niels Olesen, Britt Bang Jensen, Ellen Ariel, Laurence Miossec and Isabelle Arzul

3.4 Analysis of data

Current available detection and diagnostic methods per disease/pathogen: The data per pathogen on the current available diagnostic methods are presented as follows: First, the Clinical pathology is given, as this may be a basis for suspicion and diagnosis of the disease/pathogen. Than, Confirmatory techniques for diagnosis are presented. These techniques are used, when the disease is already there, or at least suspected. More sensitive techniques are needed for the next section, i.e. Screening techniques for the pathogen. Subsequently, Comments and recommendations on available techniques are given, and a part on What should we do for diagnosis at suspicion? The disease/pathogen may be notifiable: this is given in: EU-legislation related to techniques, and in OIE recommendations related to techniques (& ref lab OIE). The techniques are critically judged for their use in Assessment. Each part is followed by specific References.

Diseases/pathogens of fish

3.4.1 Epizootic haematopoietic necrosis virus (EHN)

EHN is a serious disease causing significant losses in redfin perch (*Perca fluviatilis*) and moderate-low mortalities in rainbow trout (*Oncorhynchus mykiss*) in Australia.

Clinical pathology

Affected fish may become lethargic and display loss of equilibrium, flared opercula and increase skin pigmentation. Gross signs include anaemia, skin, gill and fin lesions. Enlargement of the spleen and focal necrosis in liver and kidney haematopoietic portion is a common finding, while heart, pancreas, gastrointestinal tract, gill and pseudobranch are less frequently involved.

Agent description

The causal agent of EHN is a double-stranded DNA virus belonging to the *Iridoviridae* family, genus *Ranavirus*, with the type species frog virus 3 (FV3). Virions (150-180nm) show icosahedral morphology, the genome is 150-170kb and the virus replicate in both the nucleus and cytoplasm with cytoplasmic assembly (Chinchar et al., 2005). Ranaviruses have been isolated from healthy or diseased frogs, salamanders, reptiles and fish in America, Europe, Australia and Asia (Langdon et al., 1986; Wolf et al, 1968; Chinchar, 2002; Drury et al., 1995; Fijan et al., 1991; Hyatt et al., 2002; Speare & Smith, 1992; Zupanovic et al., 1998; Ahne et al., 1989; Pozet et al., 1992; Plumb et al., 1996; Grizzle et al., 2002; Chen et al., 1999).

Confirmatory techniques for diagnosis

- Cell culture isolation. Standard procedures according to the OIE manual (OIE, 2006). Several cell lines at 15-22°C.
- E.M. (Electron microscopy): confirm presence of icosahedral virions (150-180 nm in diameter) and virus inclusion bodies
- Serological tests
  - Neutralising antibodies against ranavirus have not been detected in infected animals although they are capable of producing antibodies.
- Antibody-based antigen detection methods such as
  - Immunoperoxidase test of infected cell cultures.
  - Immunoperoxidase test of histological sections
  - Antigen-capture ELISA. A validated test for detection of ranavirus in fish tissues and cell culture is described in the OIE manual.
  - Immunoelectron microscopy – Gold-labelling of sections or cell cultures
- Molecular techniques
  - PCR on cell culture or in fish tissues
  - Restriction Endonuclease Analysis (REA) on cell culture or in fish tissues.

Screening techniques for the pathogen

- Virus isolation of EHNV in cell culture from liver, kidney and spleen tissues is possible in a variety of cell lines from 15-22°C. Validated virus isolation procedures are described in the OIE Diagnostic Manual.
- Antigen-capture ELISA for detection of EHNV in tissues or in cell culture is also validated and published in the OIE Manual.

Comments and recommendations on available techniques

In the OIE Aquatic Diagnostic Manual, the different methods are compared.

For surveillance, the two methods above are recommended. Likewise for detection and confirmation, but in addition the PCR, REA and sequencing methods are listed for confirmatory identification.

For those laboratories that do not have the ELISA implemented for routine surveillance, the cell culture screening followed up with the PCR method would be a practical solution. PCR directly on tissues would be more economical, but is not validated. Primers and procedures are published and most laboratories have experience with and facilities for PCR. The published ELISA method is validated however, and this gives some advantage.

What should we do for diagnosis at suspicion?

According to the OIE Aquatic Manual, the presence of EHNV is suspected if at least one of the following criteria is met:

1) Apparently healthy finfish, which are moribund or dead in which the parenchymal tissues contain histological evidence of focal, multifocal or locally extensive liquefactive or coagulative necrosis with or without intracytoplasmic basophilic inclusion bodies and/or in which EHNV is demonstrated by the following means:

Characteristic cytopathic effect in cell culture and cell culture is positive for EHNV in immunoperoxidase test or antigen-capture ELISA

OR

Tissues positive in antigen-capture ELISA or immunoperoxidase stain or immunoelectron microscopy or PCR

And for both 1 and 2: Sequence consistent with EHNV is demonstrated by PCR-REA or PCR-sequencing.

Liver, spleen and kidney from diseased fish should be processed for virus isolation.

EU-legislation related to techniques

EHN is listed in 2006/88/EC. There is no specification of diagnostic methods in the new legislation yet.

OIE recommendations related to techniques (& ref lab OIE):

EHNV is listed by the OIE. Recommendations are given above, and detailed descriptions of tests can be found in the Diagnostic Manual of the OIE.

OIE designated experts: Alex Hyatt and Richard Whittington.

Assessment

Although surveillance for EHNV is not well established in Europe, the current surveillance scheme for VHSV and IHNV in cell culture appears to be within the recommendations by the OIE experts for EHNV. Hence we have surveyed for EHNV for many years in the EU.

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3.4.2 Red sea bream iridovirus

Red seabream iridovirus disease (RSIVD) is a serious disease firstly observed in Japan causing significant losses mainly in cultured red seabream (*Pagrus major*). Overt infections have been reported from further cultured marine fish including yellowtail (*Seriola quinqueradiata*), Japanese seabass (*Lateolabrax* sp.) and Japanese parrotfish (*Oplegnatus fasciatus*). Heavy losses associated to RSIV and RSIV-like have been reported in Japan and several Asian countries including China, Hong Kong, Korea, Malaysia, Philippines, Taiwan, Thailand, Singapore (OIE, 2006).

Clinical pathology

Clinical pathology, clinical signs: Affected fish become lethargic, exhibit severe anaemia, petechiae of the gill, and enlargement of the spleen. Gross pathology: pale gills and enlarged spleen (Wang et al., 2003).

Agent description

The causal agent of RSIV disease is RSIV, preliminarily included in the *Ranavirus* genus (Hedrick et al., 1992), has been more recently classified into the newly established genus *Megalocytivirus* as proposed by He et al. and considered as a strain of *Infectious spleen and kidney necrosis virus* (ISKNV). The virus has been replicated in a limited number of cell lines: GF and KRE-3 following incubation at 20-25 °C. The virion is 200-240 nm in diameter and is inactivated by chloroform and ether treatment. The fully sequenced genome is about 112 kbp.

Confirmatory techniques for diagnosis

- RSIV cannot be identified by neutralisation tests as the antisera generated by the immunisation of rabbits have few neutralising antibodies.
- IFAT (ISO): *Indirect fluorescent antibody test:* This test is described by, 2004, Nakajima et al., 1995, and Nakajima and Sorimachi, 1995. Samples to be taken: spleen. Specificity and sensitivity: MAb M10 can detect RSIV (Oseko et al., 2004). It does not detect ranaviruses. 'Gold' standard: abnormal enlarged cell with strong fluorescence is confirmed by IFAT. The test is standardized, and validated. Its specificity is high (RSIV and ISKNV (infectious spleen and kidney necrosis virus)) and sensitivity is also high. Tests which use polyclonal antibodies are not standardized and not validated, but these tests have also a high sensitivity.
- IPMA: Immuno Peroxidase Monolayer Assay: Nakajima et al., 1998 described an IPMA, in which Monoclonal Ab RSIV M10 is used. It is not standardized.
- Sequencing: Do et al., 2005 described the sequencing of RSIV. The test is not standardized and not validated.
- PCR (Polymerase Chain Reaction): In the OIE Aquatic Manual (2006), PCR testing is described. Samples to be tested include spleen from affected fish or supernatants from cell cultures that had developed CPE. PCR and use of nested PCR are described by Kurita et al., 1998,, 2004, Wang et al., 2003, Gibson-Kueh et al., 2004, Jeong et al., 2004, Oshima et al., 1996, and Oshima et al., 1998. The tests are not standardized and not validated.
- LAMP (Loop-Mediated Isothermal Amplification): This test is described by, 2004. It is not standardized, and not validated, but much more sensitive as the PCR.
- Histopathology, Microscopic pathology, according to Wang et al., 2003: Tissue smears: confirm presence of abnormally enlarged cells in Giemsa-stained stamp-smear of the spleen. Fixed sections: confirm presence of abnormally enlarged cells in tissues such as spleen, heart or intestine. Examination of histological sections from diseased fish may reveal abnormally enlarged cells from spleen, heart kidney, liver or intestine (OIE, 2006). The test is standardized, but not validated.
- IHC (Immuno Histo Chemistry): In this test Monoclonal Ab RSIV M10 is used. The test is standardized, but not validated (OIE, 2006)

- E.M. (Electron microscopy/cytopathology): confirm presence of virions (200-240 nm in diameter) in the enlarged cells by electron microscopy, different from ranaviruses (Inouye et al., 1992, Wang et al., 2003). This test is not standardized and not validated.

Screening techniques for the pathogen

- Virus isolation of RSIV and ISKNV is undertaken using the GF ATCC GruntFin cell line or GE-, GG-, BF-2 (Blue Gill Fry), or KRE-3 cells (Inouye et al, 1992, Nakajima et al., 1994, 1998) at 22-25°C). Spleen and/or kidney from diseased fish need to be sampled for virus isolation.Virus isolation is standardized, but not validated.
- There are no established detection methods for surveillance, because the carrier state of the agents has not yet been investigated. A tentative method would be virus isolation followed by IFAT. The nested PCR is also suitable for the purpose (Choi et al., 2001).

Comments and recommendations on available techniques

In the OIE Aquatic Manual (2006), the methods are intercompared.

For surveillance, no suitable methods were present: The methods do have application in some situations, but cost, accuracy, or other factors severely limit their application, or the methods are presently not recommended for this purpose.

The OIE (2006) recommends the following methods for **detection of RSIV**:

- Virus isolation and identification by one of the following methods
- Antibody-based assays (IFAT) of isolated virus
- Antibody-based assays (IFAT) of stamp-smear
- Polymerase Chain Reaction (PCR)
- Sequence

The OIE (2006) recommends the following methods for **diagnosis of RSIV disease:**

- Virus isolation and identification by one of the following methods
- Antibody-based assays (IFAT) of isolated virus
- Antibody-based assays (IFAT) of stamp-smear*
- PCR
- Sequence

The above methods are recommended for reasons of availability, utility, and diagnostic specificity and sensitivity.

*) Standard method with good diagnostic sensitivity and specificity. The OIE (2006) state, that, although not all of the tests above have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

The WP4 task force agrees with the recommendations of the OIE, and concludes, that there are currently no good surveillance methods for RSIV disease. However, virus isolation, and subsequently IFAT or PCR with sequencing the viral genome are good methods for presumptive (detection) and confirmatory diagnosis of RSIV.

What should we do for diagnosis at suspicion?

According to the OIE Aquatic Manual (2006), the presence of RSIV-ISKNV shall be suspected if at least one of the following criteria is met:

1) Presence of typical clinical signs and gross pathology and confirmation of abnormally enlarged cells on stamp-smear or tissue section.
2) Presence of typical clinical signs and gross pathology and confirmation of the presence of virions in abnormally enlarged cells by electron microscopy.
3) Virus isolation with specific CPE.

Presence of IFAT positive cells on stamp-smear.

Spleen and/or kidney from diseased fish need to be sampled for virus isolation. Stamp smears are made of spleen and kidney for IFAT. Internal organs are sampled for histopathology, and E.M..

EU-legislation related to techniques

RSIV is not listed in the EU legislation.

OIE recommendations related to techniques (& ref lab OIE):

RSIV is listed by the OIE. Recommendations are given above, and detailed descriptions of tests can be found in the Aquatic Manual (2006) of the OIE.

OIE reference laboratory for RSIV: Fisheries Research Agency, Kanagawa, Japan, Dr. K. Nakajima, E-mail: RSIV-lab@fra.affrc.go.jp

Assessment

Although surveillance for RSIV lacks well established and practically applicable tests, there are well established presumptive and diagnostic tests for RSIV, with, apart from BF-2, specific other cell lines, like GF, GE, GG, and KRE-3 cells. The OIE recommended tests should be used.

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Fig. 1: RSIV infection in red sea bream. (M.Sano, J. Kurita, T.Ito acknowl.).

3.4.3 Infectious Salmon Anaemia Virus

Infectious salmon anaemia (ISA) is a systemic viral infection of reared Atlantic salmon *(Salmo salar)* mainly in the marine environment, and has been reported from Norway, Canada (New Brunswick and Nova Scotia), Chile, the Faeroe Islands and USA (Maine), Faeroes islands, Atlantic coast of Canada, USA. In addition ISA virus has been reported in Chile, from Pacific Coho salmon (*Oncorhynchus kisutch*) and in Ireland in clinically healthy rainbow trout

Clinical pathology

Infectious salmon anaemia (ISA) is a disease of farmed Atlantic salmon (*Salmo salar*) (Thorud et.al. 1988) caused by infectious salmon anaemia virus (ISAV) (Falk et al.1997, Krossoy et al.1999, Mjaaland et al 1997). ISA primarily affects fish held in seawater or fish exposed to seawater. However, indications of disease outbreaks in fish held in fresh water have also been reported (Nylund et al. 1998). The disease may appear as a systemic and lethal condition characterised by severe anaemia and haemorrhages in several organs.

Agent description

ISA virus is a pleiomorphic enveloped ssRNA virus with properties consistent with those of *Orthomyx-oviridae.* ISA virus is now classified in the genus *ISAvirus* as the type species (Fauquet et al., 2005). The virus has a single stranded RNA genome and it has surface projections associated with haemagglutination receptor-destroying and fusion activity.

The ISA virus has been divided into two major clusters; the North American and the European (Devold & al., 2001) and analysis of the genomic segment 5 has supported this (Devold& al., 2006). The European cluster has further been sub-divided into three groups (EU-G1-G3) (Nylund et al., 2007). Some ISA virus isolates from North America are often referred to as "European-in-North America" as they are European-like. (Nylund & al., 2007).

Confirmatory techniques for diagnosis

The diagnosis of ISA (as a disease) was initially based on clinical signs, macro-pathological findings and histopathological evaluation of formalin-fixed paraffin-embedded tissue sections. Following the isolation of the causative agent, a number of direct methods for detection of virus and confirmation of the diagnosis have been established. These are isolation of the virus in cell culture followed by immunological identification, immunological demonstration of ISA virus antigen in tissues and PCR techniques.

Cell culture isolation of ISA virus

Diagnostic cell culture isolation of ISA virus from infected fish is usually performed using either SHK-1, TO and/or ASK-II cell lines. Recent experiences indicate that ASK-II cells should be the first choice for primary isolation (Rolland et al., 2005). ISA virus in cell culture is usually identified by an IFAT test using anti ISA virus MAbs or by PCR. A presumed low or non-virulent strain of ISA virus (HPR0) has so far proven non-cultivable. Also several clinical cases of ISA did not result in the development of CPE, and test sensitivity/specificity for cultivation is judged to be relatively low

Demonstration of ISA virus antigens

Immunohistochemistry techniques using anti-ISA virus antibodies on formalin-fixed paraffin-embedded tissue sections, tissue cryosections and tissue imprints are currently the first choice for detection of ISA virus in diseased fish. The method has a major advantage of being able to associate virus detection with known target cells and pathological lesions. The methods are rapid, relatively cheap, robust and suitable for detection of ISA virus in fish with clinical ISA. Detection of ISA virus in sub-clinically infected fish is less reliable due to restricted sensitivity.

PCR and real time PCR

RT-PCR is the method of choice for detection of ISA virus especially in sub-clinically infected fish (i.e. in ISA virus infected fish showing no signs of disease). The method is rapid, with presumed high specificity and sensitivity.

From a literature search it was obvious that only few tests for monitoring and confirmation have been validated and neither the diagnostic- or test-sensitivities or specificities are known.

Screening techniques for the pathogen

No validated laboratory methods are available for screening of populations in order to document freedom of ISA. Screening by RT-PCR and especially real time PCR has been used for re-establishing disease free status after a disease outbreak or to confirm or rule out suspicion of disease. Passive surveillance by regular clinical inspections has successfully been in force in most of Europe in order to document freedom of ISA. During a voluntary laboratory screening for ISA in Ireland by PCR suspicion of the presence of ISAV was made in clinically healthy rainbow trout. The veterinary significance of the finding remains unresolved.

A number of direct methods for detection of virus and confirmation of the diagnosis following pathology have been established. These include isolation of the virus in cell culture followed by immunological identification (Dannevig et al., 1995; Falk et.al., 1998), immunological demonstration of ISAV antigen in tissues (Falk et.al. 1998) and PCR techniques (Devold et.al., 2000; Mjaaland et al., 1997).

Comments and recommendations on available techniques

For monitoring recently developed quantitative PCR and RT-PCR seem to be the method of choice. One of the problems with PCR is that it gives no clue to understanding the biological significance of the findings, e.g. do the finding of a non-cultivatable HPRO virus have any significance for a putative outbreak of clinical ISA? As long as this question is unresolved the screening of large numbers of fish for documentation of freedom for ISA might be useless. The sensitivity of the immunochemical techniques and the cultivation seem to be rather low and therefore less suited for monitoring disease freedom.

The disease however was even before the pathogen was known effectively controlled in Norway only by clinical and pathological examinations, therefore in the EU a method for disease monitoring was based on regular clinical inspections and not on laboratory testing for the presence of virus like it is the case for viral haemorrhagic septicaemia and infectious haematopoietic necrosis, two other viral fish diseases with high impact.

Therefore it might also in future be recommended that maintenance of disease freedom should be done by careful clinical inspections combined with laboratory examinations in case of suspicion of ISA.

See also Annex 7.3 for literature on current available techniques.

What should we do for diagnosis at suspicion?

Rapid sampling of kidney tissue imprint for IFAT and or collection of tissue in formalin for immunohistochemistry

1. Cross pathology and haematocrite determination
2. Virus isolation in cell culture: collect spleen, heart and kidney and inoculate onto ASK or SHK-1 cell lines. Presence of virus can be detected by haemadsorption test using salmonid erythrocytes, while identification in case of CPE or positive haemadsorption is done by IFAT, ELISA, or PCR.

EU-legislation related to techniques

ISA is regarded as an exotic List 1 disease in the European Community (Council Directive 91/67/EC, Annex A). In the new Aquaculture Directive 2006/88/EC, ISA has become an non-exotic disease in the Community despite the fact that the disease has not appeared for more than 4 years in EU, and is only prevalent in Norway and recently in the Faeroe Islands.

OIE recommendations related to techniques (& ref lab OIE)

ISA is listed by the OIE (Manual of Diagnostic Tests for Aquatic animals, 2006). According to the OIE Manual, 2006, the following methods are suitable for surveillance (for fish without clinical signs) and/or diagnosis (of diseased fish):

- Pathology (macroscopic and histology): only for presumptive diagnosis
- IFAT on kidney imprints: only for confirmatory diagnosis
- Immunohistochemistry: only for confirmatory diagnosis
- RT-PCR (with sequencing for confirmation/ characterisation): Suitable for surveillance (not confirmatory for infectious virus), and suitable for confirmatory diagnosis together with other tests positive for ISA
- Cell culture: suitable for surveillance and for confirmatory diagnosis

OIE-reference laboratory for ISA: The appointed OIE reference laboratory for ISA is the National Veterinary Institute, Oslo, Norway while the Community Reference Laboratory for fish diseases at the National

Veterinary Institute in Aarhus, Denmark serves as the coordinating partner.

Assessment

Till now it is most likely that control and surveillance of ISA is achieved the best way by passive surveillance with inspections at regular intervals. In case of suspicion the pathogen is detected by PCR, IFAT, IHC, or ELISA. Relatively many tests are validated, even according to ISO 17025.

In future, if the biological significance of PCR findings have been established, or after the development of e.g. a combined Real Time gene array including the simultaneous detection of viral genes, viral peptides and fish antibodies against the virus, a monitoring programme based on laboratory testing of clinical healthy fish might be adopted.

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Most of the information of this section was extracted from the ISA chapter in the OIE Manual of Diagnostic Test for Aquatic Animals, 2006, and from the draft Scientific Report regarding a request from the European Commission concerning to EFSA for a scientific opinion on possible vector species and live stages of susceptible species not transmitting disease as regards certain fish diseases.

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Fig. 2: ISA infected Atlantic salmon, Faroe Islands 2002: dark liver, haemorrhages and anemia (N.J. Olesen acknowl.)

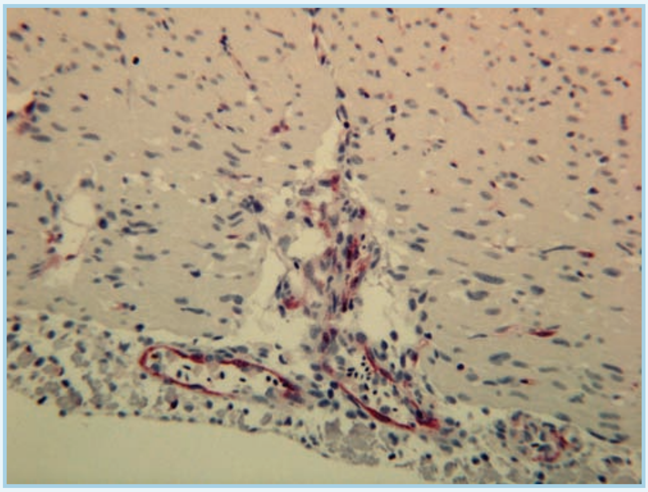


Fig. 3: ISA-IHC (immunohistochemistry) staining of endothelial cells in the heart from ISA infected Atlantic salmon (N.J. Olesen acknowl.)

3.4.4 Koi Herpes Virus

KHV disease is a acute and lethal infection of carp and koi (*Cyprinus carpio*), caused by the cyprinid herpesvirus 3 (CyHV-3), and has been found in at least 26 countries over the globe: Austria, Belgium, China, Czech Republic, Denmark, France, Germany, Hong Kong, Indonesia, Ireland, Israel, Italy, Japan, Korea, Luxembourg, Malaysia, Netherlands, Poland, S-Africa, Singapore, Switzerland, Sweden, Taiwan, Thailand UK, and USA.

Clinical pathology

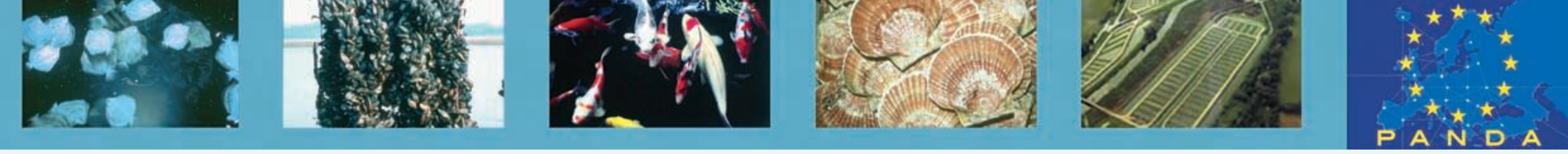
Clinical signs of KHVD include lethargy, fatigue, disorientation, erratic swimming and frequent ventilation (gasping). Fish can die within hours of the first signs appearing, but at lower temperatures the course of the disease is more protracted (Walster, 1999). Most often irregular discolouration of the gills is consistent with often severe gill necrosis. Furthermore, anorexia, enophthalmia (sunken eyes), fin erosion, superficial haemorrhaging at the base of the fins, pale, irregular patches on the skin associated with excess mucus secretion and also decreased production of mucus in patches, leaving the epidermis with a sandpaper-like texture, tumble swimming, and mortality are reported (Bretzinger et al., 1999; Haenen et al., 2004; Hoffmann et al., 2004; Antychowicz et al., 2005). The test is not standardized and not validated.

Agent description

The causal agent belongs to the *Herpesviridae* family and has been preliminary identified as cyprinid herpesvirus 3 (CyHV-3). The nucleocapsid size calculated on thin virion sections ranges between 110 and 120 nm. The reported whole genome varies between 150 kbp to 295 kbp. The virus may be replicated *in vitro* only in selected cells (KF, CCB) and with some difficulties.

Confirmatory techniques for diagnosis

- IFAT after cpe in cell culture: In these tests, rabbit antibodies against KHV are used: Hedrick et al., 2000; Pikarsky et al., 2004; Dishon et al., 2005.
- IFAT on kidney touch imprints with rabbit–anti-CNGV are described by Pikarsky et al., 2004; Perelberg et al., 2005; and Shapira et al., 2005.
- ELISA (antigen): In these tests, rabbit antibodies against KHV are used, described by: Ronen et al., 2003; Pikarsky et al., 2004; and Dishon et al., 2005.
- Dot blot assay: has been described by Gray et al., 2002.
- SDS page: has been described by Gilad et al., 2003.
- PCR & RT-PCR: Various PCR techniques have been described by Gilad et al., 2002b; Gray et al., 2002; Bercovier et al., 2005; Way et al., 2004a,b;



Hoffmann et al., 2004; Dixon et al., 2004; Pikarsky et al., 2004; Gilad et al., 2004; Antychowicz et al., 2005; Dishon et al., 2005; Ishioka et al., 2005; Yuasa et al., 2005; The tests are more or less standardized, and mostly not validated, although rings tests have been organized by the OIE reference laboratory (Dr. K.Way et al., from CEFAS, UK), which enables validation according to ISO 17025.

- Sequencing: This was done by Way et al., 2004a; Antychowicz et al., 2005; Ishioka et al., 2005; Waltzek et al., 2005; Aoki et al., 2007. Sequencing is not standardized and not validated.
- KHV gene cloning: Used as a research tool by Bercovier et al., 2005
- Analysis of RFLP (Restriction Fragment Length Polymorphism): Described by Gilad et al., 2003.
- LAMP (Loop Mediated Isothermal Amplification): This test has been described by Gunimaladevi et al., 2004;, 2005; and Yoshino et al, 2006.
- Histopathology: Ariav et al., 1999; Hedrick et al., 2000; Hoffmann et al., 2004; Pikarsky et al., 2004; Sano et al., 2004; Tu et al., 2004
- Immunohistochemistry: Research tool: Pikarsky et al., 2004;
- In Situ Hybridization: has been described by Le Deuff, et al., 2001; and Way et al., 2004a.
- Electron Microscopy (E.M.) and Transmission E.M. have been described by Ariav et al., 1999; Bretzinger et al., 1999; Hedrick et al., 2000; Neukirch & Kunz, 2001; Hoffmann et al., 2004; and Hutoran et al., 2005.

Screening techniques for the pathogen

- Virus isolation: on Cell lines: Koi fin KF-1, Common Carp Brain CCB, CFC, Koi Fin cell KFC at temp. 22-26ºC have been described by Hasegawa et al., 1997; Neukirch et al., 1999; Hedrick et al.,2000.; Way et al., 2001; Neukirch & Kunz, 2001; Gilad et al., 2002; Ronen et al., 2003; Neukirch & Steinhagen, 2003; Pikarsky et al., 2004; Sano et al., 2004; Engelsma & Haenen, 2005; Antychowicz et al., 2005. The test is not standardized, but the OIE (2007, in press) recommends a method. The test is very low sensitive compared to PCR methods.
- IFAT on kidney touch imprints with rabbit–anti-CNGV are described by Pikarsky et al., 2004; Perelberg et al., 2005; and Shapira et al., 2005.
- ELISA (antibody): Antibody testing against KHV in sera from *Cyprinus carpio* is described by Gilad et al., 2002a; Ronen et al., 2003; Adkison et al., 2005; and Memel et al., 2006.
- PCR & RT-PCR: Various PCR techniques have been described by Gilad et al., 2002b; Gray et al., 2002; Bercovier et al., 2005; Way et al., 2004a,b; Hoffmann et al., 2004; Dixon et al., 2004; Pikarsky et al., 2004; Gilad et al., 2004; Antychowicz et al., 2005; Dishon et al., 2005; Ishioka et al., 2005; Yuasa et al., 2005; The tests are more or less standardized, and mostly not validated, although rings tests have been organizeed by the OIE reference laboratory (Dr. K.Way et al., from CEFAS, UK), which enables validation according to ISO 17025.
- LAMP (Loop Mediated Isothermal Amplification): This test has been described by Gunimaladevi et al., 2004;, 2005; and Yoshino et al, 2006.

Comments and recommendations on available techniques

In the past few years, very many techniques have been developed for detection of KHV or CNGV, and diagnosis of KHV disease. Because most tests are not validated yet, it was advized by Haenen et al., 2004 to use at least 2 tests in parallel to diagnose KHV disease. In practice, at many labs it appeared, that virus isolation was not very sensitive (personal comm.). Therefore, more and more, sensitive and specific PCR methods were advized to use (OIE, 2007 in press). Today, PCR methods (Gilad et al., 2002b; or Gray et al., 2002) are used in many countries, and a yearly ring test is running since 2006, organized by one of the OIE reference laboratories, CEFAS at Weymouth. Some laboratories use a TaqMan PCR, which is even more sensitive than the regular PCR (Gilad et al., 2004). Because the communication around KHV is well organized, as it is a newly notifiable disease for both the OIE and the EU, labs lacking the test methods can get easy in touch with laboratories which do already use well established KHV tests.

What should we do for diagnosis at suspicion?

The OIE (2007, in press) recommends the following:

Definition of suspect case: A suspect case of KHVD is defined as the presence of typical clinical signs of the disease in a population of susceptible fish OR presentation of typical histopathology in tissue sections OR typical CPE in cell cultures without identification of the causative agent OR a single positive result from one of the diagnostic assays described above.

Definition of confirmed case: A confirmed case is defined as a suspect case with subsequent identification of the causative agent by one of the serological or molecular assays described above OR a second positive result from a separate and different diagnostic assay described above.

Fish material suitable for virological examination is: Asymptomatic fish (apparently healthy fish): Gill, kidney, spleen, and encephalon (any size fish). Clinically affected fish: Gill, kidney, spleen, gut and encephalon (any size fish).

EU-legislation related to techniques

KHV is listed in the list of non-exotic notifiable diseases of aquaculture animals in the new EC Aquaculture Directive 2006/88/EC. No special tests are recommended sofar, but the EU mostly follows the recommendations of diagnostic methods by the OIE (see below).

OIE recommendations related to techniques (& ref labs OIE)

KHV disease is listed by the OIE.

The OIE (Manual of Diagnostic Tests for Aquatic animals 2007, in press) recommends the following tests: First the tests are rated against purpose of use: The methods currently available for surveillance, detection and diagnosis of KHVD are listed below. The designations used in the overview indicate:

A = the method is **currently the recommended method** for reasons of availability, utility and diagnostic sensitivity and specificity;

B = the method is a standard method with good diagnostic sensitivity and specificity;

C = the method has application in some situations, but cost, accuracy or other factors severely limits its application;

D = the method is currently not recommended for this purpose.

Although not all of the tests listed as category A or B have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results makes them acceptable.

The OIE (2007) recommends for:

- **Surveillance to declare freedom from infection**:
  - PCR of tissue extracts* (C)
  - Detection of KHV antibodies in exposed fish (ELISA)** (C)
- **Presumptive diagnosis of infection or disease (detection):**
  - Gross signs (B)
  - Histopathology of tissues and organs (B)
  - Isolation of in cell culture (C)
  - Antibody-based assays to detect KHV antigen (IFAT, ELISA) (B)
  - Transmission EM of tissues (B)
  - PCR of tissue extracts* (A)
  - PCR – sequence analysis (C)
  - Detection of KHV antibodies in exposed fish (ELISA)** (C)
- **Confirmatory diagnosis of infection or disease (diagnosis)**:
  - Histopathology of tissues and organs (C)
  - Antibody-based assays to detect KHV antigen (IFAT, ELISA) (C)
  - Transmission EM of tissues (C)
  - PCR of tissue extracts* (A)
  - PCR – sequence analysis (A)

IFAT = Indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay; EM = electron microscopy; PCR = polymerase chain reaction.

**Diagnostic virologists should be aware that fish recently vaccinated against KHV may test positive by PCR. No information is currently available to indicate any genome sequence differences between the attenuated vaccine strain and wild-type (w.t.) KHV. Until this sequence information is provided, diagnostic laboratories will not be able to distinguish between w.t. and vaccine strain of KHV and this could lead to a false diagnosis.*

***Diagnostic virologists should be aware that fish recently vaccinated against KHV may test positive by ELISA. There may also be a low-level cross reaction with antibodies to CyHV-1.*

NOTE: Many diagnostic laboratories may encounter difficulties in obtaining antibodies against KHV that are suitable for use in immunodiagnostic tests. However, a limited number of monoclonal and polyclonal antibodies may be very soon available from commercial sources. It is quite likely that diagnostic kits will also soon be available from the same sources.

Reference Laboratories of the OIE for KHV:

- CEFAS, Weymouth, UK, Dr. K.Way, E-mail: K.Way@cefas.co.uk, and

- National Research Station of Aquaculture, Mie, Japan: Dr. M.Sano, E-mail: sanogen@fra.affrc.go.jp

Assessment

There have been many methods developed to detect KHV/CNGV/CyHV-3, and many diagnostic methods for KHV disease. Most of them are more or less standardized, and some are validated, based on ring trials of PCR testing, organized by CEFAS. For laboratories starting their diagnosis of KHV it is recommended, to use at least 2 test methods, to validate their tests. Virus isolation is of too low sensitivity to use for screening. PCR testing is much more fast and sensitive, with a high specificity. Detailed information on recommended tests can be found at www.oie.int in the Manual of Aquatic Animal Diseases (2007).

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Fig. 4: koi with Koi Herpes Virus Disease: enophthalmia and gill necrosis (M.Engelsma acknowl.)

3.4.5 *Streptococcus agalactiae*

(junior synonym: *Streptococcus difficile*) (warm-water streptococcosis)

Streptococcosis, caused by *Streptococcus agalactiae*, is an important bacterial disease of different fish species, like Tilapia, and can result in serious economic losses. It is at least reported from Israel, Kuwait, and the USA. The host range of *Streptococcus agalactiae* is not limited to aquatic species, but has also been isolated from warm-blooded terrestrial animals suggesting that this bacterium might be a zoonotic problem.

Clinical pathology

Streptococcus agalactiae is responsible for septicemia and mengoencephalitis in different fish species. Clinical signs vary among species of affected fish. However, the most common symptoms are high mortality, abnormal swimming behavior, C-shaped body, exophthalmia, multiple ocular lesions, haemorrhages on the body surface, enlarged liver, congestion in kidney and spleen and ascites (Duremdez et al., 2004; Eldar et al., 1994; Evans et al., 2002; Glibert et al., 2002).

Agent description

Streptococcus agalactiae (junior synonym: *Streptococcus difficile*) is a Gram-positive coccus which forms short chains. The bacterium grows with small grey 1mm colonies after 24 h and is beta-haemolytic or non-haemolytic on blood agar (Duremdez et al., 2004).

Streptococcus agalactiae is a Lancefield group B *Streptococcus.* The strain *Streptococcus difficile* is now identified as *S. agalactiae* group B, capsular type Ib (Vandamme et al., 1997).

Confirmatory techniques for diagnosis

The PCR protocols previously described in the section "screening techniques" might also be used as confirmatory techniques for identification of *Streptococcus agalactiae*, however, the PCR assay need to be validated.

Sequencing is recommended as one of the final steps for confirmatory diagnosis. Genetic similarity between *S. agalactiae* and *S. difficilis* by analysis of the 16S-23S intergenic rRNA gene sequence (371 bp) (GenBank AF064441) was reported by Berridge et al. (2001). Kawamura et al. (2005) described that *S. agalactiae* and *S. difficilis* showed very high sequence similarity in five gene sequences (The GenBank accession number can be found in the reference). Obtained sequences should be compared with available ones in GenBank.

Screening techniques for the pathogen

Tissue samples are taken from diseased fish e.g. from kidney, spleen, ascetic fluid, brain and blood. The pathogen is easily grown on different agar media: e.g. brain–heart infusion agar (BHIA), Columbia agar with 5% sheep or cattle blood, bloodagar, trypticase-soy agar (TSA) + 2% NaCl, incubated at 25°C to 37°C for 24-48 h (some incubate in air with 5% CO_2) (Duremdez et al., 2004; Eldar et al., 1994; Evans et al., 2002;Vandamme et al., 1997).

The biochemical characteristics for identification are described by following authors (Duremdez et al., 2004; Eldar et al., 1994; Vandamme et al., 1997). Routine tests for biochemical properties were done as described in different manuals of methods for general bacteriology. The bacterium shows no growth at 10°C and 45°C, in 6.5% NaCl and at pH 9.6, but grow in 40% bile.

Biochemical typing has been done by the following commercial systems: API 20 Strep, API 50 CH, Rapid ID 32 Strep, at 24 °C to 28°C (Eldar et al., 1994; Evans et al., 2002; Glibert et al., 2002; Vandamme et al., 1997).

The reference strains ATCC 13813 (non-haemolytic *S. agalactiae*) / ATCC 27956 (beta-haemolytic

S. agalactiae) and ATCC 51487 (*S. difficile*) might be included for comparative purposes. DNA-DNA hybridization of *S. agalactiae* and *S. difficile* showed relatedness of more than 75.4% (Kawamura et al., 2005).

Serological tests used for characterisation: Lancefield´s grouping of group specific carbohydrate antigen, Streptococcal grouping kit and Slidex streptokit latex B kit. *Streptococcus agalactiae* belongs to Lancefield group B (Evans et al., 2002; Vandamme et al., 1997).

Histology allows observing abnormalities but not specific to streptococcal infection. Bullminnows have been experimentally infected with a non-haemolytic group B *Streptococcus* sp. The infected fisk showed a systemic infection in the eye, liver and spleen (Rasheed et al., 1985).

Identification might be confirmed by PCR assay based on specific primers deduced from the 16S rRNA gene. These primers produced a 375-bp amplicon (Berridge et al., 2001). A specific DNA fragment (length 220 bp) was amplified using primers F1 and IMOD (Duremdez et al., 2004).

The specificity of *Streptococcus agalactiae* PCR assay was demonstrated by the fact that no specific band was amplied when related *Streptococcus* spp. or commonly encountered aquatic bacterial pathogens were examined. Limitations in primer specificity validation due to examination of a relatively small number of bacteria species (Berridge et al., 2001). PCR sensitity has not been evaluated.

A multiplex PCR-based method was designed for detection of the main pathogens involved in warm-water streptococcosis. The sensitivity of the multiplex PCR using purified DNA was 12.5 pg for *S. difficilis* (Mata et al., 2004).

Comments and recommendations on available techniques

Bacteriological culture and biochemical identification of the causal agent remain the ultimate confirmation of the disease.

Protocols for PCR are available in pre cited articles. However the techniques need to be validated and more specifically specificity and sensitivity values are needed.

What should we do for diagnosis at suspicion?

Tissue samples should be taken from diseased fish e.g. from kidney, spleen, ascetic fluid, brain and blood, and cultured and typed like described above.

EU-legislation related to techniques

Streptococcus agalactiae (junior synonym: *Streptococcus difficile*),warm-water streptococcosis is not listed by the EU. Therefore no details are given by the EU.

OIE recommendations related to techniques

Streptococcus agalactiae (junior synonym: *Streptococcus difficile*),warm-water streptococcosis is not listed by the OIE. No details are given by the OIE on techniques.

Assessment

It is advised to culture the bacterium as described above, and at least use biochemical techniques to type the bacterium further. PCR techniques need to be validated and more specifically specificity and sensitivity values are needed. Sequencing is recommended as one of the final steps for confirmatory diagnosis.

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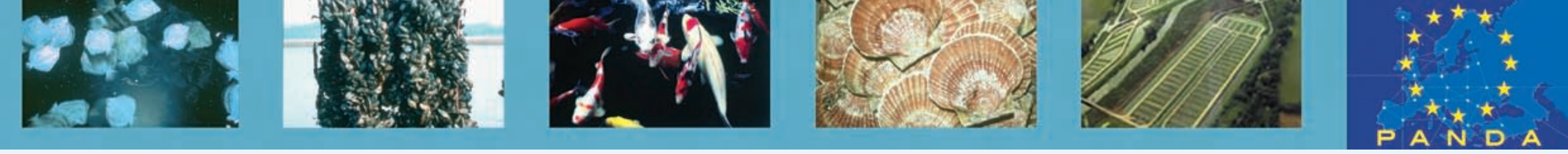

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3.4.6 *Streptococcus iniae*

(junior synonym: *Streptococcus shiloi*) (warm-water streptococcosis)

Streptococcosis, caused by *Streptococcus iniae*, can affect various freshwater and marine fish species, from both cultured and wild fish populations. It has been reported from Australia, China, Europe (Italy, Spain) Israel and the USA. The host range of *Streptococcus iniae* is not limited to aquatic species, but the bacterium has also been isolated from humans suggesting that this bacterium might be a zoonotic problem.

Clinical pathology

Streptococcus iniae is responsible for septicemia and mengoencephalitis in different fish species. Clinical signs vary among species of affected fish. However, the most common symptoms are high mortality up to 70%, exophthalmia, corneal opacity, dark pigmentation and ascites (Bachrach et al., 2001; Bromage et al., 1999; Colorni et al., 2002; Eldar et al., 1994).

Agent description

Streptococcus iniae (junior synonym: *Streptococcus shiloi*) is a Gram-positive coccus, which forms short chains. The bacterium grows with small white 1mm colonies after 48 h and is beta-haemolytic on blood agar (sheep) and partial haemolysis when the medium was supplemented with human or bovine blood (Bromage et al., 1999; Eldar et al., 1995).

Fig. 5: Nile tilapia with *Streptococcus iniae/ S. agalactiae* disease signs (Joyce Evans acknowl.).

Confirmatory techniques for diagnosis

The different PCR protocols previously described in the section "screening techniques" can also be used as confirmatory techniques for identification of *S. iniae.*

Sequencing is recommended as one of the final steps for confirmatory diagnostic. The rDNA sequence analyses from different isolates of *S. iniae* was determined and deposited in the GenBank database (accession no. AF335573 and no. AF335572) (Bachrach et al., 2001; Colorni et al., 2002). Obtained sequences should be compared with available ones in GenBank.

Screening techniques for the pathogen

Tissue samples are taken from diseased fish e.g. from kidney, spleen, brain and blood. The pathogen is easily grown on different agar media: e.g. brain–heart infusion agar (BHIA), Columbia agar with 5% sheep or cattle blood, bloodagar, incubated at 24°C to 30°C for 24-48 h (Bachrach et al., 2001; Barnes et al., 2003; Bromage et al., 1999; Eldar et al., 1994; Eldar et al., 1995; Eldar et al., 1999).

The biochemical characteristics for identification are described by following authors (Bromage et al., 1999; Eldar et al., 1994; Eldar et al., 1995; Shoemaker et al., 2001). Routine tests for biochemical properties were done as described in different manuals of methods for general bacteriology. The bacterium shows no growth at 10°C and 45°C, and no growth in 6.5% NaCl and in 40% bile, and growth at pH 9.6 (Eldar et al., 1994).

Biochemical typing has been done by the following commercial systems: API 20 Strep (profile 4562117) and API 50 CH. Incubation temperatures (between 24°C and 37°C) have been used (Bachrach et al., 2001; Barnes et al., 2003; Bromage et al. 1999; Colorni et al., 2002; Eldar et al., 1994; Eldar et al., 1995; Eldar et al., 1999).

Klesius et al. (2006) have developed a rapid and non-letal technique to detect and identify *S. iniae* using a monoclonal antibody-based indirect fluorescent antibody technique.

The reference strains ATCC 29178 (*S. iniae*) and ATCC 51499 (*S. shiloi*) might be included for comparative purposes. DNA-DNA hybridization of *S. iniae* and *S. shiloi* showed relatedness of 77% to 100% (Eldar et al., 1994; Eldar et al., 1995; Eldar et al., 1999).

Serological tests used for characterisation: Lancefield´s grouping of group specific carbohydrate antigen. Streptex system, A –F grouping (Murex Diagnostics) show no reaction (Bromage et al., 1999; Eldar et al., 1994). Serological differences have been described based on capsular antigens (Bachrach et al., 2001; Barnes et al., 2003; Kanai et al., 2006).

Histology allows observing abnormalities but not specific to streptococcal infection. Histopathology might show differences between diseases caused by *S. iniae* and *L. garviae* (Eldar & Ghittino, 1999). Main lesions found are acute suppurative meningitis and panophthalmitis. Systemic disease with multiple necrotic foci (Bromage & Owens, 2002; Colorni et al., 2002; Eldar et al., 1999; Lahav et al., 2004).

For epidemiological studies have been used restriction fragment length polymorphism (RFLP) ribotyping. DNA has been digested with the restriction enzymes *Hind*III, *Eco*RI, *Pvu*II and *Kpn*I (Bachrach et al., 2001; Eldar et al., 1997; Eldar et al., 1999).

Random amplied polymorphic DNA (RAPD) and AFLP techniques have also been used to evaluate genetic diversity in *S. iniae* (Bachrach et al., 2001; Colorni et al., 2002; Dodson et al., 1999; Eldar et al., 1997).

Identification might be confirmed by PCR assay based on specific primers (Sin-1 and Sin-2) deduced from the 16S rRNA gene sequence of *S. iniae* (Zlotkin et al., 1998). These primers produced a 300-bp amplicon and have also been used by Colorni et al. (2002). The specificity of *S. iniae* PCR assay was demonstrated by the fact that no specific band was amplied when other fish pathogen (7 different) was used as the DNA template (Zlotkin et al., 1998).

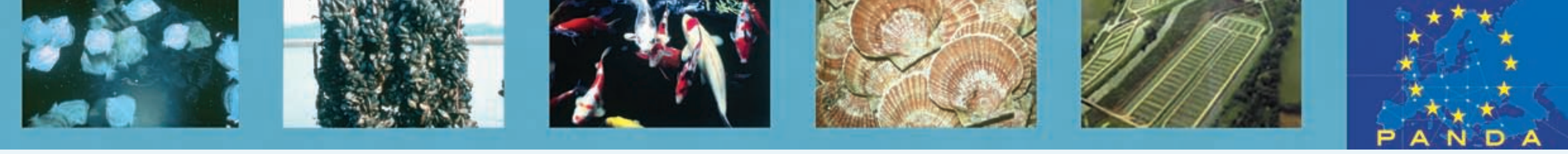
Berridge et al. (1998) have constructed PCR primers (5´144 and 3´516) from a consensus sequence of *S. iniae* 16S-23S ribosomal DNA intergenic spacer. These primers produced a 373-bp amplicon. The specificity of the selected primer pair was demonstrated by the fact that no specific band was amplied when a variety of fish and human pathogens (27 different) was used as the DNA template (Berridge et al., 1998).

A multiplex PCR-based method was designed for detection of the main pathogens involved in warm-water streptococcosis. The sensitivity of the multiplex PCR using purified DNA was 25 pg for *S. iniae* (Mata et al., 2004).

Comments and recommendations on available techniques

Bacteriological culture and biochemical identification of the causal agent remain the ultimate confirmation of the disease.

Streptococcus iniae infection of trout results in a more prolonged course with specific lesions, while the disease induced by *Lactococcus garvieae* produces a generalized disease and rapid death (Eldar & Ghittino, 1999).



Protocols for PCR are available in pre cited articles. However the techniques need to be validated and more specifically specificity and sensitivity values are needed.

What should we do for diagnosis at suspicion?

Tissue samples should be taken from diseased fish e.g. from kidney, spleen, brain and blood, and cultured and typed like described above.

EU-legislation related to techniques

Streptococcus iniae (junior synonym: *Streptococcus shiloi*), warm-water streptococcosis is not listed by the EU. Therefore no details are given by the EU.

OIE recommendations related to techniques

Streptococcus iniae (junior synonym: *Streptococcus shiloi*),warm-water streptococcosis is not listed by the OIE. No details are given by the OIE on techniques.

Assessment

It is advised to culture the bacterium as described above, and at least use biochemical techniques to type the bacterium further. PCR techniques need to be validated and more specifically specificity and sensitivity values are needed. Sequencing is recommended as one of the final steps for confirmatory diagnosis.

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Fig 6: *Streptococcus iniae* infected Tilapia showing spinal curvature "C-shaped" (Joyce Evans acknowl.).

3.4.7 *Lactococcus garvieae*

(junior synonym: *Enterococcus seriolicida*) (warm-water streptococcosis)

Lactococcosis may cause significant economic problems in various species: *Seriola quinqueradiata*, *Seriola dumerili*, *Seriola lalandi*, *Anguilla anguilla / japonica*, *Oncorhynchus mykiss*, *Oreochromis* sp., *Paralichthys olivaceous*, *Scopthalmus maximus*, *Sebastes schlegali*, *Mugil cephalus*, *Coris aygula*, and *Macrobrachium rosenbergii,* and has been reported from Australia (Tasmania, Victoria), Europe (Italy, Spain, Turkey), Israel, Japan, South Africa, and Taiwan. The host range of *Lactococcus garvieae* is not limited to aquatic species, but the bacterium has also been isolated from cows and humans suggesting that this bacterium might be a zoonotic problem.

Clinical pathology

Lactococcus garvieae is responsible for fatal septicemia and meningoencephalitis in different fish species. Clinical signs vary among species of affected fish. However, the most common symptoms are high mortality, exophthalmus, hemorrhages on opercula, fins, intestine, liver, spleen and kidney (Eldar et al., 1996; Kusuda et al., 1991).

Agent description

Lactococcus garvieae (junior synonym: *Enterococcus seriolicida*) is a Gram-positive coccus which forms short chains. The bacterium grows with small grey/white 1mm colonies after 24 h and is alpha-hemolytic on blood agar (Colorni et al., 2003; Kusuda et al., 1991).

Confirmatory techniques for diagnosis

The different PCR protocols previously described in the section "screening techniques" can also be used as confirmatory techniques for identification of *L. garviae.*

Sequencing is recommended as one of the final steps for confirmatory diagnostic. The sequence of the 1544 bp PCR amplicon of 16S rDNA from different isolates of *Lactococcus garvieae* was determined and deposited in the GenBank database (Chen et al., 2001; Chen et al., 2002). Obtained sequences should be compared with available ones in GenBank.

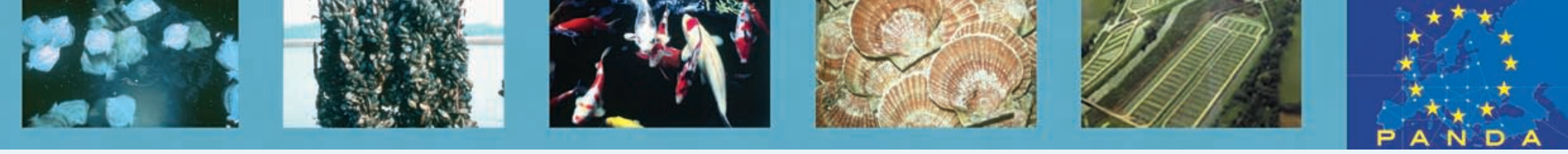
Screening techniques for the pathogen

Tissue samples are taken from diseased fish e.g. from kidney, spleen, brain and blood. The pathogen is easily grown on different agar media: e.g. brain–heart infusion agar (BHIA), Columbia agar with 5% sheep blood, bloodagar, trypticase-soy agar (TSA) + 1% NaCl, incubated at 24°C to 28°C for 24-48 h (Eldar et al. 1996; Eldar et al. 1999; Kusuda et al. 1991; Ravelo et al. 2001; Ravelo et al. 2003).

The biochemical characteristics for identification are described by following authors (Chen et al., 2002; Eldar et al., 1999; Kusuda et al., 1991; Ravelo et al., 2001). Routine tests for biochemical properties were done as described in different manuals of methods for general bacteriology. The bacterium grows at both 10°C and 45°C, and in 6.5% NaCl, and at pH 9.6, and in 40% bile.

Biochemical typing has been done by the following commercial systems: API 20 Strep, API 50 CH, Rapid ID 32 Strep, API ZYM, at 24 °C to 28°C (Chen et al., 2002; Colorni et al., 2003; Eldar et al., 1996; Ravelo et al., 2001; Vela et al., 2000).

Susceptibility to clindomycin has been used to differentiate between *Lactococcus garvieae* and



Lactococcus lactis (different results obtained) (Colorni et al., 2003; Elliott & Facklam, 1996).

The reference strain ATCC 49156 (*Enterococcus seriolicida*) / ATCC 43921 (*Lactococcus garvieae*) might be included for comparative purposes. DNA-DNA hybridization of *Enterococcus seriolicida* and *Lactococcus garvieae* showed relatedness of more than 70% (Kusuda et al., 1991; Eldar et al., 1999).

Serological tests used for characterisation: Lancefield´s grouping of group specific carbohydrate antigen. *Lactococcus garvieae* does not belong to A to H, K to N, and O (Kusuda et al., 1991) but belong to group N regarding Eldar et al. (1999). Two antigenic variants found, KG+ (non-capsulated) and KG- (capsulated) (Colorni et al., 2003; Ooyama et al., 2002).

Histology allows observing abnormalities but not specific to streptococcal infection. The *Lactococcus garvieae* infected trout presented acute meningitis, with exudate on brain surface, severe peritonitis with fat necrosis, and pseudomembrane-like formation on the intestine (Eldar & Ghittino, 1999).

For epidemiological studies have been used restriction fragment length polymorphism (RFLP) ribotyping. DNA has been digested with the restriction enzymes *Hin*dIII and *Eco*RI (Eldar et al., 1999). Random amplied polymorphic DNA (RAPD) technique has also been used to evaluate genetic diversity in *Lactococcus garvieae* (Colorni et al., 2003; Ravelo et al., 2003).

Molecular typing with pulsed-field gel electrophoresis (PFGE) and digested with *Apa*I has been used by Vela et al. (2000).

Identification might be confirmed by PCR assay based on specific primers (pLG-1 and PIG-2) deduced from the 16S rRNA gene (Zlotkin et al., 1998). These primers produced an 1100-bp amplicon and have been used by several authors (Chen et al., 2001; Chen et al., 2002; Colorni et al., 2003; Eldar et al., 1999; Vela et al., 2000). The PCR assay can be used to differentiate between *Lactococcus garvieae* and *Lactococcus lactis.*

The specificity of *Lactococcus garviae* PCR assay was demonstrated by the fact that no specific band was amplied when *L. lactis* or any other fish pathogen (5 different) was used as the DNA template (Zlotkin et al., 1998).

PCR sensitivity was evaluated by testing 10-fold dilutions of *L. garviae.* The PCR assay was positive down to the dilution corresponding to 4 CFU (Zlotkin et al., 1998).

A dihydropteroate synthase gene has been used as target for PCR (Aoki et al., 2000). The PCR primer set amplified a 709 bp DNA fragment from *L. garviae.* The total procedure from the point of DNA extraction can be performed in less than 4 h.

A multiplex PCR-based method was designed for detection of the main pathogens involved in warm-water streptococcosis. The sensitivity of the multiplex PCR using purified DNA was 30 pg for *L. garviae* (Mata et al., 2004).

Comments and recommendations on available techniques

Bacteriological culture and biochemical identification of the causal agent remain the ultimate confirmation of the disease.

Lactococcus garvieae infection of trout produces a generalized disease and rapid death, while the disease induced by *Streptococcus iniae* results in a more prolonged course with specific lesions (Eldar & Ghittino, 1999).

Protocols for PCR are available in pre cited articles. However the techniques need to be validated and more specifically specificity and sensitivity values are needed.

What should we do for diagnosis at suspicion?

Tissue samples should be taken from diseased fish e.g. from kidney, spleen, brain and blood, and cultured and typed like described above.

EU-legislation related to techniques

Lactococcus garvieae (junior synonym: *Enterococcus seriolicida*) warm-water streptococcosis is not listed by the EU legislation. Therefore no details are given by the EU.

OIE recommendations related to techniques

Lactococcus garvieae (junior synonym: *Enterococcus seriolicida*) warm-water streptococcosis is not listed by the OIE. No details are given by the OIE on techniques.

Assessment

It is advised to culture the bacterium as described above, and at least use biochemical techniques to type the bacterium further. PCR techniques need to be validated and more specifically specificity and sensitivity values are needed. Sequencing is recommended as one of the final steps for confirmatory diagnosis.

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3.4.8 *Trypanoplasma salmositica*

Trypanoplasma (*Cryptobia*) *salmositica* is a blood parasite, that causes cryptobiosis in salmonids and other fish species, and has been reported from North America. Severity of the disease and mortality rates vary significantly between species and stocks of salmon (Woo, 2003).

Clinical pathology

Trypanosoma salmositica multiplies readily in susceptible fish, causes anaemia, and mortality is variable and may be up to 100% in untreated fish (Woo & Pynton, 1995; Ardelli & Woo, 2001). The clinical signs of salmonid cryptobiosis are anorexia, exophthalmia, abdominal distension with ascites, general oedema, splenomegaly and a microcytic hypochromic anemia (Woo, 1979, Woo 2006 (book chapters)).

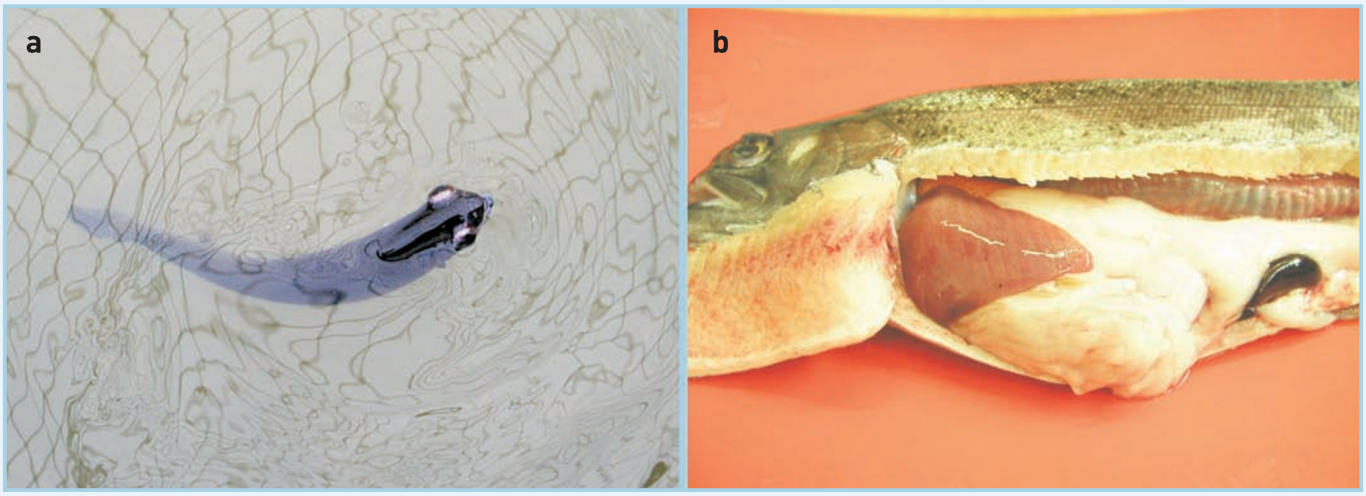

Fig. 7: Warm-water streptococcosis: *Lactococcus garvieae* **a**) Rainbow trout with exophthalmia, **b**) Rainbow trout with enlarged spleen, thickened swimbladder, and haemorrhages in liver and muscles. Other signs: haemorrhages internal organs, meningoencephalitis, septicemia. (A. Manfrin, IZSV, Padova acknowl.)

Agent description

Trypanosoma salmositica (syn. *Cryptobia salmositica*) is a pathogenic haemoflagellate of *Oncorhynchus* spp. in rivers and streams on the Pacific coast of North America (Woo, 1998). The pathogen can also survive on the body surface of fish because it has a contractile vacuole to osmoregulate when the fish is in fresh water (Woo, 2003).

Confirmatory techniques for diagnosis

- Fresh preparations of gill/body mucus or intestinal fluid or blood/ascites: Woo & Poynton, 1995; Woo 2006 (book chapters). The test is standardized.
- Parasite isolation: This is done experimentally in HMEM + 10% (v/v) FBS at 5º & 10ºC; carp: SNB-9 diphastic blood agar with vitamins & ABs at 25º; TDL 15 with 10% FBS, 1% goldfish setum and 17 mM Hepes; DEAE-cellulose method, as described by Ardelli & Woo, 1998; Woo, 1979 (see book chapters Woo); Nohynkova, 1984 (see book chapters); Li & Woo (unpublished (see Woo, 1995, book chapters); Woo et al., 1987 (see book chapters).
- Fixed smear of gill/body mucus or intestinal fluid or blood/ascites is Giemsa stained, and read like described by Woo & Poynton, 1995; Woo 2006 (book chapters). The test is standardized.
- Haematocrit centrifuge technique, like described by Woo, 1969 (see Woo, 1995, book chapters); Woo, 2001 is standardized and highly sensitive. Parasites are > 1 week post infection detectable.
- Clotting technique: According to Strout, 1962 (see book).
- In vitro haemolysis of fish erythrocytes: According to Zuo & Woo, 2000.
- Monoclonal Antibody has been developed by Feng & Woo, 1996a; Verity & Woo 1996.
- Monoclonal antibody characterization: Described by Feng & Woo, 1996b.
- Monoclonal antibody probes have been developed by Woo & Poynton, 1995; Woo 2006.
- IFAT (antigen typing): This test is described by Woo, 1995 & 2006 (see book chapters).
- Immuno-substrate enzyme technique (MISET for detection of antibodies): This test is described by Woo, 1990 (see book chapters). The test is standardized.
- Metallo- & Cystein proteases test has been developed by Zuo & Woo, 1998.
- 200 kD glycoprotein characterization: Described by Feng- & Woo, 1998a & 1998b.
- Antigen-capture ELISA for detection of parasite: Antibodies used: MAb (MAb-007;against 47 kD antigen) to detect parasite antigen in blood; Described by Verity & Woo, 1993/1996; and Woo, 2001. This test is not species specific, but is standardized and has a high sensitivity.
- Antibody-capture ELISA for detection of antibodies in the fish blood: This test has been described by Sitja-Babodilla & Woo 1994. The test is standardized.

- Immunological technique for serodiagnosis, like described by Woo, 1995 & 2006; Ardelli & Woo, 2002.
- SDS-Page: According to Woo & Thomas, 1991; and Zuo & Woo, 1997.
- Polypeptide and antigen profiles: These have been described by Woo & Thomas, 1991; and Chin et al., 2004.
- DNA probe has been developed by Li & Woo 1996. It is used for confirmative species identification and is highly specific.
- Histopathology: This is described by Bahmanrokh & Woo 2001.
- Electron Microscopy: Paterson & Woo, 1983 described it.

Screening techniques for the pathogen

- Antigen-capture ELISA for detection of parasite: Antibodies used: MAb (MAb-007;against 47 kD antigen) to detect parasite antigen in blood; Verity & Woo, 1993/1996; Woo, 2001. This test is not species specific, but is standardized and has a high sensitivity.
- Antibody-capture ELISA for detection of antibodies in the fish blood: This test has been described by Sitja-Babodilla & Woo 1994. The test is standardized.

Comments and recommendations on available techniques

There are very many tests developed on *Trypanoplasma salmositica*, by a relative small group of experts. Although the number of screening tests is low, the number of confirmation tests is high, of which some are used only experimentally. Some tests are standardized. It is recommended, to use more than 1 confirmative test, when there is no or minor experiece with the parasite.

What should we do for diagnosis at suspicion?

When the clinical pathology is like described above, fresh preparations of gill/body mucus or intestinal fluid or blood/ascites should be taken for parasite isolation. Additionally fixed smears of gill/body mucus or intestinal fluid or blood/ascites are Giemsa stained, and read. Additional confirmation tests should be used to type the haemoflagellate further, according to references mentioned above.

EU-legislation related to techniques:

Trypanoplasma salmositica (syn. *Cryptobia salmositica*) is not listed by the EU, and therefore no recommendations are made by the EU. It is an exotic pathogen to the EU.

OIE recommendations related to techniques:

Trypanoplasma salmositica (syn. *Cryptobia salmositica*) is not listed by the OIE (Aquatic Animal Health Code and Manual, 2006 version).

Assessment

There is a bright variety of tests described in literature for the diagnosis of *Trypanoplasma salmositica*. Nevertheless, exact typing will need some specialistic skills. To use more than 1 confirmative test is recommended.

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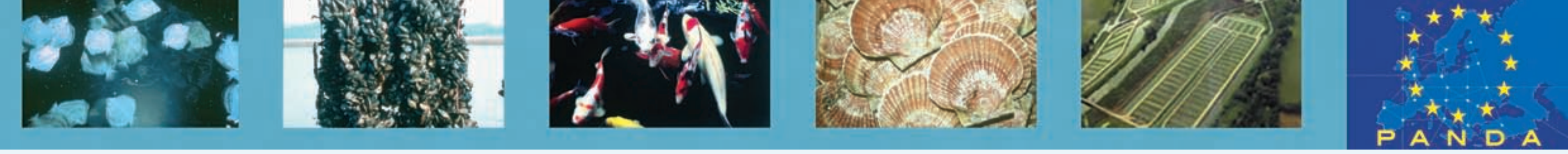

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Fig. 8: *Cryptobia salmositica* (P.T.K. Woo, acknowl.).

Fig. 9: Exopthalmia in *Cryptobia salmositica* infected fish (P.T.K. Woo, acknowl.).

3.4.9 *Ceratomyxa shasta*

Ceratomyxosis is caused by a myxosporean, *Ceratomyxa Shasta*, which may cause high mortalities in salmonids. Losses are reported in juvenile fish, both hatchery-reared and in wild, as well as in pre-spawning adults. The disease has been reported from Canada (NW Pacific) and USA (NW Pacific).

Clinical pathology

Clinical disease signs include lethargy, darkening of the body surface, abdominal distension and hemorrhaging in the area of the vent (Conrad & Decew, 1966; Bartholomew et al., 1989c). These signs develop as the parasite invades the intestinal tract, causing an inflammatory reaction and necrosis. Mortalities up to 90% have been recorded in rainbow trout *(Oncorhynchus mykiss)* and sea-run cutthroat trout *(O.clarki)* (Tipping, 1988).

Agent description

Ceratomyxosis is a disease in salmonid fish that results from infection by the myxozoan *Ceratomyxa shasta* (Bartholomew, 1998). *C. shasta* is a myxosporean protozoan parasite with a spore size of 14-23 m long x 6-8 m wide (Bartholomew *et al.*, 1989). It has a complex life cycle with both vertebrate and invertebrate hosts, involving the requirement to use the polychaete worm *Manayunkia speciosa* as an alternate host (Bartholomew *et al.*, 1997).

Confirmatory techniques for diagnosis

- Fresh preparate: Confirmation if spores detected (Bartholomew, 2003b). This test is standardized, but not validated.
- Fixed smear: Intestinal scraping, fluid or ascites Giemsa stain: Confirmation if spores detected (Woo, 1999 (book); Bartholomew, 2003a, b). The test is not standardized, nor validated.
- Isolation: Intestinal scraping, fluid or ascites; intestinal lavage: Confirmation if spores detected (Coley et al., 1983; Bartholomew 2003a)
- IFAT (antigen): with monoclonal antibodies (Bartholomew et al., 1989b), not commercially available (Bartholomew 2003a; Bartholomew et al., 2004). The test is not standardized, nor validated.
- IPMA: with monoclonal antibodies, not commercially available (Palenzuela and Bartholomew, 2002). The test is not standardized, nor validated.
- PCR: Specific primers for amplification of parasite DNA from intestinal (or other) tissue; non-lethal assay developed. (Fox et al., 2000; Palenzuela et al., 1999; Palenzuela and Bartholomew, 2002; Bartholomew 2003 a, b; Bartholomew et al., 2004). Standardized as confirmation protocol in the USFWS -AFH/FHS Inspection Protocols. The test is not validated. No cross-reaction reported. Sensitivity to 0.01 spore.
- Quantitative PCR (stand., very sensitive): Specific primers and probe for amplification of parasite DNA from tissues and water (Hallett and Bartholomew, 2006). The test is standardized, but not validated. No cross-reaction reported, Sensitive to 0.0001 spore.

- Histopathology: Standard procedures using entire intestine or posterior portion: Confirmation if spores detected; presumptive if presporogonic myxozoan stages present; provides measure of infection severity (Bartholomew, 2003a; Bartholomew et al., 2004). The test is standardized, but not validated.
- IHC: With monoclonal antibodies, not commercially available. The test is not standardized, nor validated.
- ISH: DNA probes have been developed; primarily a research tool (Palenzuela and Bartholomew, 2002). The test is standardized, but not validated.
- Non-lethal PCR: Protocol modified to use intestinal swab (Fox et al., 2000).
- E.M.: Developed as a research tool; not recommended for diagnostics (Bartholomew et al, 1989c, 1997; Yamamoto & Sanders, 1979)

Screening techniques for the pathogen

- Clinical pathology: Anorexia, lethargy, darkening, swollen abdomen, ascites, exophthalmia, mortality; Destroys tissue of intestine and other internal organs (Woo, 1999 (book); Palenzuela and Bartholomew, 2002, Bartholomew, 2003a). This test is not standardized, nor validated.
- Fresh preparate: Intestinal scraping, fluid or ascites: presumptive if presporogonic myxozoan stages present (Bartholomew, 2003b): This test is standardized as screening test in the USFWS - AFH/FHS Inspection Protocols.
- Fixed smear: Intestinal scraping, fluid or ascites Giemsa stain: presumptive if presporogonic myxozoan stages present (Woo, 1999 (book); Bartholomew, 2003a, b). The test is not standardized, nor validated.
- Isolation: Intestinal scraping, fluid or ascites; intestinal lavage: presumptive if presporogonic myxozoan stages present (Coley et al., 1983; • PCR: Specific primers for amplification of parasite DNA from intestinal (or other) tissue; non-lethal assay developed. (Fox et al., 2000; Palenzuela et al., 1999; Palenzuela and Bartholomew, 2002; Bartholomew 2003 a, b; Bartholomew et al., 2004). Standardized as confirmation protocol in the USFWS -AFH/FHS Inspection Protocols. The test is not validated. No cross-reaction reported. Sensitivity to 0.01 spore.
- Quantitative PCR: Specific primers and probe for amplification of parasite DNA from tissues and water (Hallett and Bartholomew, 2006). The test is standardized, but not validated. No cross-reaction reported, Sensitive to 0.0001 spore.
- Non-lethal PCR: Protocol modified to use intestinal swab (Fox et al., 2000).

Comments and recommendations on available techniques

Although *Ceratomyxa shasta* is a parasite sofar restricted to 1 area of the world, various test methods have been developed by a small group of scientists, to diagnose the parasite, and even screen fish populations for presence of *C.shasta*. The tests are described in literature, and therefore are well established for use, although not validated under ISO norms.

What should we do for diagnosis at suspicion?

Make a fresh preparate: Intestinal scraping, fluid or ascites: presumptive if presporogonic myxozoan stages are present. This is a standardized screening test in the USFWS -AFH/FHS Inspection Protocols. Make a fixed smear and/or isolate the pathogen, like described above. Use confirmation tests or send the smears to an expert from the reference list.

EU-legislation related to techniques

Ceratomyxa shasta is not listed by the EU, and therefore no recommendations are made by the EU. It is an exotic pathogen.

OIE recommendations related to techniques

Ceratomyxa shasta is not listed by the OIE (Aquatic Animal Health Code and Manual, 2006 version).

Assessment

There are various detection and confirmation methods developed for the parasite *Ceratomyxa shasta* by a small group of specialists. The tests are published, but still need validation.

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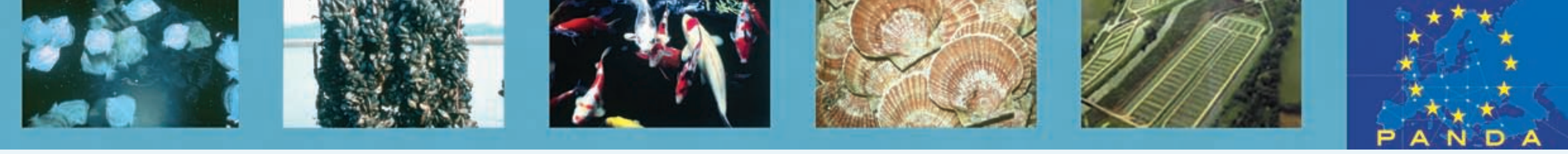
Fig 10: Spore of *Ceratomyxa shasta* (J.Bartholomew acknowl.)

3.4.10 *Neoparamoeba perurans*

Neoparamoeba perurans is identified as the agent of serious amoebic gill disease (AGD) in Atlantic salmon *(Salmo salar)* reared in sea pens in Tasmania, Australia, Atlantic and coho salmon *Oncorhynchus kisutch* farmed on the west coast of the USA, Atlantic salmon farmed in Ireland and Scotland, turbot farmed in Spain (Young et al, 2007). *Neoparamoeba pemaquidensis* and *Neoparamoeba brachiphila* have been isolated by culture from fish with AGD but could not be detected in histological sections using species-specific in situ hybridisation (Young et al, in press).

Clinical pathology

Neoparamoeba perurans causes gill disease with severe multifocal hyperplastic lesions (Young et al., 2007a), and has a possible effect on respiratory and acid-based physiology (Powell & Nowak, 2003; Adams & Nowak, 2003; Adams et al., 2004). The test is not standardized, nor validated.



Agent description

Neoparamoeba perurans is a parasomal amoeboid protozoan.

Confirmatory techniques for diagnosis

- Gill histopathology: The gill shows severe multi-focal hyperplastic lesions, and there is a possible effect on the respiratory and acid-based physiology, as described by Young et al in press, Harris et al, 2004; Morrison et al., 2004; Clark et al., 2003; Adams et al., 2004, and Dyková et al., 2000. The test is standardized and validated.
- Fresh preparation: taken from the gills and screened by light microscopy. Shows presence of amoebae. The test is used: For AGD suspicion at farms (Morrison et al., 2004, Dyková et al., 2000, 2005).
- Fixed smear: A Giemsa stain may be used for confirmation, shows parasome (Zilberg et al, 1999).
- Parasite isolation and identification: This can be done on malt-yeast-seawater agar (MYS), according to; Tan et al., 2002, Morrison et al., 2005, Dyková et al., 2005. The test is standardized and validated. However, no cultures of *Neoparamoeba perurans* have been obtained. Fresh isolation from the gills results in almost 100% *Neoparamoeba perurans* (Morrison et al., 2005, Young et al 2007).
- IFAT (antigen): with mucus smears or the cultured parasite. The test is at least used for confirmation. (Douglas-Helders et al., 2003; Tan et al., 2002).The test is standardized and validated, however not species-specific.
- Immuno dot blot of mucus (stand.): To estimate distribution of the parasite on gills and in water, described by Douglas-Helders et al., 2003. This test is not species-specific.
- Sequencing: Strain typing, phylogeny, described by Young et al., in press a.
- PCR on clonal cultures: It is an 18S ribosomal-based PCR (by Young et al in press a, Fiala and Dyková, 2003; Wong et al., 2004; and Morrison et al., 2005).
- Nested PCR: described by Douglas-Helders et al., 2003, it is used experimentally only.
- Immuno Cyto Chemistry (ICC): Gills are processed according to Morrison et al., 2004;. The test is standardized and validated, but not species specific.
- In situ hybridisation – the only species specific test on tissue sections (Young et al, 2007)
- Electron Microscopy: According to Dyková et al., 2000, 2005.

Screening techniques for the pathogen

- Fresh preparation: taken from the gills and screened by light microscopy. The test is used: For AGD on the farms (Morrison et al., 2004, Dyková et al., 2000, 2005).
- Gill histopathology: The gill shows disease with severe multifocal hyperplastic lesions, and there is a possible effect on the respiratory and acid-based physiology, as described by Young et al, 2007, Harris et al, 2004; Morrison et al., 2004; Clark et al., 2003; Adams et al., 2004, and Dyková et al., 2000. The test is standardized and validated.
- PCR on clonal cultures: It is an 18S ribosomal-based PCR (by Fiala and Dyková, 2003; Wong et al., 2004; and Morrison et al., 2005).

Comments and recommendations on available techniques

Although literature on *Neoparamoeba perurans* is rather new, there have been various tests developed sofar. The confirmation methods seem to be the most developed, although some tests are still in an experimental phase. For screening specialist skills are needed.

What should we do for diagnosis at suspicion?

Given the clinical pathology, take gill tissue for histopathology and make a fresh preparate of the gills, to screen for the parasite by light microscopy. If there is a suspicion, confirmation tests, like PCR may be used.

EU-legislation related to techniques

Neoparamoeba perurans is not listed by the EU, and therefore no recommendations are made by the EU. It is present in the EU.

OIE recommendations related to techniques:

Neoparamoeba perurans is not listed by the OIE (Aquatic Animal Health Code and Manual, 2006 version).

Assessment

Given the clinical pathology, take gill tissue for histopathology and make a fresh preparation of the gills, to screen for the parasite by light microscopy. If there is a suspicion, confirmation tests, like PCR may be used.

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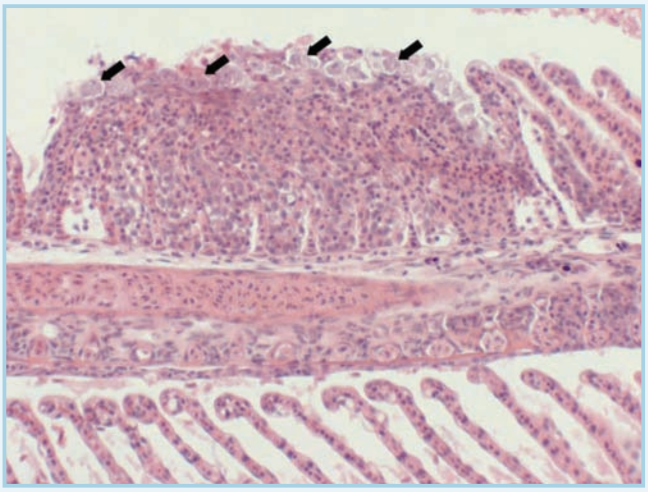

Fig. 11: Gill lesions with amoebae of *Neoparamoeba perurans*, HE stain (Neil Young acknowl.)

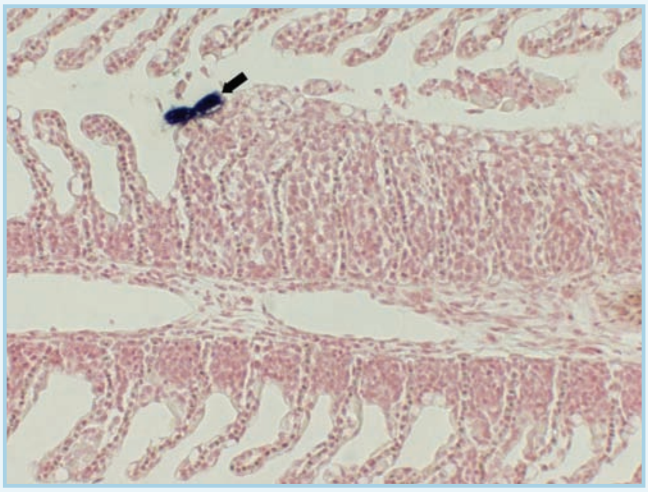

Fig. 12: In situ Hybridization (ISH) of gill lesions with amoebae of *Neoparamoeba perurans* (Neil Young acknowl.)

3.4.11 *Parvicapsula pseudobranchicola*

Parvicapsula pseudobranchicola is a myxozoan parasite of Atlantic salmon, and causes parvicapsulosis, pseudobranch infections associated with low-grade to significant mortalities. It is a problem in Norwegian salmon farming (Nylund et al., 2005).

Clinical pathology

Parvicapsula pseudobranchicola infections in farmed Atlantic salmon in Norway are associated with surfacing, lethargy, disorganised swimming, darkening, eye bleedings, cataracts, exophthalmia, cheesy covering of pseudobranchs, vision impairment (Karlsbakk et al., 2002).

Agent description

Parvicapsula pseudobranchicola is a myxozoan. Diagnosis has relied on the detection of *Parvicapsula* spores, with the pseudobranch being the preferred organ but a developed PCR protocol has shown greater sensitivity than light microscopy (Nylund *et al.*, 2005). A comparison of the sequence of the ssu rDNA from *P. pseudobranchicola* with that of other myxozoans has shown that it groups closely together with *P. unicornis* and *P. asymmetrica*. The closest relative to this group is *P. minibicornis* (Nylund *et al.*, 2005). The sizes of *Parvicapsula* sp. mature spores are 7 to 10 × 3 to 5 m (Yasutake and Elliott, 2003).

Confirmatory techniques for diagnosis

- Parasite isolation: From the pseudobranchs myxosporean disporous parasite with asymmetrical curved spores in sutural view that measure 11.1-13.8 µm in length; for Parv.kareii sp.n. and Parv.anisocaudata sp.n. as described by Karlsbakk et al., 2002; Zhao et al., 2000.
- Haematology: This has been described from a *Parv.minibicornis* induced infection by Wagner et al., 2005.
- Sequencing: has been described for phylogenetic study of *Tetracapsula renicola n sp* by Nylund et al., 2005; Kent et al., 2000
- PCR (highly sensitive): The pseudobranch is tested with PCF3/PCR3 primers: 203 bp product; PCR for *Parv.minibicornis*, as described by Nylund et al., 2005; St-Hilaire et al., 2002; Jones et al., 2003, 2004, and Nylund et al., 2005. It has a higher sensitivity than light microscopy.
- Histopathology: This has been described for *Parv.minibicornis;* for *Parv.*sp.; for *Parv.minibicornis;* for *Tetracapsula renicola n sp;* of *Parv.spinachiae*; for Parv.sp., by St-Hilaire et al., 2002; Yasutake & Elliott, 2003; Jones et al., 2003, 2004; Kent et al., 2000; Køie, 2003; Landsberg, 1993; and Sterud et al., 2003.
- ISH: This test was developed for *Parvicapsula minibicornis*, by Jones et al., 2004.

Screening techniques for the pathogen

- Clinical pathology:
- Fresh preparate: From the pseudobranchs, myxozoan trophozoites and typical Parvicapsula spores of *Parvicapsula minibicornis* and *Parv.renalis* nov sp, as described by Karlsbakk et al., 2002; Kent et al., 1997; and Landsberg, 1993.
- Parasite isolation: from the pseudobranchs myxosporean disporous parasite with asymmetrical curved spores in sutural view that measures 11.1-13.8 µm in length; for *Parv.kareii* sp.n. and *Parv.anisocaudata* sp.n. as described by Karlsbakk et al., 2002; Zhao et al., 2000.

Comments and recommendations on available techniques

The disease problems in Atlantic salmon with *Parvicapsula pseudobranchicola* are relatively new. Few publications have appeared sofar on diagnostic methods. The validation staus is not known. Depending on the severity of the disease it causes more tests will be developed in future. It is advisable, to contact Norwegian experts at suspicion of this parasite in salmon in other European countries.

What should we do for diagnosis at suspicion?

If the clinical pathology in Atlantic salmon is like described above, the pseudobranchs should be sampled to make a fresh smear and isolate the parasite. If myxosporean parasites are seen like the figures in literature, further typing with molecular biological methods is recommended, parallel to histopathology.

EU-legislation related to techniques

Parvicapsula pseudobranchicola is not listed by the EU, and therefore no recommendations are made by the EU. It is present in Norway.

OIE recommendations related to techniques

Parvicapsula pseudobranchicola is not listed by the OIE (Aquatic Animal Health Code and Manual, 2006 version).

Assessment

Diagnosis has relied on the detection of *Parvicapsula* spores, with the pseudobranch being the preferred organ (Nylund et al., 2005). The detection and diagnostic tests for *Parvicapsula pseudobranchicola* are being developed by a small group of experts. It is advised to contact them at suscpicion. Depending on the severity of the disease the parasite causes more specific tests will be needed and probably developed.

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3.4.12 *Gyrodactylus salaris* (Gyrodactylosis)

Gyrodactylosis is a disease of Atlantic salmon (*Salmo salar*) caused by the freshwater parasite *Gyrodactylus* *salaris*. All stages of salmon, including adult spawners, in freshwater, can be infected, but disease and mortality has only been observed in pre-smolt stages (OIE, 2006). The parasite has been reported from Bosnia, Denmark, Finland, France, Germany, Norway, Portugal, Russian Federation, Spain and Sweden, as well as possibly the Czech Republic, Georgia and Ukraine.

Clinical pathology

In the early disease phase, increased flashing (fish scratch their skin on the substrate) is typical. Later, fish may become greyish due to increased mucus production and the fins may be eroded. Diseased fish are lethargic and are usually found in slower-moving water. Mortalities in farmed fish may be 100% if not treated while population reductions as high as 98% of salmon have been observed in rivers (OIE, Manual of Diagnostic Tests for Aquatic animals, 2006).

Agent description

Gyrodactylosis is a disease of Atlantic salmon (*Salmo salar*) caused by the viviparous freshwater parasite *Gyrodactylus salaris* (Platyhelminthes; Monogenea). Morphology and morphometry are important for identification of *G. salaris*, since it is morphologically similar to other *Gyrodactylus* spp. As many as 14 different characters measured from the marginal hooks, anchors, and ventral bars can be used for characterization (OIE, 2006).

Confirmatory techniques for diagnosis

- Morphometry: chaetotaxy, morphology, sclerites: Malmberg, 1970; Shinn et al., 1998, 2000, 2001, 2004; Bakke et al., 2004; Lindenstrom et al., 2003; Shinn et al., 1995; Mo, 1991a,b,c;
- PCR: primers: Meinila et al.,2002; Collins & Cunningham, 2000; Cunningham et al., 1995b Cunningham, 1997, Matejusova *et al.*, 2001
- Sequencing: Strain typing, phylogeny: Collins et al., 2004; Meinila et al., 2004; Cunningham et al., 2001, 2003; Matejusova & Cunningham, 2004; Lindenstrom et al., 2003; Hansen et al., 2003; Sterud et al., 2002; Matejusova et al., 2001; Cunningham & Mo, 1997; Cunningham et al., 1995a, b;
- RFLP(Restricted Fragment Length Polymorphism): Test according to description by Cunningham et al., 1995b; Cunningham 1997, and Cunningham et al., 2001.
- Histopathology: According to findings of Sterud et al., 1998; and Appleby et al., 1997.
- Histochemistry: Test described by Buchmann & Bresciani, 1997
- Scanning Electron Microscopy: According to Shinn et al., 2000; Buchmann & Bresciani, 1997; and Shinn et al., 2001.
- Statistical classifiers: Kay et al., 1999.

Screening techniques for the pathogen

- Clinical pathology of wild and artificial infections has been described by Lindenstrom et al., 2003; Sterud et al., 2002; Sterud et al., 1998; Appleby et al., 1997; and Buchmann & Bresciani, 1997.
- Fresh preparate: Light microscopy: described by OIE, 2006; Buchmann & Bresciani, 1997, Malmberg, 1970
- Isolation of the parasite: Malmberg, 1970; Shinn et al., 1998, 2000, 2001, 2004; Bakke et al., 2004; Lindenstrom et al., 2003; Shinn et al., 1995; Mo, 1991a,b,c;
- Morphometry: chaetotaxy, morphology, sclerites: Malmberg, 1970; Shinn et al., 1998, 2000, 2001, 2004; Bakke et al., 2004; Lindenstrom et al., 2003; Shinn et al., 1995; and Mo, 1991a,b,c.
- RFLP (Restricted Fragment Length Polymorphism): Test according to description by Cunningham et al., 1995b; Cunningham 1997, and Cunningham et al., 2001.

Comments and recommendations on available techniques

There are many well established and validated tests for ISAV. The OIE (2006) recommends, that the same methods to detect and identify *Gyrodactylus salaris*, respectively, must be used independently of purpose.

What should we do for diagnosis at suspicion?

The Manual of Diagnostic Tests for Aquatic animals, 2006 recommends:

Definition of suspect case: Observation of *Gyrodactylus* specimen(s) on fins or skin of Atlantic salmon or rainbow trout in skin scrapings or by stereomicroscopic examination.

Definition of confirmed case: Morphological identification of *Gyrodactylus* specimen(s) to *G. salaris* based on structures of the attachment organ or genetic identification of *Gyrodactylus* specimen(s) to *G. salaris* based on molecular methods (ITS, IGS and COI). However, a combination of both methods is recommended.

EU-legislation related to techniques

Gyrodactylus salaris is not listed by the EU, and therefore no recommendations are made by the EU. Prevention against introduction of the pathogen is possible via so called *additional measures*, related to the EU legislation, via national legislation.

OIE recommendations related to techniques (& OIE ref lab)

Gyrodactylus salaris is listed by the OIE (Manual of Diagnostic Tests for Aquatic animals, 2006). The OIE recommends:

Field diagnostic methods based on clinical signs like flashing, reduced activity, and stay of fish in low current areas

Clinical methods, based on clinical signs, in combination with water temperature (outbreaks most common in spring and in periods when the water temperature is 7-17°C), scrapings (wet mounts) from skin or fins.

Agent detection and identification methods: Detection of *Gyrodactylus* and identification of *G. salaris* is a two-step process. Firstly, parasite specimens are observed using optical equipment and secondly, parasites are identified, usually on an individual basis using other equipment and methods.

Optical method: Optical equipment must be used to detect *Gyrodactylus*. Fresh fins should be examined under a binocular dissecting microscope with good illumination. Ethanol (70%)-preserved *Gyrodactylus* specimens may be used for optical identification as well. Details of sampling and preservation are described in the Manual.

Gyrodactylus salaris identification based on morphology and morphometry of sclerites in the attachment organ: Identification of *Gyrodactylus* species is based on morphology and morphometry of marginal hooks, anchors (hamuli) and bars in the opisthaptor (the attachment organ)with good preparation according to Malmberg (1970). Malmberg's ammonium picrate glycerine (APG) method is commonly used for preparing whole mounts of small Monogenea (1957). Alternatively to the APG-method, live or ethanol-preserved specimens can be placed in a drop of proteinase K on a slide and covered with a cover-slip for a few hours (depending on the temperature). After digestion of the parasite soft parts, the opisthaptoral sclerites are suitable for species identification, according to the morphology and morphometry tables, present in the Manual of Diagnostic Tests for Aquatic animals of the OIE (2006).

Gyrodactylus salaris identification based on DNA analysis:

PCR amplification of the internal transcribed spacer (ITS), ITS sequencing and sequence analysis, and analysis of the ribosomal RNA gene intergenic spacer region are described in detail in the Manual.

According to the OIE (2006), diagnostic/detection methods to declare freedom are the same as those mentioned above.

The Reference laboratory for the OIE: National Veterinary Institute, Fish Health Section, Dr T.A. Mo, Ullevålsveien 68, P.O. Box 8156 Dep., 0033 Oslo NORWAY Tel: (47.23) 21.61.10 Fax: (47.23) 21.61.01; E-mail: tor-atle.mo@vetinst.no

Assessment

In the OIE Manual of Aquatic Animal Diseases, the detection and confirmation of *G.s.* is described. Although typing to the genus level of *Gyrodactylus* is possible without molecula methods, specific typing to *G.salaris* requires specific skills, or molecular techniques.

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www.disease-watch.com/documents/CD/inex/html/fp001gyr.htm

Fig. 13: *Gyrodactylus salaris.* (© Crown copyright, reproduced with permission of CEFAS Lowestoft)

3.4.13 *Aphanomyces invadans* (Epizootic Ulcerative Syndrome)

Epizootic ulcerative syndrome (EUS) is caused by the fungus *Aphanomyces invadans* and is an economically devastating fish disease of many different fish species in southern, south-eastern and western Asia, occurring as a seasonal epizootic condition of wild and farmed freshwater and estuarine fish. Outbreaks of ulcerative disease in the USA have been shown to be very similar to EUS in Asia (OIE, 2006).

Clinical pathology

Aphanomyces invadans causes disease, characterised by the presence of invasive *Aphanomyces* infection and necrotising ulcerative lesions typically leading to a granulomatous response (OIE, 2006,Australian lab website; Yanong, 2003; Hawke et al. 2003).

Agent description

Aphanomyces invadans is a fungus, an oomycete known as *Aphanomyces invadans* or *A. piscicida*, which causes epizootic ulcerative syndrome (EUS) or Red Spot Disease (RSD) or Mycotic Granulomatoses (MG) or Ulcerative Mycosis (UM) or epizootic granulomatous aphanomycosis (EGA) (Baldock et al., 2005) in more than 50 fish species. *Aphanomyces invadans* is a peronomycete fungus (Order Saprolegniales), which shows asexual spore morphogenesis. The aseptate mycelium is 11.7 µm -16.7 µm in culture but it is narrower (ca 8.3µm) in fish tissues. Motile secondary zoospores are the infectious stages (EFSA, 2007).

Confirmatory techniques for diagnosis

- Fresh preparate: In the preparate, hyphae can be seen (OIE Manual 2006; Yanong, 2003).
- Fixed smear: As described by Blazer et al., 2002.
- Haematology: Is described for common carp, experimental only, by Harikrishnan et al., 2005
- Fungus isolation: This can be done at Czapek Dox agar with Penicillin G and oxolinic acid or Glucose/peptone medium/agar with penicillin K and oxolinic acid; or Peptone/ Yeast/ Glucose (PYG) agar with 200µg/ml streptomycin and 100µg/ml ampicillin;See Thompson, Miles: described culture with macrophages; ROIE Manual 2006, Lilley et al., 1998; Blazer et al., 1999, 2002; Hawke et al., 2003; Kiryu et al., 2002, 2003, 2005; Kurata et al., 2000; Lilley et al, 1997a,b; Thompson et al., 1999; Johnson et al., 2004; Miles et al., 2001.
- Typing by growth charachteristics: Slow growing, fails to grow at 37°C on GPY (glucose peptone yeast) agar, according to Lilley et al., 1997a, 1998; Blazer et al., 2002.
- ELISA/test for antibodies: Described by Thompson et al., 1999; and Miles et al., 2001.
- Monoclonal antibodies: Used for immunohistochemistry, by: Miles et al., 2003.
- Macrophage response: Described by Thompson et al., 1999.
- Immuno dot test: Monoclonal antibody based: Described by Devaraja et al., 2004.

- Gel electrophoresis: Described by Lilley et al, 1997a,b.
- Western blot: Described by Lilley et al, 1997a,b; Thompson et al., 1999.
- Hemagglutination: including hemolytic activity of *A.invadans*: Kurata et al., 2000
- Sequencing: Has been done by Blazer et al., 2002; Hawke et al., 2003; and Lilley et al., 2003.
- PCR test: As described by Blazer et al., 2002; Hawke et al., 2003; Lilley et al., 2003; Phadee et al., 2004; Vandersea et al., 2006.
- Histopathology: H&E and Grocott's stain: Typical granulomas of skin and invasive hyphae are seen; sequential histopathology (Catap & Munday); descriptions in: OIE Manual 2006, Blazer et al., 1999, 2002; Catap & Munday, 2002; Hawke et al., 2003; Johnson et al., 2004; Kiryu et al., 2002; Yanong, 2003
- ICC (Immunocytochemistry): This was done with polyclonal sera and with peroxidase or fluorescein (Lilley et al, 1997a,b), or with monoclonal antibody (Miles et al., 2003: Vandersea et al., 2006)
- ISH (In situ hybridization): a fluorescent peptide nucleic acid in situ hybridization (FISH): by Vandersea et al., 2006.
- Electron Microscopy: Scanning E.M.: As described by Kiryu et al., 2003; and Thompson et al., 1999.
- Pyrolysis mass spectrometry (PyMS): This test is used experimentally for phylogenetic studies and confirmation: by Lilley et al., 2001.

Screening techniques for the pathogen

- Clinical pathology: Affected fish show loss of appetite, darkening, floating below water surface, hyperactivity with jerky swim pattern. Red spots may be observed on body surface, head, operculum or caudal peduncle. Large red or grey shallow ulcers, often with brown necrosis, are observed in the later stages. Large superficial lesions occur on the flank or dorsum. Most species other than striped snakeheads and mullet will die at this stage. In highly susceptible species the lesions extend and may lead to complete erosion of the posterior part of the body, or necrosis of the soft and hard cranium tissue, so that the brain is exposed in the living fish (OIE Manual 2006; Callinan et al., 2005; Hawke et al., 2003; Kiryu et al., 2003; Johnson et al., 2004; Yanong, 2003)
- Fungus isolation: At Czapek Dox agar with Penicillin G and oxolinic acid or Glucose/peptone medium/agar with penicillin K and oxolinic acid; or Peptone/ Yeast/ Glucose (PYG) agar with 200µg/ml streptomycin and 100µg/ml ampicillin; Thompson, Miles: culture with macrophages. Descriptions in OIE Manual 2006, Lilley et al., 1998; Blazer et al., 1999, 2002; Hawke et al., 2003; Kiryu et al., 2002, 2003, 2005; Kurata et al., 2000; Lilley et al, 1997a,b; Thompson et al., 1999; Johnson et al., 2004; Miles et al., 2001.
- According to the OIE (2006), the method for surveillance of susceptible fish populations for declaration of freedom from EUS is examination of the gross clinical signs and sampling of the diseased fish only for isolation of *A. invadans* or for histopathology examination to demonstrate absence of the *A. invadans.*

Comments and recommendations on available techniques

The clinical signs of EUS are not very specific. Therefore, isolation and confirmative testing is necessary. Histopathology is one of the main techniques. The OIE recommends the PCR testing as confirmation test for this fungus. Many of the other developed tests are used experimentally. As EUS is notifiable for the OIE and recently also for the EU, it is expected, that more tests will be developed in the nearby future.

What should we do for diagnosis at suspicion?

The OIE (2006) has the following definitions:

- *Definition of suspect case:* A suspect case of EUS disease is defined as the presence of typical clinical signs of the disease in a population of susceptible fish OR presentation of typical histopathology in tissue sections OR isolation of the slow growing *Aphanomyces* without identification of the causative agent OR a single positive result from one of the diagnostic assays described above (OIE, 2006). It means, that specimens from ulcers are taken for a fixed smear, and fungus isolation. After hyphae are seen, and growth results by isolation, confirmative tests are done. In parallel, ulcers and surrounding tissue are sampled for histopathology.
- *Definition of confirmed case:* A confirmed case of EUS is defined as a suspect case that has produced typical mycotic granulomas in affected tissues or organs with subsequent identification of the causative agent by one of the assays described above OR a second positive result from a separate and different diagnostic assay described above.

EU-legislation related to techniques

EUS is listed in the list of exotic notifiable diseases of aquaculture animals in the new Aquaculture Directive 2006/88/EC. No special tests are recommended sofar, but the EU mostly follows the recommendations of diagnostic methods by the OIE (see below).

OIE recommendations related to techniques (& ref lab OIE)

From the OIE Manual, 2006: The methods currently available for surveillance, detection, and diagnosis of EUS are listed below. The designations used indicate:

A = the method is the **recommended** method for reasons of availability, utility, and diagnostic specificity and sensitivity;

B = the method is a standard method with good diagnostic sensitivity and specificity;

C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and

D = the method is presently not recommended for this purpose.

These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation (see Chapter 1.1.2 of OIE, 2006), their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

The OIE (2006) recommends for:

- Surveillance to declare freedom from infection:
  - Direct observation of the oomycete hyphae in muscle or internal organs under microscope (C)
  - Histopathology of tissues and organs (B)
  - Isolation of *A. invadans* and confirmatory identification (C)
- Presumptive diagnosis of infection or disease (detection):
  - Gross signs (B)
  - Direct observation of the oomycete hyphae in muscle or internal organs under microscope (B)
  - Histopathology of tissues and organs (A)
  - Isolation of *A. invadans* and confirmatory identification (A)
- Confirmatory diagnosis of infection or disease (diagnosis):
  - Direct observation of the oomycete hyphae in muscle or internal organs under microscope (C)
  - Histopathology of tissues and organs (A)
  - Isolation of *A. invadans* and confirmatory identification (A)
  - PCR of pure isolate of *A. invadans* (A)

The antibody based assays to detect *A. invadans* antigen (IFAT, ELISA), Transmission EM of tissues, PCR of tissue extracts are currently not recommended by the O.I.E. to use, as they are all given the code D.

(*IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay; EM = electron microscopy; PCR = polymerase chain reaction)*

Reference laboratory for EUS for the OIE: Inland Aquatic Animal Health Research Institute (AAHRI), Dept. of Fisheries, Bangkok, Tailand, Dr. S. Kanchanakhan, E-mail: sudat@fisheries.go.th

Assessment

There are many tests for the detection and diagnosis of *Aphanomyces invadans*. Especially there are many conformative tests. Recommended is for screening histopathology, for presumptive diagnosis of EUS the clinical pathology, fresh preparates, fungus isolation and histopathology, and for confirmation histopathology, isolation with identification, and a PCR of the pure isolate of the fungus.

As EUS is an exotic disease newly listed for the EU, training of National Reference Laboratories will be necessary, to be able to diagnose EUS in case of suspicion.

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Fig. 14: EUS in bluegill (John Hawke and Al Camus acknowl.)

Fig. 15: EUS in channel catfish (John Hawke and Al Camus acknowl.)

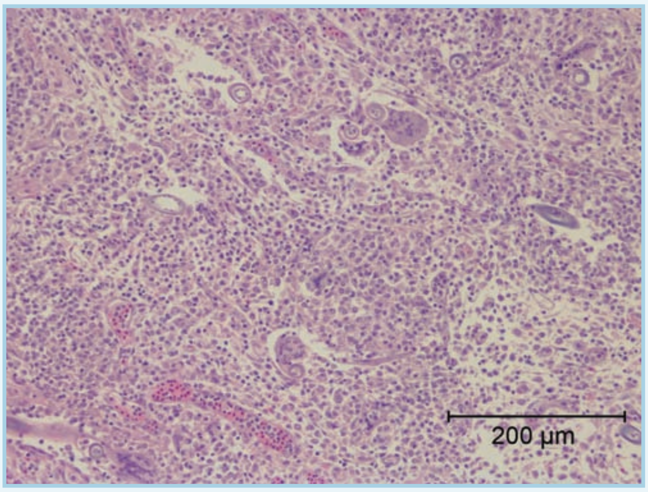


Fig. 16: EUS in fish muscle tissue at the border of a lesion (John Hawke and Al Camus acknowl.)

Diseases/Pathogens of molluscs

3.4.14 Introduction on mollusc diseases/pathogens

Molluscs, including wild and cultivated ones, live in the open water. It means that firstly they can get infected with all kinds of pathogens, as they filter the water and secondly, treatments can't be used because of their potential impact on the environment. Moreover molluscs do not produce antibodies and thus it is not possible to use vaccine. Considering global mollusc aquaculture, transfers and introductions are highly significant and currently recognised as a major source of epizootics and mass mortality outbreaks. In this context, prophylactic measures are the only available way to avoid pathogen spread. In an area free of a disease, the key point is to avoid any introduction of infected stocks. For that purpose, standards, guidelines and recommendations are provided at international, regional and national levels. However, we have to keep in mind that transfers are not the unique route of disease introduction or emergence; other hazards may exist such as introduction of pathogens through ballast waters. In an infected zone, goal is likely to reduce the impact of diseases. This needs better understanding of diseases and host-pathogen relationships.

Histopathology and cytology (tissue imprints) are still widely used in diagnostic laboratory for mollusc diseases. Histopathology provides general information including physiological aspects and general health condition and allows screening the presence of several pathogens in all the organs present in the slide. Imprints are very cheap and quick to perform. However these techniques are sometimes not sufficient notably when confirmation is required or for some diseases like bacterial and viral infections. Nevertheless, efforts have been made to improve diagnostic methods for diseases of molluscs and molecular techniques - including PCR and *in situ* hybridization - have been developed during the last ten years. Unfortunately, most of the time, these new techniques are not validated.

In this chapter, the tests used for diagnosis of disease hazards of molluscs are presented. In Table 4.1.b an overview is presented of the susceptible European species per pathogen and the current test methods used or that should be used by European laboratories. One by one we will present the data. References for all mollusc diseases/pathogens are given in a joint reference list 3.4.20.

Additionally, the task force added data on diagnosis and detection of non-WP listed diseases/pathogens of mollscs. These data deal about Herpesvirus of oyster (OsHV-1), *Bonamia ostreae*, and *Marteilia refringens*, and can be found in Annex 7.4.1-7.4.3, and their evaluation in Annex 7.5.

3.4.15 *Nocardia* spp. (Pacific oyster nocardiosis – *Nocardia crassostreae*)

Nocardia crassostreae is an Actinomycete bacterium that causes disease in the oysters *Crassostreae gigas* and *Ostrea edulis*. The extent of associated mortalities has not been accurately measured but estimated at about 35% in some localities. In British Columbia (Canada), European flat oysters *Ostrea edulis* cultured alongside infected *C. gigas* have been found infected by *N. crassostreae* but mortality rate is unknown. Recently, a few flat oysters and Pacific oysters from The Netherlands (Lake Grevelingen) have been found infected by *N. crassostreae* during mortalities due to poor environmental conditions (Engelsma et al., submitted).

Clinical pathology

Nocardia crassostreae (Actinomycete bacteria) causes infection in the oysters *Crassostreae gigas* and *Ostrea edulis*. The bacteria can be found all year as bacterial foci primarily in gonadal follicles, vesicular connective tissue, gills, heart and adductor muscle, but they can finally invade every tissue. They are usually associated with mortalities during the late summer and fall.

Agent description

Nocardia crassostreae is an Actinomycete bacteria in the Nocardia otitidiscaviarum rRNA sub-group.

Confirmatory techniques for diagnosis

- Sequencing of 16S rRNA can be used after culture of bacteria on Brain Heart Infusion (BHI) agar plates (Friedman, Beaman et al. 1998). The GenBank/EMBL accession numbers for the sequences reported in Friedmann et al. paper are: U92799 and U92800.
- A new PCR technique incorporating a lysozyme treatment after proteinase K digestion of tissue has been recently developed (Bower, Goh et al. 2005). This PCR technique uses two primers specific of *Nocardia crassostreae*.
- An alternate technique is to get a specific pattern after PCR-randomly amplified polymorphic DNA fingerprinting from DNA extracted from bacterial culture (Isik and Goodfellow 2002).
- Previous ISH technique developed by using a primer as a probe is non species specific and so has no value as a confirmatory technique.

Screening techniques for the pathogen

- Imprints of tissue – particularly mantle or adductor muscle with yellow-green pustules – show Gram-positive, acid-fast, branched colonies of filamentous bacteria.
- Histology is the current screening technique. Dense clumps of Gram-positive and PAS-positive (Friedman, Beattie et al. 1991), branching, beaded, basophilic bacteria surrounded by haemocytes can be seen in most organs. Though Hematoxylin and Eosin staining does not specifically stain the bacteria, colonies can be easily distinguished from surrounding tissue.

Comments and recommendations on available diagnostic techniques

Culture of *Nocardia crassostreae* on special media is needed for DNA extraction and sequencing. The last PCR technique (Bower, Goh et al. 2005) is now species specific and so can confirm a suspected case of Nocardia crassostreae infection. The PCR technique seems to be more sensitive than histology for the very low or early infections but need to be validated. Sequencing after bacteria culture is too costly to be used as routine confirmatory technique.

What should we do for diagnosis at suspicion?

Nocardia crassostreae infection can be suspected when green or yellow pustules are visible on the mantle or adductor muscle but these are not specific symptoms and can also be observed on oysters infected by other organisms (e.g. *Mikrocytos mackini*). Imprints of pustules can be stained by a Gram technique. Small pieces of tissue including pustules or immediately around should be excised and frozen or fixed in ethanol for a PCR test. Remaining of the oyster can be fixed in Davidson's for histopathology examination.

EU-legislation related to techniques

Not listed by the EU legislation.

OIE recommendations related to techniques (& ref lab OIE)

Not listed by the OIE Manual of Diagnostic Tests for Aquatic Animals (2007 version) nor by the Aquatic Animal Health Code (2007 version).

Assessment

For confirmation, imprints of pustules can be stained by a Gram technique, and isolation of the bacterium may also be done. Small pieces of tissue including pustules or immediately around should be excised and frozen or fixed in ethanol for a PCR test. Remaining of the oyster can be fixed in Davidson's for histopathology examination. Histology is the current screening technique.

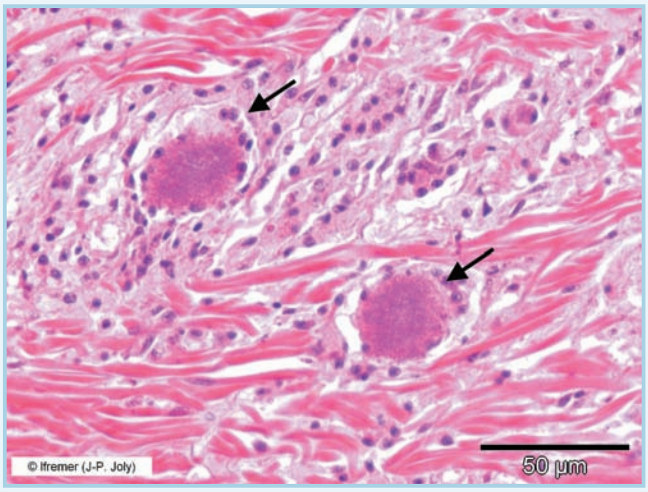


Fig. 17: *Nocardia crassostreae* bacterial colonies in the adductor muscle of *Ostrea edulis* (H&E staining)(IFREMER acknowl.)

3.4.16 *Candidatus Xenohaliotis californiensis* (withering syndrome)

The proposed new genus and species "*Candidatus* Xenohaliotis californiensis" is a rickettsia causing the disease known as "withering syndrome" of abalones, responsible for mortality since the mid 1980s among natural and cultured populations of abalones from the West coast of the USA (California) and Mexico (Baja California). Up to 95-100% of natural populations of black abalone *Haliotis cracherodii* from California islands near San Francisco disappeared in the late 1980s. Susceptible known species are: *Haliotis cracherodii* (black abalone), *H. rufescens* (red abalone), *H. corrugata* (pink abalone), *H. fulgens* (green abalone), and *H. sorenseni* (white abalone).There are suspicions that *H. discus hannai* and *H. midae* could be infected too. "*Candidatus* Xenohaliotis californiensis" has also been recently diagnosed in the European abalone *Haliotis tuberculata* from Spain, and suspected to be present in two hatcheries from Ireland and France (Balseiro et al., 2006).

Clinical pathology

Infected abalones are discoloured and weakened and can be detached easily from the substrate. They do not attempt to right themselves when turned upside down.

Agent description

Candidatus Xenohaliotis californiensis is a proposed new genus and new species of intracellular prokaryote with morphological characteristics of the class Proteobacteria, order Rickettsiales and family Rickettsiaceae, occurring in the epithelium of the intestinal tract.

Confirmatory techniques for diagnosis

- A PCR reaction using species specific primers (RA 5.1 and RA 3.6) has been developed (Andree, Friedman et al. 2000). This technique appears to be more sensitive than histology technique (Balseiro, Aranguren et al. 2006).
- An *in situ* hybridisation test using 4 probes designed from the small-subunit of ribosomal DNA has also been developed (Antonio, Andree et al. 2000).

Screening techniques for the pathogen

- Squash preparation of gastrointestinal tract can be stained by Hoechst solution (10 $\mu g.ml^{-1}$ of bisBenz-imide in distilled water) and observed with epifluorescent ultraviolet light and filters (356 nm excitation and 465 nm emission). Large bacterial inclusions in gut epithelium appear bright blue.

- In histology, colonies of bacteria appear in large intracellular colonies in the epithelium of the digestive tract (picture below) and particularly in the enzymes secreting cells of the digestive diverticula.

Comments and recommendations on available diagnostic techniques

Sensitivity and specificity of the PCR test are in the process of being assessed.

What should we do for diagnosis at suspicion?

Techniques to be used on all post-larval stages but preferably on oldest animals. Difficulties or impossibility to recover from an upside-down position, together with weakness, retraction of the mantle and the foot in severe cases, are symptomatic – though not specific - of withering syndrome of abalones. Usually necrotic parts of the foot tissue can also be visible.

Tissue squash of the post-oesophagus part of digestive tract stained by a modified Giemsa stain (e.g. Hemacolor) or by a fluorescent stain for nucleic acid is an easy and quick technique that can be used for presumptive diagnosis (Moore et al., 2001). Other parts of the animal can be fixed 24h in Davidson's fixative for further histology study and ISH technique to confirm the pathogen presence. Alternatively, small pieces of tissue (post-oesophagus, digestive gland and foot) can be sampled for the confirmatory PCR test.

EU-legislation related to techniques

Candidatus Xenohaliotis californiensis is listed by the EU legislation (Council Directive 95/70 EC).

OIE recommendations related to techniques (& ref lab OIE)

Candidatus Xenohaliotis californiensis is listed by the OIE Manual of Diagnostic Tests for Aquatic Animals (2007 version) and Aquatic Animal Health Code (2007 version).

The OIE recommends:

- PCR technique for **surveillance**
- Histopathology, ISH and PCR techniques as **presumptive** techniques
- ISH and SSU rDNA sequencing as **confirmatory** techniques (along with PCR when it is used with histopathology)

Reference lab for the OIE: Friedman Shellfish Laboratory, Washington, USA, Prof. C. Friedman: E-mail: carolynf@u.washington.edu

Assessment

It is recommended to follow the recommendations of the OIE.

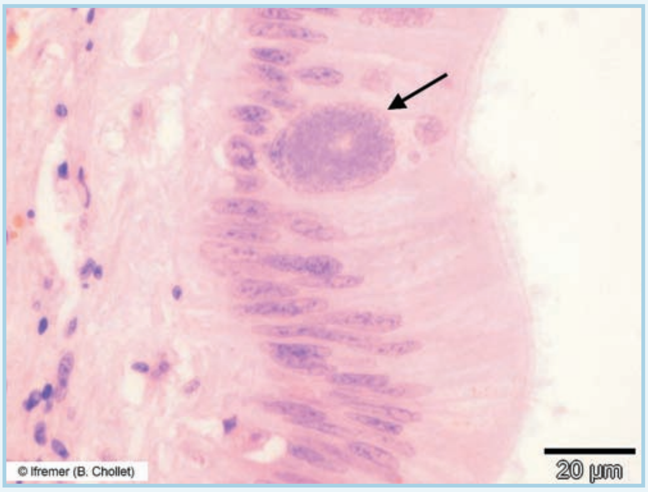


Fig. 18: Cytoplasmic vacuole containing "*Candidatus* Xenohaliotis californiensis" bacteria inside the gut epithelium of the abalone *Haliotis cracherodii* (H&E staining)(B.Chollet, Ifremer, acknowl.).

3.4.17 *Perkinsus olseni/atlanticus*

Perkinsus olseni/atlanticus is a parasitic dinoflagellate of clams. The sometimes massive aggregation of *Perkinsus* cells and haemocytes form lesions that may interfere with respiration and other physiological processes such as reproduction (fertility/fecundity, when large lesions occur in the gonads), growth and/or survival and thus have an impact on fishery productivity. Infection in *Ruditapes decussatus* has been associated with extensive mortalities in clam breeding areas located on the south coast of Portugal. However, on the Galician coast of Spain, perkinsosis did not appear to affect the energetic physiology of infected *R. decussatus* at about 15 °C but, Villalba and Casas (2001) speculated that higher temperatures may impact on disease severity. In France, a recent two-year study (2004-2005) showed that both cultivated and natural populations along the Atlantic and Mediterranean coasts are infected, prevalence being higher in the South. No abnormal mortalities were reported in these populations.

Susceptible hosts in Europe (non-exhaustive list) are: *Ruditapes philippinarum, Ruditapes decussates, R. rhomboids, Venerupis aurea, Venerupis pullastra,* and *Crassostrea gigas.*

Natural hosts in the world are: *Haliotis rubra, H. laevigata, H. scalaris,* and *H. cyclobates.*

Experimental hosts are: *Venerupis senegalensis* (= *pullastra*), *Pinctada sugillata*, and *Anadara trapezia.*

Clinical pathology

In heavily infected clams, *Perkinsus olseni/atlanticus* frequently induces the formation of white or light brown nodules on the gills, foot, gut, digestive gland, kidney, gonad and mantle.

Agent description

Perkinsus olseni/atlanticus is a pathogenic dinoflagellate of clams.

Confirmatory techniques for diagnosis

- A species specific PCR technique based on the amplification of part of the rRNA non transcribed spacer (NTS) region has been developed (Robledo J. A., Coss C. A. et al. 2000). Forward sequence (PA690F): 5′ ATG CTA TGG TTG GTT GCG GAC C 3′. Reverse sequence (PA690R): 5′ GTA GCA AGC CGT AGA ACA GC 3′. Expected amplicon of 690-bp. Specificity: PCR tested with *P. marinus* DNA extracted from *Crassostrea virginica* and *Perkinsus* sp. DNA extracted from *Macoma balthica* is negative. Sensitivity: lowest limit of detection of *P. olseni* isolated DNA is 0,01 µmol of NTS DNA.
- Sequencing of the NTS can also be used to assess the species of Perkinsus observed in clams (Murrell A., Kleeman S. N. et al. 2002).
- A pair of primers has also been designed from the intergenic spacer (ITS) sequence between the 5S and the 18S rRNA to produce a PCR-based diagnostic test (de la Herran R., Garrido-Ramos M. A. et al. 2000). Forward sequence (PK1): 5′ ACC AGT CAC CAC AGG GCG TAA T 3′. Reverse sequence (PK2): 5′ GTA GCG TGC TCT GAT GAT CAC T 3′. Expected amplicon of 554 bp. Tested with *P. olseni* extracted from infected *Ruditapes decussatus*.

Screening techniques for the pathogen

- The standard diagnostic technique for *Perkinsus* sp. diagnosis in molluscs is the culture of host tissue (usually gills) in the "Ray's Fluid Thioglycolate Medium" (RFTM). This technique has been adapted for *Perkinsus olseni* diagnosis in whole clams (Almeida M., Berthe F. et al. 1999). The biggest limitation of the technique is the time it needs to get hypnospores that can be visualised after staining by Lugol's iodine (usually between 5 and 7 days of incubation in RFTM).
- Histology can be used as a screening technique. Trophozoites are often large (up to 40 µm) and can easily be visualised by histology. In most clams, infection is usually associated with an infiltration of numerous haemocytes into the surrounding tissues. Encapsulation and phagocytosis is common.

Comments and recommendations on available diagnostic techniques

The first PCR technique has only been validated against *P. marinus* and *Perkinsus* sp. from *Macoma balthica*. Specificity and sensitivity: the technique amplifies only DNA from *P. olseni* and can detect 0.01 amol of cloned *P. olseni* NTS DNA in the presence of 1 µg of clam DNA. The second PCR technique has not been validated. Sequencing is too costly to be routinely used in diagnosis but can be used to confirm the species.

What should we do for diagnosis at suspicion?

White nodules can be observed on the surface of the mantle, digestive gland and gill tissues of highly infected clams. In case of suspicion of perkinsosis due to *Perkinsus olseni*, gills are placed in Ray's Fluid Thioglycolate Medium for 5 to 7 days. In parallel a piece of gills should be fixed in ethanol for molecular analysis (PCR/ sequencing).

EU-legislation related to techniques

Listed by the EU legislation.

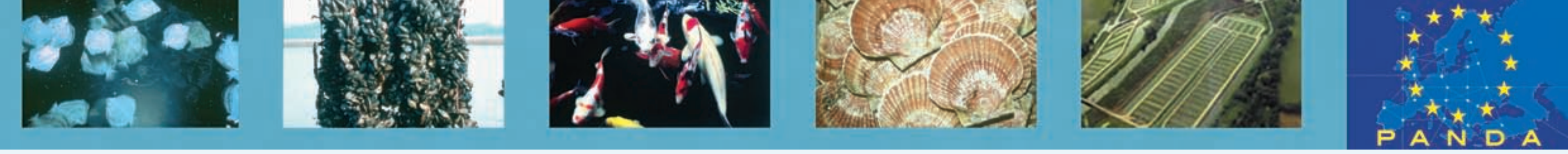
OIE recommendations related to techniques (& ref lab OIE)

Perkinsosis due to *Perkinsus olseni* is listed by the OIE Code (2007 version) and the Manual of Diagnostic Tests for Aquatic Animals (2007 version).

The OIE recommends:

- RFTM culture of tissue for **surveillance**
- PCR technique for **presumptive** diagnostic
- DNA sequencing for **confirmatory** diagnostic

Reference lab for the OIE: Virginia Institute of Marine Science, Gloucester Point, USA, Dr. E.M. Burreson: E-mail: gene@vims.edu



Assessment

It is recommended to follow the recommendations of the OIE.

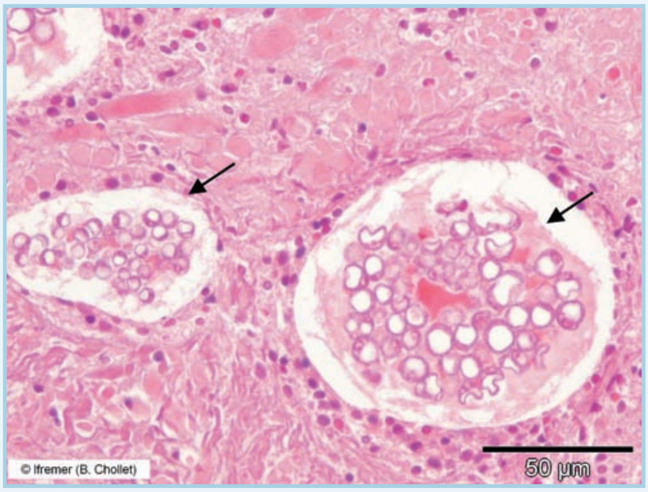


Fig. 19: Muscle tissue from the mantle of *Ruditapes philippinarum* showing trophozoites of *Perkinsus olseni* (arrows). Note the haemocytes surrounding the parasites (H&E staining)(B. Chollet acknowl.).

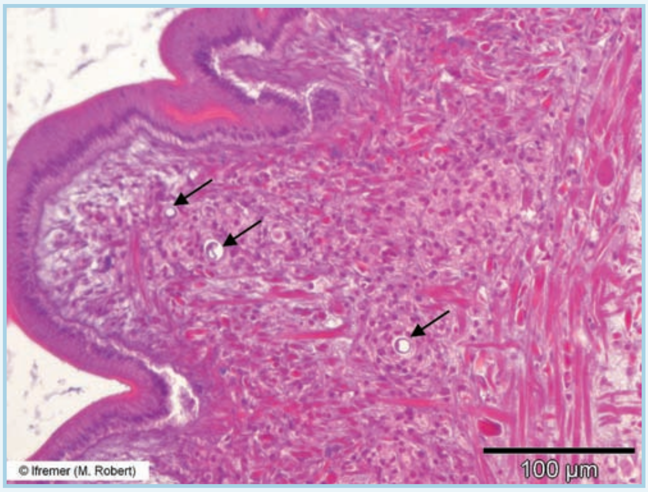


Fig. 20: Mantle of *Ruditapes philippinarum* showing trophozoites of *Perkinsus olseni* (arrows). Note the numerous haemocytes surrounding the parasites (H&E staining)(M. Robert, Ifremer acknowl.).

3.4.18 *Perkinsus marinus*

Perkinsus marinus is a parasitic dinoflagellate which causes disease of economic importance in *Crassostrea virginica*. *Crassostrea gigas* is the susceptible host in Europe, and can be infected but does not develop the disease (Calvo, Luckenbach et al. 1999).

Clinical pathology

Dead of gaping oysters are the main clinical signs; thin, watery tissue and pale digestive gland are the gross signs. However these signs are not specific to infection with *Perkinsus marinus*. *Crassostrea gigas* can be infected but do not develop the disease (Calvo, Luckenbach et al. 1999).

Agent description

Perkinsus marinus is a pathogenic dinoflagellate of oysters. Recent investigations indicate that this parasite may not belong in the Phylum Apicomplexa where it was initially classified but rather it seems to be more closely related to the Dinoflagellida.

Confirmatory techniques for diagnosis

- A PCR technique based on the amplification of a part of the rRNA non transcribed spacer (NTS) region has been developed (Marsh A. G., Gauthier et al. 1995; Robledo J. A., Gauthier et al. 1998). A set of primers (PmarITS-70F and PmarITS-600R) for two species specific standard or real-time PCR techniques has also been designed from the intergenic spacer sequence (ITS) (Audemard, Reece K. S. et al. 2004). These last two techniques can detect less than a cell DNA of P. marinus in the reactive medium.
- DNA sequencing of the ITS region can be done to identify the species, by comparing the ITS region nucleotide sequences with reference sequences deposited in the GenBank database (http://www.ncbi.nih.gov/entrez/).

Screening techniques for the pathogen

- The standard diagnostic technique for *Perkinsus* sp. detection in bivalves is the culture of host tissue (usually gills) in the "Ray's Fluid Thioglycolate Medium" (RFTM). The biggest limitation of the technique is the time it needs to get hypnospores that can be visualised after staining by Lugol's iodine (usually between 5 and 7 days of incubation in RFTM).
- Histology can be used as a screening technique. Sections of tissue should include digestive gland and gills. Positive result is the occurrence of spherical cells about 2-10 µm in diameter with a large vacuole and an eccentrically displaced nucleus (see picture below). Cells are often phagocyted by haemocytes.
- In advanced infection only, smears can also be used as a screening technique. Collect haemolymph with a syringe inserted into the

adductor muscle, place a drop of haemolymph on a slide and smear. Presence of spherical cells about 2-15 µm in diameter with a large vacuole and an eccentrically displaced nucleus indicates the presence of *Perkinsus* sp.

Comments and recommendations on available diagnostic techniques

The NTS PCR assay has been validated against fluid thioglycollate culture (Robledo J. A., Gauthier et al. 1998). The ITS PCR assay has not been validated against fluid thioglycollate culture. However the ITS primers are recommended over the NTS assay because they are more likely to amplify all *Perkinsus marinus* strains (Audemard, Reece K. S. et al. 2004).

What should we do for diagnosis at suspicion?

In case of suspicion of perkinsosis due to *Perkinsus marinus*, oyster tissues including heart, rectum, piece of gill and mantle are placed in Ray's Fluid Thioglycolate Medium for 5 to 7 days. In parallel piece of tissues should be fixed in ethanol for molecular analysis (PCR/ sequencing).

EU-legislation related to techniques

Perkinsus marinus is listed by the EU.

OIE recommendations related to techniques (& ref lab OIE)

Infection with *Perkinsus marinus* is listed by the OIE Code (2007 version) and by the OIE Manual of Diagnostic Tests for Aquatic Animals (2007 version).

The OIE recommends:

- RFTM culture of tissue for **surveillance**
- PCR technique for **presumptive** diagnostic
- PCR technique and DNA sequencing for **confirmatory** diagnostic

Reference lab for the OIE: Virginia Institute of Marine Science, Gloucester Point, USA, Dr. E.M. Burreson: E-mail: gene@vims.edu

Assessment

It is recommended to follow the recommendations of the OIE.

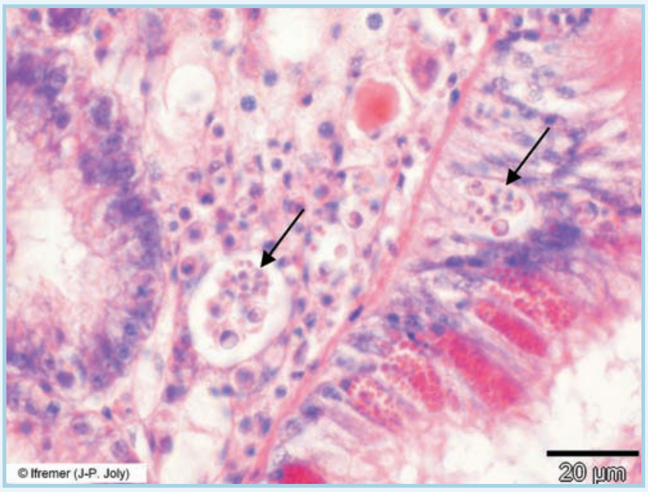


Fig. 21: *Perkinsus marinus* cells observed in conjonctive tissue of digestive gland from *Crassostrea virginica* (H&E staining)(J.-P. Joly acknowl.).

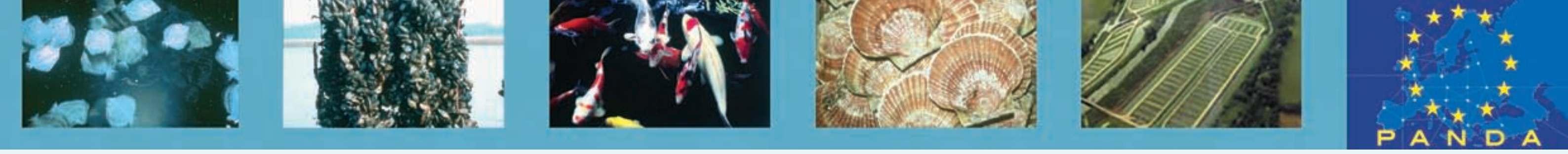
3.4.19 *Marteilioides* spp. (*M. chungmuensis*: Marteilioidosis)

Marteilioides chungmuensis is responsible for marteilioidosis, an oocyte infection of Pacific oysters *Crassostrea gigas*. The disease is reported in Korea and Japan. Reported prevalences fluctuate according to the diagnostic tool, the area and to the season. Highest prevalences are reported during spawning and the gonadal regeneration period. Recent works showed that male can also be infected but the parasite seems to be excluded from male oysters without initiating sporulation.

The Iwagaki oyster, *Crassostrea nippona* has been showed to be susceptible to the disease when transplanted to an enzootic area. Parasites similar to *Marteilioïdes chungmuensis* were described in *Crassostrea echinata* (Australia) and *Ruditapes philippinarum* (Korea). However, molecular parasite characterization has not been performed yet. Some of the infections described as "oyster egg diseases" may be attributed to M. chungmuensis. The susceptible known species are *Crassostrea gigas* and *Crassostrea nippona*.

Clinical pathology

Infected eggs are released or retained within the follicle, leading to visible distension of the mantle surface and thus to marketability loss of infected oysters. Infection can also cause spawning failure by delaying spawning and destroying ripe oyster oocytes.



Agent description

Marteilioides spp. (*M. chungmuensis* is a protistan of the phylum Paramyxea, which infects cytoplasm of mature oocytes of Pacific oysters, and affects significantly the reproductive output of infected female oysters. The vegetative stages have amoeboid primary cell that cleave internally to form secondary cells (sporonts) and sporulation consists of sporonts that produce a single pluricellular spore and then degenerate such that the spore is enveloped by a cytoplasmic residuum and the plasmalemma of the sporont.

Confirmatory techniques for diagnosis

- The PCR protocol previously described in the section "screening techniques" can also be used as a confirmatory technique.
- An *in situ* hybridization (ISH) protocol has also been developed using three Dig-labelled oligonucleotide probes MCSP-05, MCSP-06 and 6-R (Itoh et al. 2003). No non-specific binding was observed when tests were performed with other Paramyxean like *Marteilia refringens* and *M. sydneyi*. ISH can help to detect immature stages of the parasite which are more difficult to detect in traditional histological sections.
- Transmission electron microscopy is time consuming and cannot be applied in routine but is recommended when *Marteilioides* like parasites are described in a new host species. Moreover, transmission electron microscopy can help to differentiate *M. chungmuensis* from another member of this genus: *M. branchialis* (found in Australia). This last one is characterized by the presence of two concentric cells (rather than three) within the spore. In addition, *M. chungmuensis* in *Crassostrea gigas* contains only two to three sporonts per primary/stem cell compared with two to six for *M. branchialis.* Multivesicular bodies resembling those of *Marteilia* sp. are present in *M. branchialis* stem cells but absent from those of *M. chungmuensis*.
- Sequencing is recommended as one of the final steps for confirmatory diagnostic. Targeted region is SSU rDNA. Obtained sequences should be compared with available ones in gene banks.

Screening techniques for the pathogen

- Smears or imprints can be realised using nodules sometimes present on infected gonad. *Marteilioides chungmuensis* is observed in the cytoplasm of infected ova or sometimes extracellularly, liberated from the ova. The parasite can be present under different stages.
- Stem (primary) cells contain secondary cells. These may, in turn, contain developing sporonts, giving rise to a single tertiary cell by endogenous budding. Each tertiary cell forms a tricellular spore by internal cleavage.
- Histology allows observing parasites inside ovocytes. Different parasite stages can be observed and are similar to those reported for smears (see picture below). The parasite is quite easy to recognize because of its specific cellular localisation and because of its size. However, initial infection and primary cells can be more difficult to detect.
- A nested-PCR using primers OPF-2-OPR-2 and OPF-3-OPR-3 and amplifying 672 and 447 bp of the SSU rDNA respectively was developed to detect the parasite in *Crassostrea gigas* (Itoh et al. 2003). The detection limit of this technique has not been determined yet. However, by comparing histology and PCR results, this last technique allows to detect the parasite in some oysters found negative by the first one suggesting that PCR is more sensitive than histology. These primers could not amplify other Paramyxean like *Marteilia refringens* and *M. sydneyi*.

Comments and recommendations on available diagnostic techniques

Protocols for PCR and *in situ* hybridization are available in pre cited articles. However both techniques need to be validated and more specifically specificity and sensitivity values are lacking.

What should we do for diagnosis at suspicion?

Abnormal masses with a nodular appearance can be observed in highly infected individuals. Nodules, if present, should be used for smears which enable the rapid detection of *Marteilioïdes chungmuensis*. A piece of gonad should also be fixed in ethanol for molecular analysis and the remaining gonad tissue can be fixed in Davidson's for histological examination.

EU-legislation related to techniques

Marteilioïdes chungmuensis is not listed by the EU legislation.

OIE recommendations related to techniques

Marteilioïdes chungmuensis is not listed by the OIE.

Assessment

It is recommended to use histology, cytology and PCR for screening, and PCR, DNA sequencing, ISH (in situ hybridization), and/or Transmission Electron Microscopy for confirmation, see Table 5.1.b.

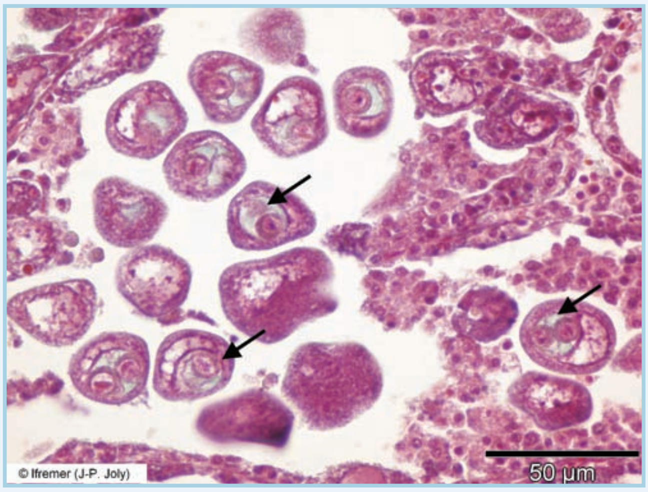


Fig. 22: Gonad of *Crassostrea gigas* showing M*arteilioides chungmuensis* inside ovocytes (arrows). Note the compression of ovocyte nucleus (Masson trichrome staining)(J.-P. Joly acknowl.).

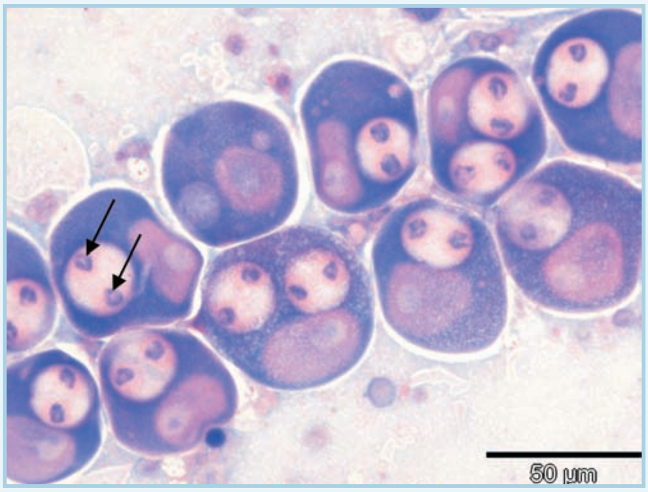


Fig. 23: Gonad imprint of a Pacific oyster *Crassostrea gigas* from Korea showing ovocytes infected by *Marteilioides chungmuensis*. Arrows: secondary cells (Hemacolor staining)(IFREMER acknowl.).

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Diseases/Pathogens of crustaceans

3.4.21 Introduction to crustacean diseases/pathogens

The following pathogens: 4 viruses, a bacteria and a fungus are all significant pathogens to farmed commercial crustaceans. A rickettsia-like organism (*Coxiella cheraxi*) was reported at least one time and only in Australia to mortalities in crayfish. Except for the bacterial disease (Gaffkemia) of the lobster *Homarus americanus* all the other pathogens are listed by the OIE and 3 of them (TSV, YHV and WSSV) are listed as "Notifiable diseases to the OIE".

One by one we will present the data. References for all mollusc diseases/pathogens are given in 3.4.27 in a joint reference list. Additionally, the task force added data on diagnosis and detection of non-WP listed diseases/pathogens of crustaceans: Data on gaffkemia (*Aerococcus viridans*) and Crayfish plague by *Aphanomyces astaci* can be found in Annexes 7.4.4 and 7.4.5, and their evaluation in Annex 7.5.

3.4.22 Yellowhead disease (YHD)

Yellowhead Virus causes mass mortality in shrimp. Only genotype 1 (considered as the true agent of Yellowhead disease) can cause mortalities in *Penaeus monodon, Litopenaeus vannamei, L. stylirostris, Farfantepenaeus aztecus, Fa. duorarum, Macrobrachium sintangene, Palaemon styliferus* and *P. serrifer*.

There are variations in the susceptibility of different penaeid species to disease. Yellowhead disease can cause up to 100% mortality in infected *P. monodon* ponds within 3 days of the first appearance of clinical signs (OIE, 2006). The pathogen has been reported from Asia, Australia, Bangladesh, China PR, India, Indonesia, Malaysia, Philippines, Sri Lanka, Taiwan, Thailand, USA (Texas,) and Vietnam.

Clinical pathology

Mass mortality in shrimp: Moribund shrimp may cessate to feed, congregate at pond edges near the surface, showing a bleached overall appearance and a yellowish discoloration of the cephalothorax. Often, whitin a few days, total crop loss may occur.

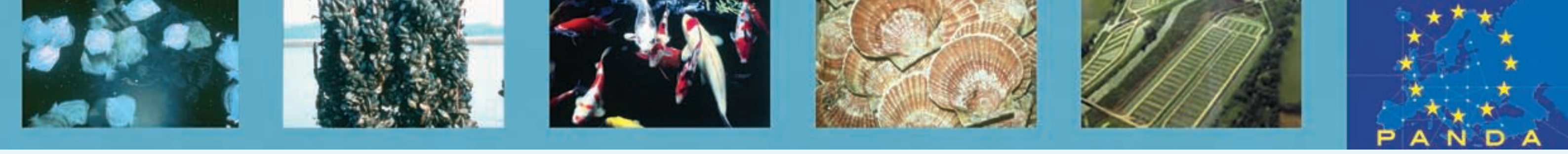
Agent description

The disease is due to an enveloped, rod-shaped virus, 40-60 nm in diameter and 150-200 nm length, developing in cytoplasm of cell from tissues of ectodermal and mesodermal origin. The agent, YHV (Yellowhead virus), was classified as a single species in the genus Okavirus, family Roniviridae, order Nidovirales (OIE, 2006). GAV (Gill associated virus) and 4 other genotypes occur commonly in *P.monodon*, but never associated with disease.

The viral genome contains a single molecule of ssRNA, 26,235 nt long, structured in 4 ORF. Only genotype 1 (considered as the true agent of Yellowhead disease) can cause mortalities in *Penaeus monodon, Litopenaeus vannamei, L. stylirostris, Farfantepenaeus aztecus, Fa. duorarum, Macrobrachium sintangene, Palaemon styliferus* and *P. serrifer.*

Confirmatory techniques for diagnosis

- Of course, the above RT-PCR can be use, but more sophisticated RT-nested PCR are available using different sets of primers which are specific for GAV and YHV, or differentiating all the known genotypes in the Yellow Head complex (GAV and YHV).
- Tang and Lightner (1999) reported the use of ISH (in situ hybridization) for YHV detection. The probe is constructed by PCR-labeling using the following primers:



- – YHV1051F: 5′-ACA TCT GTC CAG AAG GCG TC-3′
- – YHV1051R: 5′-GGG GGT GTA GAG GGA GAG AG-3′

- Observation of YHV or GAV particles in TEM (transmission electron microscopy) is considered too as a confirmatory diagnostic method.

Screening techniques for the pathogen

- In histology, prominent features correspond to a generalized multifocal necrosis associated with pycnosis and karyorrhexy. Lesions in affected tissues are characterized by the presence of basophilic cytoplasmic inclusions in of mesodermic or epidermic cells in origin.
- RT-PCR can be used as a screening technique using the following primers:
  - – 10F: 5′-CCG CTA ATT TCA AAA ACT ACG-3′
  - – 144R: 5′-AAG GTG TTA TGT CGA GGA AGT-3′
  - – Diagnostic is considered positive when obtaining a 135 bp amplicons.

Comments and recommendations on available techniques

All the diagnostic techniques reported above are highly specific. The use of ISH has the advantage to present both a high specific diagnostic confirmed by tissular and cellular location of viral lesions.

What should we do for diagnosis at suspicion?

Follow the recommendations of the OIE (2006), under confirmatory diagnosis, see below.

EU-legislation related to techniques

Yellowhead disease is newly listed by the EU as a crustacean exotic disease (Annex 4, 2006/88/EC)

OIE recommendations related to techniques (& ref lab OIE)

The disease is notifiable to the OIE and listed by the OIE Manual of Diagnostic Tests for Aquatic Animals (2006). The methods (OIE, 2006) currently available for surveillance, detection, and diagnosis of YHV are listed, see below.

The designations used indicate:

A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity;

B = the method is a standard method with good diagnostic sensitivity and specificity;

C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and

D = the method is presently not recommended for this purpose.

These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

The OIE recommends for:

- Surveillance
  - – PCR (A)
- Presumptive diagnosis
  - – Histopathology (A)
  - – Antibody based assays (A)
  - – PCR (A)
  - – DNA Probes *in situ* (A)
- Confirmatory diagnosis
  - – Histopathology (B)
  - – Transmission electron microscopy (A)
  - – Bioassay (B)
  - – Antibody based assays (B)
  - – PCR (A)
  - – DNA Probes *in situ* (A)
  - – Sequencing (A)

For all other combinations of the above tests with the goals of testing the OIE gives C or D, so, less to non acceptable. See OIE, 2006.

To declare freedom: Two steps PCR negative results are required. A positive result must be confirmed by sequencing.

OIE reference laboratory: Australia Animal Health Laboratory (AAHL), Geelong, Dr. P. Walker: E-mail: peter.walker@csiro.au

Assessment

All the diagnostic techniques reported above are highly specific. The use of in situ hybridization (DNA probes in situ) has the advantage to present both a high

specific diagnostic confirmed by tissular and cellular location of viral lesions.

3.4.23 White spot disease (WSD or WSSD), White spot syndrome (WSS)

Whit Sport Syndrome Virus causes White Spot Disease in all decapod crustaceans from marine to freshwater sources (comprising brackishwater sources), from eggs to broodstock.

Clinical pathology

The disease is characterized by the presence of white spots on the carapace of diseased animals, with a high degree of colour variation with a predominance of reddish and pinkish discoloured shrimp, reduction in feed intake, increasing lethargy, movement of moribund shrimp to the water surface and pond/tanks edges, and consequent attraction of shrimp-eating birds (OIE, 2006).

Agent description

The disease is due to an enveloped, ovoid virus, 270-290 nm long and 120-150 nm in diameter. One extremity of the envelope forms a long tail-like structure (appendage), characteristic of the agent. The rod shaped nucleocapsid, is 300-350 nm long and 65-70 nm in diameter.

The genome is a single circular molecule of dsDNA, about 300 kbp (Marks et al., 2005). Virions develop in hypertrophied nuclei in infected cells from tissues of ectodermal and mesodermal origin. White spots on the carapace of diseased animals are due to calcified deposits due to calcium metabolism modifications of infected cells of the sub-cuticular epithelium. The rod shaped nucleocapsid, is 300-350 nm long and 65-70 nm in diameter. The agent was classified as a single species (White spot syndrome virus 1) in the genus Whispovirus, family Nimaviridae.

Confirmatory techniques for diagnosis

- By TEM, evidencing typical WSSV particles and nucleocapsids directly in blood samples is relatively easy.
- Of course, PCR reactions as mentioned above constitute excellent confirmatory techniques after histological examination.
- ISH (Nunan & Lightner, 1997), using a probe obtained by labeling PCR (First-step PCR as above), increases the security in the diagnostic validity by double checking the sensitivity of the probe and the specific location of symptoms.

Screening techniques for the pathogen

- Presence of white spots under the cuticle can be a good method for field diagnostic. Unfortunately, this sign cannot be often evidenced in numerous cases.

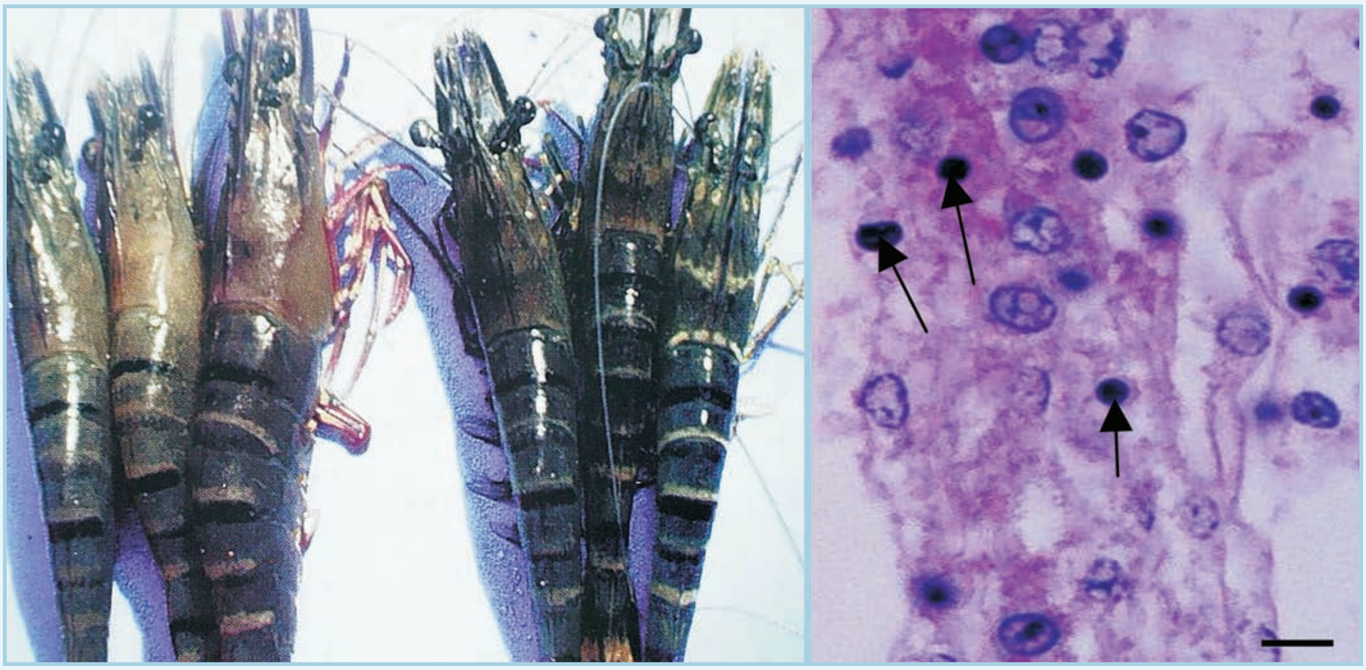
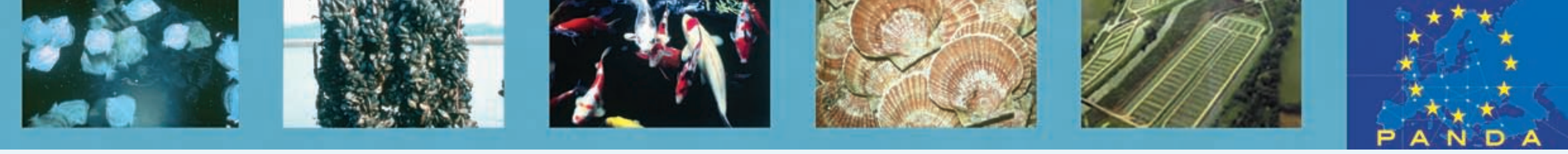


Fig. 24: Left: Clinical signs of the disease in 3 YHV infected *P. monodon* and 3 healthy shrimp. Right: YHV infected gills. H & E staining. Dark lesions are in cytoplasm of infected cells (arrows). H 1 E. Bar = 30 µm (D.V. Lightner acknowl.)



- Histology is available for routine diagnostic by demonstrating the presence of hypertrophied nuclei in target tissues such as sub-cuticular epithelium, connective tissue, hematopoietic and lymphoid organs, gills, etc.
- Moreover, these enlarged nuclei are highly Feulgen positive.
- By PCR, it is suggested to use one step or two-step PCR, the second particularly to detect WSSV in carrier stages. The protocol was described by Lo *et al*. (1996) using the following primers:

  First-step PCR: 146F1: 5′-ACT ACT AAC TTC AGC CTA TCT AG-3′

  146R1: 5′-TAA TGC GGG TGT AAT GTT CTT ACG A-3′

  The WSSV specific amplicons obtained has a size of 1447 bp.

  Second-step PCR (nested): 146F2: 5′-GTA ACT GCC CCT TCC ATC TCC A-3′

  146R2: 5′-TAC GGC AGC TGC TGC ACC TTG T-3′

  After the second step the amplicons obtained has a size of 941 bp.

Comments and recommendations on available techniques

Beside these PCR detection methods and dot-blot or in situ hybridization techniques here mentioned and suggested by the OIE Manual of Diagnostic tests for Aquatic Animals (2006), alternative protocols and other primer sets or probes have been reporters by numerous other investigators in this field.

What should we do for diagnosis at suspicion?

Follow the recommendations of the OIE (2006), under confirmatory diagnosis, see also below.

EU-legislation related to techniques

White spot disease is listed by the EU as a crustacean non exotic disease (Annex 4, 2006/88/EC)

OIE recommendations related to techniques (& ref lab OIE)

White spot disease is notifiable to the OIE and listed by the OIE Manual of Diagnostic Tests for Aquatic Animals (2006).

The disease is notifiable to the OIE and listed by the OIE Manual of Diagnostic Tests for Aquatic Animals (2006). The methods (OIE, 2006) currently available for surveillance, detection, and diagnosis of WSD are listed, see below.

The designations used indicate:

A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity;

B = the method is a standard method with good diagnostic sensitivity and specificity;

C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and

D = the method is presently not recommended for this purpose.

These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

The OIE recommends for:

- Surveillance
  - PCR (B for post larvae, A for juveniles and adults)
- Presumptive diagnosis
  - Histopathology (A)
  - Antibody based assays (A)
  - PCR (A)
  - DNA Probes *in situ* (A)
- Confirmatory diagnosis
  - Bioassay (B)
  - Histopathology (B)
  - Transmission electron microscopy (A)
  - Antibody based assays (B)
  - PCR (A)
  - DNA Probes *in situ* (A)
  - Sequencing (A)

For all other combinations of the above tests, including gross signs, with the goals of testing the OIE gives C or D, so, less to non acceptable. See OIE, 2006.

To declare freedom of WSD: Two-step PCR and sequencing are the prescribed methods and negative results are required.

OIE reference laboratories:

- Dept. of Life Science, Institute of Zoology, National Taiwan University, Dr. G. Chu-Fang Lo: E-mail: gracelow@ccms.ntu.edu.tw
- Aquaculture Pathology Section, Dept. of Vet. Science, University of Arizona, Tucson, USA, Prof. D. Lightner, E-mail: dvl@u.arizona.edu

Assessment

The techniques recommended by the OIE are recommended to use.

3.4.24 Taura Syndrome (TS)

Taura Syndrome Virus causes serious disease in shrimp: The principal host species are *Litopenaeus vannamei* and *L. stylirostris*; but hosts to TSV include too: *L. setiferus, L. schmitti, Penaeus monodon, Metapenaeus ensis, Fenneropaenaeus chinensis,*

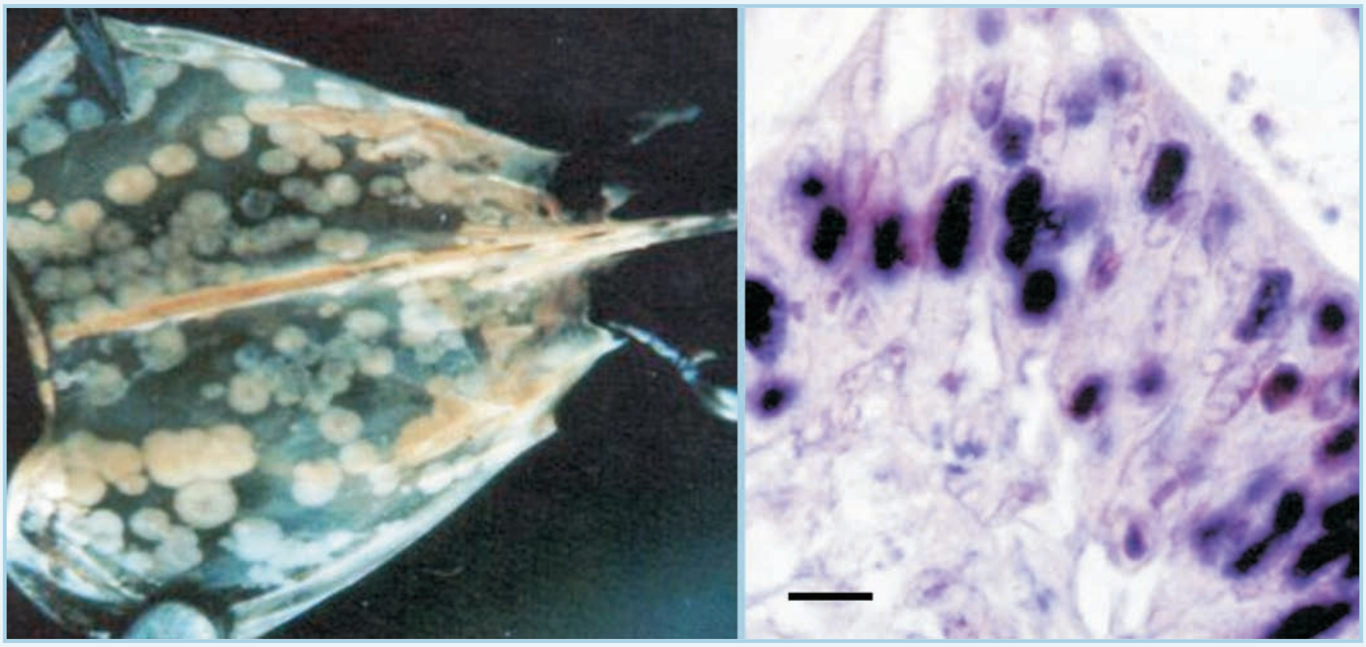


Fig. 25: Left: Clinical signs of White Spot Syndrome on the cephalothorax (D.V. Lightner acknowl.). Right: ISH of infected sub-cuticular epithelium; infected nuclei are strongly labeled with the DIG probe and are interspersed within healthy nuclei. Bar = 50 µm (J.R. Bonami acknowl.)

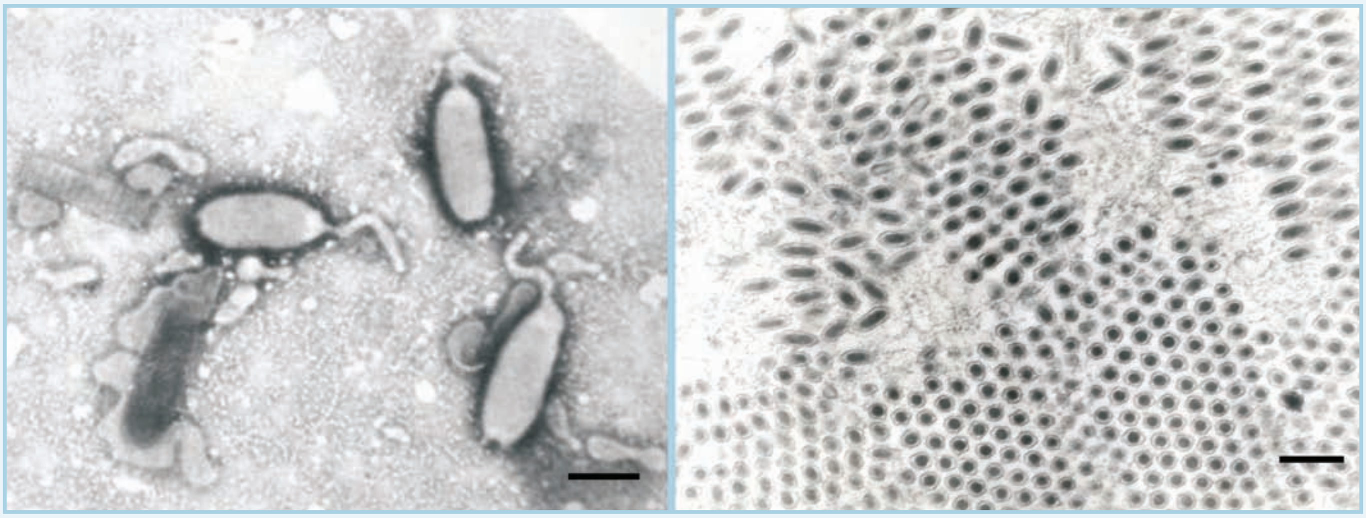


Fig. 26: WSSV enveloped virions exhibiting the tail-like structure and nucleocapsids. PTA. Bar = 100 nm.WSSV infected nucleus showing cross and longitudinal sections of virions and their arrangement in nucleoplasm. TEM. Bar= 500 nm.(J.R. Bonami acknowl.)

Marsupenaeus japonicus, Farfantepenaeus aztecus and *Fa. duorarum*.

Clinical pathology

Taura syndrome is a disease of shrimp. The clinical pathology is described more in detail in the OIE Manual of Diagnostic tests for Aquatic Animals (2006): TS disease has three distinct phases

- Acute phase: Gross signs include expansion of the red chromatophores giving the affected shrimp a general, overall pale reddish coloration and making the tail fan and pleopods distinctly red, with signs of focal epithelial necrosis in the cuticular epithelium, soft shells, and an empty gut. Numerous birds can be seen if shrimp are larger than 1 gram (OIE, 2006).
- Transition (recovery) phase: During the transition phassome shrimp in affected ponds show random, multifocal, irregularly shaped melanised cuticular lesions. These melanised spots are haemocyte accumulations indicating the sites resolving TS lesions in the cuticular epithelium. Such shrimp may or may not have soft cuticles and red-chromatophore expansion, and may be behaving and feeding normally (OIE, 2006).
- Chronic phase after molting, persistently infected shrimp show no obvious signs of disease, but, *L. vannamei* that are chronically infected with TSV may be less resistant to normal environmental stressors (i.e. sudden salinity reductions) than uninfected shrimp (OIE, 2006).

Agent description

The disease is due to a small icosahedral virus, 32 nm in diameter and no enveloped. The genome consists of a single piece of a linear, positive sense, molecule of ssRNA, 10,205 nt long (excluding a poly-A tail) divided in 2 ORF. The agent develops in cytoplasm of tissues of ectodermic and mesodermic origin.

The TSV was listed among the unassigned species of the Dicistroviridae family. At least three genotypic groups of TSV and two antigenic variants have been identified (OIE, 2006).

Confirmatory techniques for diagnosis

- Both protocols of RT-PCR and real-time RT-PCR described in the next section can be used as confirmatory techniques.

Screening techniques for the pathogen

- Histopathology and *in situ* hybridization are available for juveniles and adults. But for all development stages, the most recommended method is RT-PCR, amplifying a conserved genomic sequence of 231 nt (Nunan *et al*., 1998) using the following primers 9992F: AAG-TAG-ACA-GCC-GCG-CTT-3' and 9195R: TCA-ATG-AGA-GCT-TGG-TCC-3'.
- Real time RT-PCR method using TaqMan chemistry was described by Tang *et al*. (2004). Primers were selected from ORF1 region: TSV1004F: TTG-GGC-ACC-AAA-CGA-CAT-T-3' and TSV1075R: GGG-AGC-TTA-AAC-TGG-ACA-CAC-TGT-3'; labeled TaqMan probe, TSV-P1: CAG-CAG-TGA-CGC-ACA-ATA-TTC-GAG-CAT-C-3'.

Comments and recommendations on available techniques

DNA probes can be used for detection by in situ hybridization.

What should we do for diagnosis at suspicion?

Follow the recommendations of the OIE (2006), under confirmatory diagnosis, see also below.

EU-legislation related to techniques

Taura syndrome is newly listed as a crustacean exotic disease (Annex 4, 2006/88/EC).

OIE recommendations related to techniques (& ref lab OIE)

Taura syndrome (TS) is notifiable to the OIE and listed by the OIE Manual of Diagnostic Tests for Aquatic Animals (2006).

The methods (OIE, 2006) currently available for surveillance, detection, and diagnosis of TS are listed, see below.

The designations used indicate:

A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity;

B = the method is a standard method with good diagnostic sensitivity and specificity;

C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and

D = the method is presently not recommended for this purpose.

These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

The OIE recommends for:

- Surveillance
  - Histopathology (B, not for larvae)
  - DNA Probes *in situ* (B, only for juveniles and adult shrimp)
  - RT-PCR (A)
- Presumptive diagnosis
  - Gross signs (B)
  - Histopathology (A)
  - Antibody based assays (B)
  - DNA Probes *in situ* (A)
  - RT-PCR (A)
- Confirmatory diagnosis
  - Histopathology (A)
  - Antibody based assays (B)
  - DNA Probes *in situ* (A)
  - RT-PCR (A)
  - Sequencing (A)

For all other combinations of the above tests and bioassays, direct light microscopy, and transmission electron microscopy, with the goals of testing the OIE gives C or D, so, less to non acceptable. See OIE, 2006.

To declare freedom: 2 years of history of negative test results for TSV using RT-PCR on samples of appropriate type and size.

OIE reference laboratory: Aquaculture Pathology Section, Dept. of Vet. Science, University of Arizona, Tucson, USA, Prof. D. Lightner, E-mail: dvl@u.arizona.edu

Assessment

Follow the OIE recommended tests, as mentioned above.

3.4.25 Infectious hypodermal and haematopoietic necrosis (IHHN)

IHHN virus causes serious disease in shrimp: Principal host species include *L. stylirostris, L. vannamei* and *P. monodon*, the principal cultivated penaeid, but most penaeid species can be infected (Lightner, 1996).

Clinical pathology

IHHN is a viral disease of shrimp. The clinical pathology is described more in detail in the OIE Manual of Diagnostic tests for Aquatic Animals (2006):

Gross signs are not IHHN specific. Juvenile shrimp might show a marked reduction in food consumption,

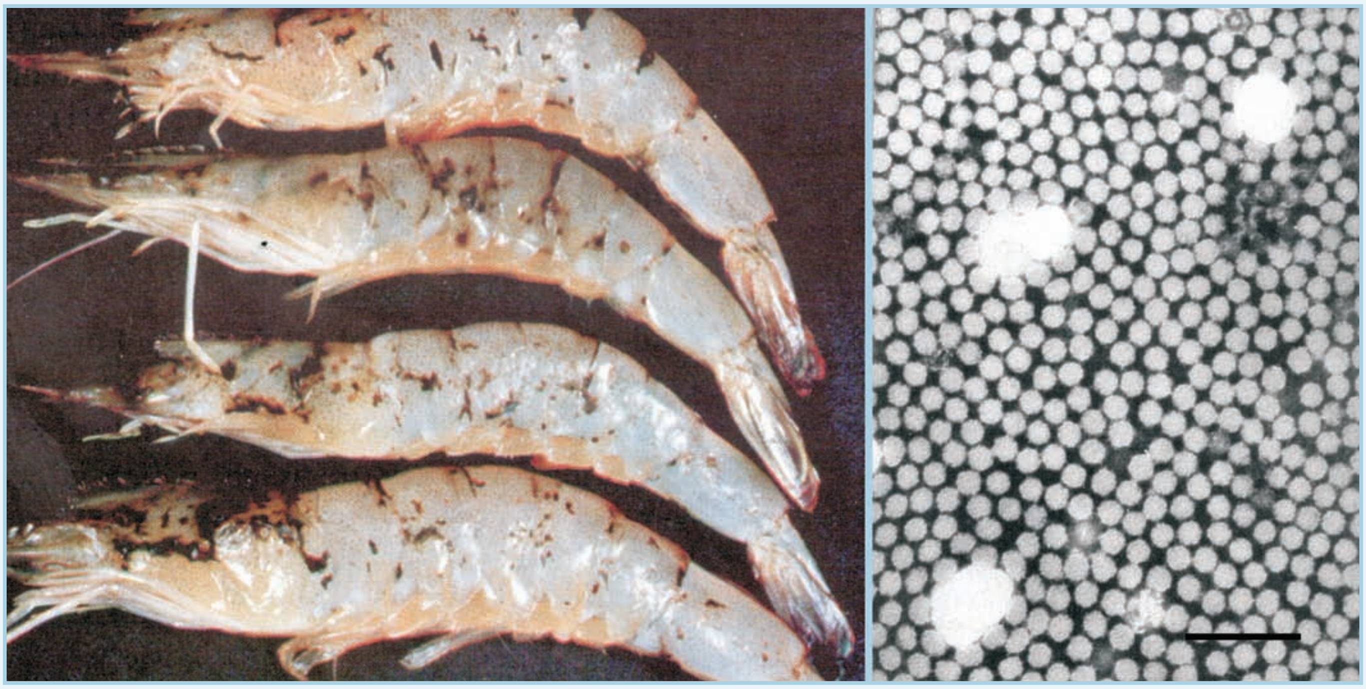
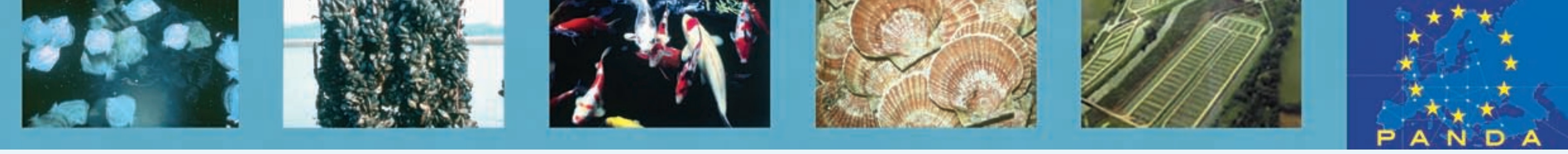


Fig. 27: Left: Clinical signs of *L.vannamei* infected with TSV (D. V. Lightner acknowl.). Right: Purified TS virions. PTA. Bar = 100 nm



followed by changes in behaviour and appearance, slow rising in tanks, to become motionless, then roll-over and slowly sink (ventral side up) to the tank bottom, for several hours until exhausted, or attacked by other shrimp. *Litopenaeus stylirostris* at this stage of infection often have white or buff-coloured spots in the cuticular epidermis, especially at the junction of the tergal plates of the abdomen, giving such shrimp a mottled appearance. This mottling later fades in moribund *L. stylirostris* as such individuals become more bluish. In *L. stylirostris* and in *P. monodon* with terminal phase IHHNV infections, moribund shrimp are often distinctly bluish in colour, with opaque abdominal musculature. Chronic disease might occur in infected populations of juvenile or older *L. vannamei*, which might display a bent or otherwise deformed rostrum, a deformed 6th abdominal segment, wrinkled antennal flagella, cuticular roughness, 'bubble-heads', and other cuticular deformities. Populations of juvenile shrimp with RDS display disparate growth with a wide distribution of sizes and many smaller than expected ('runted') shrimp (OIE, 2006).

Agent description

The etiological agent, the IHHNV, is a small icosahedral particle, 20-22 nm in diameter, containing a genome formed by a single linear molecule of ssDNA with an estimated size of about 4.1 kbp. The virus is located both in nucleus and cytoplasm of infected cells.

The agent (called *Penaeus stylirostris* densovirus: PstDNV) is considered as a tentative species in the genus Brevidensovirus, subfamily Densovirinae, family Parvoviridae (Fauquet *et al.*, 2005). Principal host species include *L. stylirostris, L. vannamei* and *P. monodon*, the principal cultivated penaeid, but most penaeid species can be infected (Lightner, 1996).

Confirmatory techniques for diagnosis

- Both histopathology,
- PCR and
- ISH methods are considered as confirmatory methods in the IHHNV diagnostic, even though they can be used as screening techniques.
- Of course, for low degree of infection one-step or better double-step PCR are suggested.

Screening techniques for the pathogen

- Histopathology can be used by evidencing of prominent intra-nuclear, Cowdry type A inclusion bodies. Dot blot and ISH have been successfully developed from cloned genomic fragments (Mari *et al.*, 1993).
- Several PCR methods are available for IHHNV detection, but 2 primers sets are the most suitable for detection of all the known variants of IHHN:
  - 392F/R: GGG-CGA-ACC-AGA-ATC-ACT-TA-3' / ATC-CGG-AGG-AAT-CTG-ATG-TG-3'. Amplicon: 392 bp
  - 389F/R: CGG-AAC-ACA-ACC-CGA-CTT-TA-3' / GGC-CAA-GAC-CAA-AAT-ACG-AA-3'. Amplicon: 389 bp

Comments and recommendations on available techniques

Diagnostic by histology is the more difficult because symptoms are difficult to observe and this technique needs well trained people. Moreover, during the first steps of the disease, or in case of low degree of infection, diagnostic with this method is very difficult. For this reason probes and PCR methods are the most used in diagnostic laboratories.

What should we do for diagnosis at suspicion?

Follow the recommendations of the OIE (2006), under confirmatory diagnosis, see also below.

EU-legislation related to techniques

IHHN is not listed by the EU. Therefore, no techniques are given by the EU.

OIE recommendations related to techniques (& ref lab OIE)

IHHN is listed by the OIE Manual of Diagnostic Tests for Aquatic Animals (2006).

The methods (OIE, 2006) currently available for surveillance, detection, and diagnosis of IHHN are listed, see below.

The designations used indicate:

A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity;

B = the method is a standard method with good diagnostic sensitivity and specificity;

C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and

D = the method is presently not recommended for this purpose.

These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

The OIE recommends for:

- Surveillance
  - DNA Probes *in situ* (B, only for juveniles and adult shrimp)
  - PCR (A)
- Presumptive diagnosis
  - Histopathology (A)
  - DNA Probes *in situ* (A)
  - PCR (A)
- Confirmatory diagnosis
  - Histopathology (A)
  - DNA Probes *in situ* (A)
  - PCR (A)
  - Sequencing (A)

For all other combinations of the above tests and gross signs, bioassays, direct light microscopy, transmission electron microscopy, and antibody based assays, with the goals of testing the OIE gives C or D, so, less to non acceptable. See OIE, 2006.

To declare freedom: 2 years of history of negative test results for IHHNV using PCR on samples of appropriate type and size.

OIE reference laboratory: Aquaculture Pathology Section, Dept. of Vet. Science, University of Arizona, Tucson, USA, Prof. D. Lightner, E-mail: dvl@u.arizona.edu

Assessment

Follow the OIE recommended tests, as mentioned above.

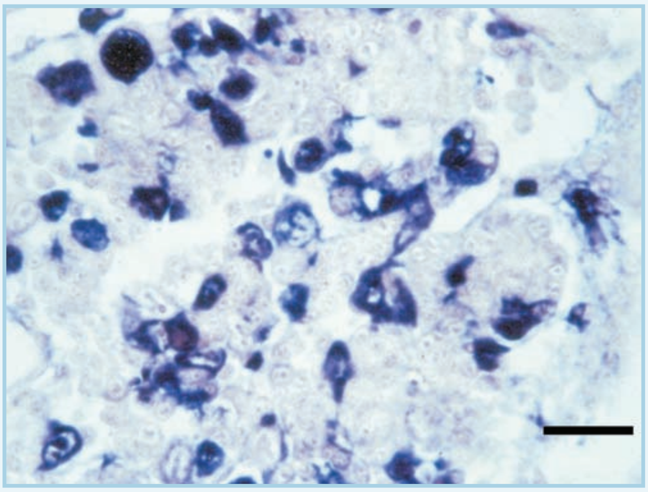


Fig. 28: IHHN infected lymphoid organ of **L. stylirostris**. **In situ** hybridization (ISH); note the strong labeling with the Dig probe of infected nuclei and cytoplasm. Bar = 100 µm (J.R.Bonami acknowl.)

3.4.26 Coxiella cheraxi

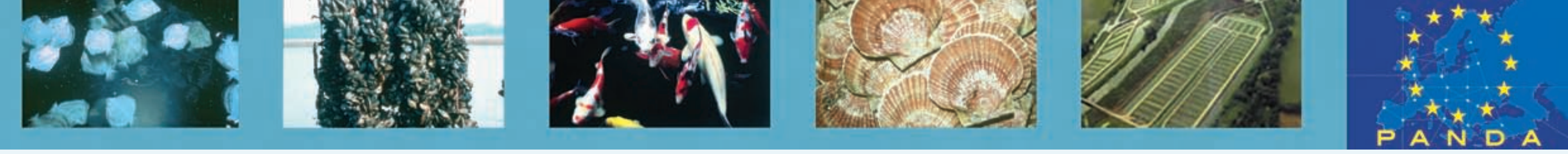
As reported by Edgerton and Prior (1999), rickettsia-like organisms were observed in hepatopancreas of the red claw *Cherax quadricarinatus*. Tan and Owens (2000) proposed to classify this agent as a new species of the genus *Coxiella* and to name it *Coxiella cheraxi* without more evidence at the level of the nomenclature. As biological data reported were scarce it was difficult to know the micro-organism reported by Edgerton and Prior (1999) was the same reported and investigated by Tan and Owens (2000). To date, it was mentioned only in Australia.

Clinical pathology

The disease is characterized by lethargy in the red claw *Cherax quadricarinatus* just before death. Carapace became reddish and important necrosis is noted at the eye level and hepatopancreas. Experimentally, after injection of healthy animals maintained at 28°C, mortality appears the second day, and all animals are dead within 20 days. Infection by oral route produces 30 % mortality within 28 days.

Agent description

In 2000, Tan & Owens have described a micro-organism from the Australian red claw crayfish *Cherax quadricarinatus*. It was cultivated in yolk sac of 6 days-old embryonated eggs and incubated at 36°C. Sequence of the 16S rRNA is close to the sequence of *Coxiella burnetti* (95.6 % homology). Data concerning bacteriological characters of *Coxiella cheraxi* are scarce. It develops in cytoplasmic basophilic vacuoles of the hepatopancreas. The vacuoles contain Gram



negative polymorphic bacteria, often coccoids, with a size of 0.2 – 0.4 µm.

Confirmatory techniques for diagnosis

None reported.

Screening techniques for the pathogen

None reported.

Comments and recommendations on available techniques

As no data are available on characterization of this micro-organism, morphological observations, tissue location, clinical signs of diseased animals (hepatopancreas deeply necrosed) and culture in embryonnated eggs can only suggest a possible relationship with the strain described and reported by Tan & Owens (2000).

What should we do for diagnosis at suspicion?

Cultivate the bacterium from lesions of the red claw, in yolk sac of 6 days-old embryonated eggs, incubated at 36°C (Tan & Owens, 2000).

EU-legislation related to techniques

Coxiella cheraxi is not listed by the EU. Therefore, no techniques are given by the EU.

OIE recommendations related to techniques (& ref lab OIE)

Coxiella cheraxi is not listed by the OIE. There is no reference lab for the disease.

Assessment

There are hardly any tests available sofar for this very rarely occurring bacterium. Cultivation is the method to use, followed by sequence of the 16S rRNA.

3.4.27 References of crustacean pathogens/diseases:

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Brock *et al.*, 1995. An overview of Taura syndrome, an important disease of farmed *Penaeus vannamei*. In: *Swimming through troubled water*, Browdy & Hopkins eds., WAS, Baton Rouge, Louisiana, 84-94.

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Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. & Ball, L.A. (2005). Virus Taxonomy. Classification and Nomenclature of Viruses. Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier, Academic Press, 1259 pp.

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Jimenez, 1992. Sindrome de Taura (Resumen). In: *Acuacultura del Ecuador*. Camara Nacional de Acuacultura, Guayaquil, Ecuador, 1-16.

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Spann *et al.*, 2003. Detection of Gill associated virus (GAV) by *in situ* hybridization during acute and chronic infections in *Penaeus monodon* and *Penaeus esculentus* shrimp. *Dis.Aquat.Org*, 56, 1-10.

Stewart, J.E. & Zwicker, B.M. (1974 a). A comparison of various vaccines for inducing resistance in the lobster (*Homarus americanus*) to the bacterial infection, gaffkaemia. *J. Fish.Res. Bd Can.*, 31, 1887-1892.

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Tang and Lightner, 2001. Detection and quantification of infectious hypodermal and hematopoietic necrosis virus in penaeid shrimp by real-time PCR. *Dis.Aquat.Org.*, 44, 79-85.

Tang *et al.*, 2002. *In situ* detection of Australian gill associated virus with a yellow head virus gene probe. *Aquaculture*, 205, 1-5.

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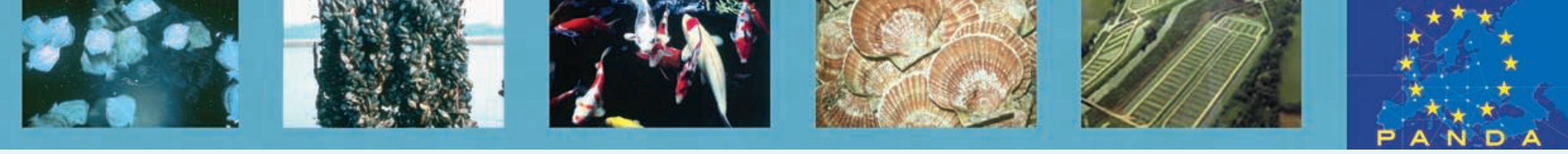

Diseases/Pathogens of amphibians

3.4.28 Amphibian ranavirus

Ranaviral disease has been seen in captive amphibians and in epizootics in wild amphibians in North America and the United Kingdom, and possibly Canada. Apart from causing high rates of mortality in amphibians, some members of this genus can also infect fish and reptiles, resulting in morbidity and mortality (OIE, 2006).

Clinical pathology

Ranaviruses cause a systemic infection in amphibians (Daszak et al., 2003). No specific clinical signs are consistently associated with infection.

Agent description

Ranaviruses belong to the *Iridoviridae* family, genus *Ranavirus*, with the type species Frog virus 3 (FV3). They have been isolated from healthy or diseased frogs, salamanders, reptiles and fish in America, Europe, Australia and Asia (Drury et al., 1995; Bovo, pers.comm.; Fijan et al., 1991; Chinchar, 2002; Hyatt et al., 2002; Speare and Smith, 1992; Wolf et al., 1968; Zupanpovic et al., 1998; Langdon et al., 1986; Ahne et al., 1989; Pozet et al., 1992; Plumb et al., 1996; Grizzle et al., 2002; Chen et al., 1999). Ranaviruses have large (150-180 nm), icosahedral virions, a double-stranded DNA genome 150-170 kb, and replicate in both the nucleus and cytoplasm with cytoplasmic assembly (Chinchar et al., 2005). They possess common antigens that can be detected by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence, but no effective neutralising antibodies have been produced to assist identification.

Confirmatory techniques for diagnosis

Validated tests for ranavirus in amphibians are scarse. Reference is therefore made largely to the OIE diagnostic manual methods for EHNV, which is also a ranavirus.

- Cell culture isolation. Standard procedures according to the OIE manual (OIE, 2006). Several cell lines at 15-22°C.
- E.M. (Electron microscopy): confirm presence of icosahedral virions (150-180 nm in diameter) and virus inclusion bodies
- Serological tests
  - Neutralising antibodies against ranavirus have not been detected in infected animals although they are capable of producing antibodies.
  - ELISA for detection of serum antibodies in toads (Whittington RJ and Speare R (1996).
- Antibody-based antigen detection methods such as
  - Immunoperoxidase test of infected cell cultures.
  - Immunoperoxidase test of histological sections
  - Antigen-capture ELISA. A validated test for detection of ranavirus in fish tissues and cell culture is described in the OIE manual.
  - Immunoelectron microscopy – Gold-labelling of sections or cell cultures
- Molecular techniques
  - PCR on cell culture or in tissues
  - Restriction Endonuclease Analysis (REA) on cell culture or in tissues.

Screening techniques for the pathogen

- Virus isolation of ranavirus in cell culture from liver, kidney and spleen tissues is possible in a variety of cell lines from 15-22°C. Validated virus isolation procedures for EHNV are described in the OIE Diagnostic Manual (2006).
- Antigen-capture ELISA for detection of EHNV in tissues or in cell culture is also validated and published in the OIE Manual.

Comments and recommendations on available techniques

In the OIE Aquatic Diagnostic Manual, the different methods are compared.

For surveillance, the two methods above are recommended. Likewise for detection and confirmation, but in addition the PCR, REA and sequencing methods are listed for confirmatory identification.

For those laboratories that do not have the ELISA implemented for routine surveillance, the cell culture screening followed up with the PCR method would be a practical solution. PCR directly on tissues would be more economical, but is not validated. Primers and procedures are published and most laboratories have experience with and facilities for PCR. The published ELISA method is validated however, and this gives some advantage.

What should we do for diagnosis at suspicion?

- Characteristic cytopathic effect in cell culture and cell culture is positive for ranavirus in PCR OR
- Tissues positive in PCR

And for both points: Sequence consistent with ranavirus is demonstrated by PCR-REA or PCR-sequencing.

Liver, spleen and kidney from diseased amphibians should be processed for virus isolation.

EU-legislation related to techniques

Amphibian ranavirus is not listed by the EU. EHN was not listed in the 91/67/EC, but is listed in 2006/88/EC, as exotic fish virus. No specification of diagnostic methods are given yet in the new legislation.

OIE recommendations related to techniques (& ref lab OIE):

EHN as a ranavirus is listed by the OIE (2007). Detailed descriptions of tests for EHNV can be found in the Diagnostic Manual of the OIE (2006).

The methods (OIE, 2006) currently available for surveillance, detection, and diagnosis of EHN are listed, see below.

The designations used indicate:

A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity;

B = the method is a standard method with good diagnostic sensitivity and specificity;

C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and

D = the method is presently not recommended for this purpose.

These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

The OIE recommends for:

- Surveillance
  - Cell culture (A)
  - Antigen-capture ELISA (A)
- Presumptive diagnosis
  - Histopathology (B)
  - Cell culture (A)
  - Antigen-capture ELISA (A)
- Confirmatory diagnosis
  - Transmission E.M. (B)
  - Immuno E.M. (B)
  - Cell culture (B)
  - Antigen-capture ELISA (B)
  - PCR-REA (A)
  - PCR - Sequence analysis (A)

For all other combinations of the above tests and gross signs, immunoperoxidase, and antibody-capture ELISA, with the goals of testing the OIE gives C or D, so, less to non acceptable. See OIE, 2006.

OIE reference laboratories:

- Australian Animal Health Laboratory, CSIRO, Geelong,. Australia, Dr. A. Hyatt, E-mail: alex.hyatt@csiro.au
- Faculty of Vet. Science, University of Syney, Camden, Australia, Prof. R. Whittington, E-mail: richardw@camden.usyd.edu.au

Assessment

Ranavirus grow easily in cultures of fish cell lines. The published PCR probes appear to recognise most ranaviruses. A combination of those 2 methods seems to be appropriate.

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3.4.29 Batrachochytrium dendrobatidis (amphibian chytridiomycosis)

Chytridiomycosis is a pandemic fungal disease of wild amphibians caused by *Batrachochytrium dendrobatidis.* It has caused loss of amphibian populations (many species) across 5 continents. It has been reported from Africa, Asia, Australia, Central America, Europe (exact distribution unknown), Japan, New Zealand, South America and USA (OIE, 2006).

Clinical pathology

Batrachochytrium dendrobatidis causes cutaneous mycosis (fungal infection of the skin), or more specifically chytridiomycosis, in wild and captive amphibians. First described in 1998, the fungus is the only chytrid known to parasitise vertebrates. *B. dendrobatidis* can remain viable in the environment (especially aquatic environments) for weeks on its own, and may persist in latent infections (www.issg.org/database/species/ecology). The fungus has been isolated from many amphibian species, frogs, salamanders, and shrimps (Rowley et al., 2006).

Agent description

Batrachochytrium dendrobatidis is a non-hyphal parasitic chytrid fungus that has been associated with population declines in endemic amphibian species in upland montane rain forests in Australia and Panama.Recent reorganisation of the Chytridiomycota has placed *Batrachochytrium dendrobatidis* in a new order, Rhizophydiales. However, lack of data has left the amphibian chytrid fungus *incertae sedis* without a family (Amphibian Diseases Home Page;

http://www.jcu.edu.au/school/phtm/PHTM/frogs/ampd is.htm). The life cycle of *Batrachochytrium dendrobatidis* is a simple progression from zoospore to the growing organism, called a thallus, which produces a single zoosporangium (Berger *et al.*, 2005). Zoospores are discharged through an inoperculate opening and they exhibit monocentric or colonial growth. It is thought that *Batrachochytrium dendrobatidis* is a recently emerged clone supported by epidemiological data showing that chytridiomycosis has been introduced into many countries from a common source and there is evidence that Africa is the origin (Berger *et al.*, 2005)

Confirmatory techniques for diagnosis

- Immunoperoxidase test: An indirect test was described by Berger et al., 2002.
- PCR test: These were described by Annis et al., 2004; Garner et al., 2006; and Retallick et al., 2006.
- RT quantitative PCR: This test was done on skin swabs, a TaqMan PCR, by Boyle et al., 2004, furthermore PCR by Kriger et al., 2006a, b; and Retallick et al., 2006.
- Sequencing: Parts of the genome were sequenced by Morehouse et al., 2003; and Annis et al., 2004.
- Polyclonal antibodies: Polyclonal antibodies of sheep and rabbit were produced and described by Berger et al., 2002.
- Histopathology: The histopathology of *Batrachochytrium dendrobatidis* infections in various amphibians was described by: Berger et al., 1998 (Fig.1), Longcore et al., 1999; Nichols et al., 2001; Parker et al., 2002; Davidson et al., 2003; Daszak et al., 2004; Hanselmann et al., 2004; Rachowicz & Vredenburg, 2004; Kriger et al., 2006b; Pasteris et al., 2006; Puschendorf et al., 2006; and Puschendorf & Bolanos, 2006.
- Quantitative histopathology: This test was described by Berger et al., 2005b.
- Light microscopy: Described by Rachowicz & Vredenburg, 2004; Berger et al., 2005a; Garner et al., 2006
- Immunocytochemistry (IHC): This technique was used in skin of amphibians (Van Ells, 2003); The IHC and modified Hollande's Trichrome stain was described by Olsen et al., 2004.
- Transmission Electron Microscopy: Described by Longcore et al., 1999; Berger et al., 2005a
- Scanning Electron Microscopy: Described by Berger et al., 2005a

Screening techniques for the pathogen

- Clinical pathology: Lesions consist of abnormal epidermal sloughing and more rarely of epidermal ulcers. Haemorrhages in the skin, muscle or eye, hyperemia (inflammation) of digital and ventrum skin, and congestion of viscare may occur (www.issg.org/database/species/reference; Berger et al., 1999). Further clinical pathology is described by Bradley et al., 2002; Parker et al., 2002; Rachowicz & Vredenburg, 2004.
- Fungal morphology: by identification of characteristic intracellular flask-shaped sporangia (spore containing bodies) and septate thalli. The fungus grows in the superficial keratinized layers of the epidermis (known as the stratum corneum and stratum granulosum). The normal thickness of the stratum corneum is between 2µm to 5µm, but a heavy infection by the chytrid parasite may cause it to thicken to up to 60 µm. The fungus also infects the mouthparts of tadpoles (which are keratinised) but does not infect the epidermis of tadpoles (which lacks keratin) (www.issg.org/database/species/ecology).
- Fresh preparate: For a direct count in skin sloughs by Weldon & Du Preez, 2006. Fresh preparates by Nichols et al., 2001.
- Fungus isolation: This was described by Longcore et al., 1999; Bradley et al, 2002; Boyle et al., 2003; Davidson et al., 2003; Annis et al., 2004; Growth on autoclaved snakeskin, 1% keratin agar, and best in tryptone or peptonized milk (Piotrowski et al., 2004); and Pasteris et al., 2006.

Comments and recommendations on available techniques

Diagnosis of *Batrachochytrium dendrobatidis* requires specialized skills. The fungus is full under attention, as it is an emerging pathogen. Histopathology is an important tool to diagnose the disease. Molecular methods need to be implemented to be able to type the fungus. There is still much to develop further related to *Batrachochytrium dendrobatidis* diagnosis.

What should we do for diagnosis at suspicion?

The chytrid can be diagnosed by routine histology of skin specimens preserved in formalin or ethanol. Examination of unstained skin scrapings is a quick method, but requires greater expertise in identifying organisms (Berger et al., 1999). Chytrid culture from fresh specimens requires specialised methods (Longcore et al., 1999) and is difficult; so most diagnoses are made using histology.

EU-legislation related to techniques

Batrachochytrium dendrobatidis is not listed by the EU, and therefore no recommendations are made by the EU. It is present in Norway.

OIE recommendations related to techniques

Batrachochytrium dendrobatidis is not listed by the OIE (Aquatic Animal Health Code and Manual, 2006 version).

Assessment

As *Batrachochytrium dendrobatidis* is an emerging pathogen, through international imports of amphibians e.g., countries should be prepared to diagnose the disease. Histopathology is important, with addition of molecular biological methods. At suspicion, it is advized to ask a 2nd opinion on the diagnosis to experienced specialists of the reference list.

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Fig. 29: *Batrachochytrium dendrobatidis* zoospore (1000x) (Dr Frank Mutschmann acknowl.)

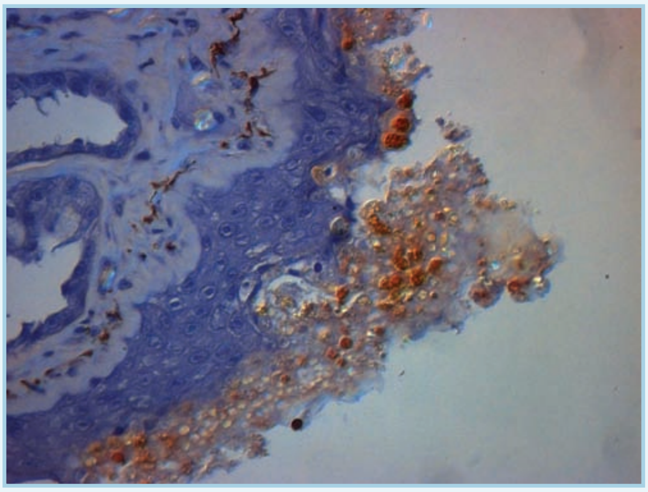


Fig. 30: Immunoperoxidase stained *Batrachochytrium dendrobatidis* in *Litoria caerulea* (Australian Green Tree Frog) (Dr Frank Mutschmann acknowl.)

section 4

General discussion

4.1 Evaluation of available tests and recommendations for improvement

In section 4, many tests and techniqueas are named with references. From these data an evaluation has been made, on which test would be the best used for which purpose, see the Table below.

Table 4.1a: Summary of WP4 results: current screening/diagnostic methods (among others, see for details the specific paragraphs), and their evaluation

*for references and details see specific table per pathogen/groups of pathogens; ab = antibody; ag = antigen; E.M. = electron microscopy; histo = histopathology; ICC = immunocytochemistry; IFAT = immunofluorescence; IHC = immunohistochemistry; IPMA = immuno peroxidase monolayer assay; ISH = in situ hybridization; LAMP = loop-mediated isothermal amplification; RFLP = restricted fragment length polymorphism; SN-test = serumneutralisation test; VI = virus isolation;

Disease/ Pathogen	Confirmatory technique (well established)	Screening technique (well established)	Evaluation
EHNV	IFAT, IPMA, ELISA (virus & serol.), SDS-page; PCR, IHC some are ISO9001	Clin.pathol., VI, IFAT, IPMA, ELISA (ag & ab); some are ISO9001	• Many good tests for screening and confirmation • RANA-project has organized a ring test. • Diagnosis of EHNV is not yet established at NRL's: • advised to extrapolate ring test to NRL's of EU, because of listed EHNV in 2006/88/EC : training needed. • PCR is now validated in Finland.
RSIV	IFAT (ISO); IPMA; sequencing; PCR; LAMP; histo; IHC; E.M.	Clin.pathol., VI	• Useful tests for screening and confirmation. • RSIV is not listed or tested in the EU yet • Cell culture (BF-2 a.o.) can be used to isolate the virus • Implementation of confirmative tests needed in Europe, via CRL Annual Meetings.
ISAV	RT-PCR; IFAT; IPMA; histo; ISH; ELISA (ab); haemabsorptio n; SN-test; E.M.	Clin.pathol., VI, RT-PCR, haematology	• The disease and pathogen are well documented in literature • Many good tests exist for screening and confirmation • There are no training needs.

Continued

Table 4.1a: Summary of WP4 results: current screening/diagnostic methods (among others, see for details the specific paragraphs), and their evaluation (continued)

Disease/ Pathogen	Confirmatory technique (well established)	Screening technique (well established)	Evaluation
KHV	IFAT (after cpe and with kidney imprints); ELISA (ag & ab); PCR & RT-PCR; sequencing; histo; ISH; LAMP; E.M.;	Clin.pathol., VI (low sensitive); ELISA (ab); PCR & RT-PCR; LAMP	• Many good tests exist. • PCR ring test is organized by the OIE ref lab (CEFAS) • Tests get more sensitive, but latent carriers of KHV possibly cannot be detected yet. • Sequence of the marker vaccine is secret → PCR positive results of field strains cannot be distinguished from those of the vaccine strain of KHV. • The (TaqMan) PCR is the test of choice, to be validated by the ring test. • There are training needs on KHV detection and diagnosis, especially in Eastern Europe.
Strepto-coccus agal-actiae	Clin.pathol.; isolation; biochemical typing; serology; PCR; DNA sequencing; DNA-DNA hybridization; Sherman criteria (some are validated)	Clin.pathol.; isolation;	• Useful tests for identification, but time consuming • Disease problems with this pathogen increase → fast and specific tests needed. • 16S RNA typing is important: needs validation, which means ring testing. Which lab is going to take this task is not defined yet.
Strepto-coccus iniae	Clin.pathol.; isolation; biochemical typing; serology; PCR; DNA sequencing; DNA-DNA hybridization; Sherman criteria (some are validated)	Clin.pathol.; isolation;	• Useful tests for identification, but time consuming • Disease problems with this pathogen increase → fast and specific tests needed. • 16S RNA typing is important: needs validation, which means ring testing. Which lab is going to take this task is not defined yet.

Continued

Table 4.1a: Summary of WP4 results: current screening/diagnostic methods (among others, see for details the specific paragraphs), and their evaluation (continued)

Disease/ Pathogen	Confirmatory technique (well established)	Screening technique (well established)	Evaluation
Lacto-coccus garviae	Clin.pathol.; isolation; biochemical typing; serology; PCR; DNA sequencing; DNA-DNA hybridization; Sherman criteria (some are validated)	Clin.pathol.; isolation;	• Useful tests for identification, but time consuming • Disease problems with this pathogen increase → fast and specific tests needed. • 16S RNA typing is important: needs validation, which means ring testing. Which lab is going to take this task is not defined yet.
Trypano-soma salmo-sitica	Fresh preparate and fixed smear of mucus/fluid (standardized); Haematocrit centrifuge technique (standardized, highly sensitive); IFAT (ab); MISET (ab); Antigen-capture ELISA (standardized, highly sensitive); Antibody capture ELISA (standardized)	Clin.pathol. (suspicion); Antigen-capture ELISA; Antibody capture ELISA	• Little experience with this pathogen in Europe • Very few specialists around the world. • Molecular biological methods for this parasite lack. • Training is needed, in clinics, detection methods and confirmative methods. Which lab takes the lead in the EC?
Cerato-myxa shasta	fresh preparate (standardized); fixed smear; isolation; IFAT (ag); IPMA; PCR (standardized); quantitat.PCR (standardized, very sensitive); histo (standardized); IHC; ISH (standardized); non-lethal PCR	Clin.pathol. (suspicion); isolation; PCR; quantit.PCR; non-lethal PCR	• Little experience with this pathogen in Europe • Very few specialists around the world. • Molecular biological methods for this parasite lack. • Training is needed, in clinics, detection methods and confirmative methods. Which lab takes the lead in the EC?

Continued

Table 4.1a: Summary of WP4 results: current screening/diagnostic methods (among others, see for details the specific paragraphs), and their evaluation (continued)

Disease/ Pathogen	Confirmatory technique (well established)	Screening technique (well established)	Evaluation
Neopar-amoeba perurans	gill histo (standardized & validated); fres preparate; fixed smear (standardized and validated); parasite isolation and identification (stand.&valid.); IFAT (ab) (stand.&valid.); immuno dot blot of mucus (standard.); sequencing; PCR on clonal cultures; ICC (stand.&valid.)	Clin.pathol.(standardized at farm level); gill histo (standardized & validated)	• Little experience with this pathogen in Europe • Very few specialists around the world. • Molecular biological methods for this parasite lack. • Training is needed, in clinics, detection methods and confirmative methods. Which lab takes the lead in the EC?
Parvi-capsula pseudo-branchi-cola	parasite isolation; sequencing; PCR (highly sensitive); histo; ISH	Clin.pathol.; parasite isolation	• Only experience in Norway with this salmon pathogen • Very few specialists around the world. • Although there is a PCR, it should be validated by other methods, which lack. • Training is needed, in clinics, detection methods and confirmative methods. Which lab takes the lead in the EC?
Gyro-dactylus salaris	morphometry; sequencing; RFLP	Clin.pathol.; isolation; morphometry; RFLP	• good tests available • diagnostic workshop was there for all NRL's of the EC • Possibly interest in the later accessed EU-members states of especially Eastern Europe to do a diagnostic training related to this parasite.

Continued

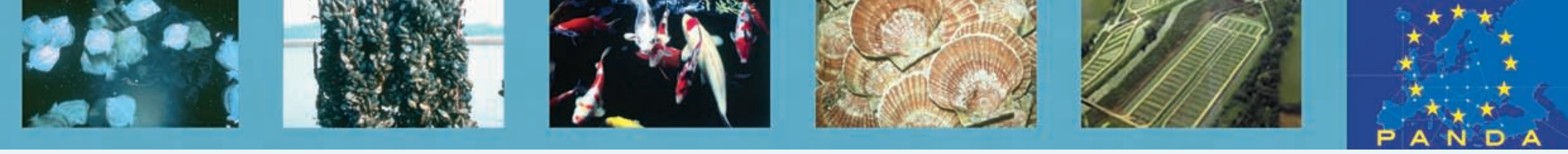


Table 4.1a: Summary of WP4 results: current screening/diagnostic methods (among others, see for details the specific paragraphs), and their evaluation (continued)

Disease/ Pathogen	Confirmatory technique (well established)	Screening technique (well established)	Evaluation
Aphanomyces invadans	Fresh preparate; fixed smear; ELISA (ab); Western blot; haemagglutination; sequencing; PCR; histo; IHC; ISH; E.M.; pyrolysis mass spectrometry	Clin.pathol.; isolation; PCR	• This fungus causes disease with very specific clinics • That makes a possible suspicion very doubtful. • Only 1 lab in Europe specialized (CEFAS). • From May 2008 all NRL's should be able to diagnose EUS: urgently training needed in clinical pathology and diagnosis.
Mollusc diseases	see attached separate table below	see attached separate table	• The NRL network with the CRL keeps close contact on the available diagnostic methods on mollusc disease diagnosis. • Especially histopathology training for new pathogens or diseases is needed and organized by the CRL, who look after the quality of diagnosis at NRL's through the Annual NRL meeting and workshops.
Crust. Yellow head	PCR (standardized, highly specific and sensitive); ISH (stand., highly spec & sens); E.M. (blood; low sensitive)	Immunoblot (specific, but low sensitive); PCR; histo (standardized low spec & sens)	• good tests available internationally • most EU countries are not yet familiar with them • There are no CRL-NRL meetings on crustacean diseases yet. • As the disease is listed in 2006/88/EC, urgently training is needed in detection and diagnostic methods. • A CRL will be appointed soon by the EU, and will need to train the NRL´s for crustacean diseases.
Crust. White spot	PCR (highly specific and sensitive); ISH (highly spec & sens); E.M. (blood; low sensitive); mini array detection (spec & sens)	Dot blot assay (specific, but low sensitive); PCR; histo (low spec & sens); LAMP (specific and sens); mini array detection (spec & sens)	• good tests available internationally • most EU countries are not yet familiar with them • There are no CRL-NRL meetings on crustacean diseases yet. • As the disease is listed in 2006/88/EC, urgently training is needed in detection and diagnostic methods. • A CRL will be appointed soon by the EU, and will need to train the NRL´s for crustacean diseases.

Continued





Table 4.1a: Summary of WP4 results: current screening/diagnostic methods (among others, see for details the specific paragraphs), and their evaluation (continued)

Disease/ Pathogen	Confirmatory technique (well established)	Screening technique (well established)	Evaluation
Crust. Taura	RT-PCR (stand., highly spec & sens); ISH (highly spec & sens);	Dot blot assay (specific, but low sensitive); histo (low spec & sens);	• good tests available internationally • most EU countries are not yet familiar with them • There are no CRL-NRL meetings on crustacean diseases yet. • As the disease is listed in 2006/88/EC, urgently training is needed in detection and diagnostic methods. • A CRL will be appointed soon by the EU, and will need to train the NRL´s for crustacean diseases.
Crust. IHHNV	Dot blot assay (specific, but low sensitive); PCR and qPCR(standardized, highly specific and sensitive); ISH (stand., highly spec & sens);	Dot blot assay (specific, but low sensitive); histo (high spec & low sens); PCR and qPCR(standardized, highly specific and sensitive);	• good tests available internationally • most EU countries are not yet familiar with them • There are no CRL-NRL meetings on crustacean diseases yet. • As the disease is listed in 2006/88/EC, urgently training is needed in detection and diagnostic methods. • A CRL will be appointed soon by the EU, and will need to train the NRL´s for crustacean diseases.
Crust. *Coxiella cheraxi*	Crust. Coxiella cheraxi 16S rRNA partial sequence; E.M.	Isolation; 16S rRNA partial sequence	• No specialists present in Europe • Training needed, but no specific tests are available
Amphib. Irido-viridae Rana virus	IFAT; ELISA (ag and ab); SN-test; sequencing; PCR, RT-PCR; RFLP; histo; IHC; E.M.; a.o.	Clin.pathol.; VI	• only diagnosed at 1 or 2 labs in Europe • Urgently training is needed: the RANA-project outcome should be extrapolated (ring test e.g.), and training in diagnosing these viruses should be parallel to that of EHNV.
Amphib. *Batra-cho-chytrium dendro-batidis*	Fresh preparate; IPMA; sequencing; PCR; RT-PCR; histo; ICC; E.M.	Clin.pathol.; isolation	• There is no known lab in Europe yet diagnosing it. • As the disease is emerging, there should be at least one national lab to be trained to diagnose the disease: clinics, isolation, and testing for confirmation.

Table 4.1.b: Summary of recommended tests advized to use for Mollusc diseases/pathogens and needed additional tests:

S = screening technique; C = confirmatory; well established technique

Infectious agent	Diagnostic techniques								
	Histology	Cytology	PCR	PCR-RFLP	DNA Sequence	ISH	TEM	Culture	Comments
Nocardia crassostreae	3: S	0: S	5: S + C	2	0: C	5: C	1	0: C	PCR and ISH are genus specific. Culture needed for sequencing
Candidatus Xenohaliotis californiensis	3: S	0: S	5: S	2	0: C	5: C	1	2	
Perkinsus olseni	3: S	2	0: S	2	4: C	1	1	3: S	PCR needed for sequencing. Culture: RFTM. only genus specific.
Perkinsus marinus	3: S	2	0: S	2	5: C	1	1	3: S	PCR needed for sequencing. Culture: RFTM. ISH only genus specific.
Marteilioides chungmuensis	3: S	0: S	5: S + C	2	0: C	5: C	0: C	2	PCR more specific and sensitive than histology but not strictly validated

0	Technique not or seldom used by EU NRLs or the CRL	3	Techniques used by most NRLs
1	Technique exists but is not useful	4	Techniques that should be used by NRLs (or NRLs refer to CRL for diagnosis)
2	Technique not adapted or not relevant	5	Techniques (mostly used outside Europe) that could be adopted by EU NRLs

4.2 Priority list for improvement of tests/testing per pathogen/disease, and skills

Per pathogen/disease, suggestions for improvement are given below:

A: highest priority

B: medium priority

C: low priority

D: no priority

- EHNV (A):
  - Validation needed for screening and confirmatory techniques
  - Getting to use them urgently at NRL's including the needed biologics (training)
  - NRL's to participate in ring test
- RSIV (B):
  - Getting to use the tests at NRL's including the needed biologics (training)
- ISAV (A):
  - Ring test needed for experienced NRL's in Europe
  - Training in tests possibly needed for NRL's of newly accessed E-European countries
- KHV (A):
  - Develop more sensitive tests to trace latent carriers
  - Test needed to distinguish field strain and vaccine strain
  - More labs to participate in ring testing, as organized by CEFAS
  - Training in tests, for national and regional labs inside and outside the EC
- *Streptococcus agalactiae (B)*:
  - Fast and specific tests needed
  - 16S RNA typing needs validation
  - Ring test needed
  - Training needed of at least NRL's
- *Strepococcus iniae(B)*
  - Fast and specific tests needed
  - 16S RNA typing needs validation
  - Ring test needed
  - Training needed of at least NRL's
- *Lactococcus garviae(B)*
  - Fast and specific tests needed
  - 16S RNA typing needs validation
  - Ring test needed
  - Training needed of at least NRL's
- *Trypanosoma salmositica(C)*
  - Molecular biological techniques needed for confirmation
  - All NRL's should have the test methods ready (training)
- *Ceratomyxa shasta (C)*
  - No additional techniques needed
  - Training needed in clinics, detection methods and confirmative methods
- *Neoparamoeba perurans (C)*
  - No additional techniques needed
  - Training needed especially for salmonid producing countries in clinics, detection methods and confirmative methods
- *Parvicapsula pseudobranchicola(C)*
  - No additional techniques needed
  - Training needed especially for salmonid producing countries in clinics, detection methods and confirmative methods
- *Gyrodactylus salaris (B)*
  - No additional techniques needed
  - Training needed especially for NRL's of newly accessed EC member states
- *Aphanomyces invadans (A)*
  - Getting to use the available tests (clinical pathology and diagnosis)urgently at NRL's including the needed biologics (training), CEFAS as teaching laboratory
  - NRL's to participate in ring test, to be organized in future
- Mollusc diseases (B):
  - The CRL (Ifremer, La Tremblade, France) organizes yearly a NRL meeting with regular workshops on diagnostic methods.
  - The training needs are thereby solved in the NRL & CRL group

  - The NRL's do already participate in ring testing on the most important exotic and non-exotic, and emerging pathogens of mollusca.
- Yellowhead (A)
  - Urgently needed: training for all (new) NRL's for crustacean diseases: clinical pathology and diagnosis
  - Implementation of these tests at NRL's
- White spot syndrome (A)
  - Urgently needed: training for all (new) NRL's for crustacean diseases: clinical pathology and diagnosis
  - Implementation of these tests at NRL's
- Taura syndrome (A)
  - Urgently needed: training for all (new) NRL's for crustacean diseases: clinical pathology and diagnosis
  - Implementation of these tests at NRL's
- IHHN (A)
  - Urgently needed: training for all (new) NRL's for crustacean diseases: clinical pathology and diagnosis
  - Implementation of these tests at NRL's
- *Coxiella cheraxi (C)*
  - 1 lab at EC level should be able to diagnose the disease/pathogen (training)
  - Testing not necessary at NRL level.
- Ranavirus (A)
  - Urgently needed: training for amphibian disease labs: clinical pathology and diagnosis
  - Outcome EC RANA project important to take into account
- *Batrachochytrium dendrobatidis (B)*
  - Training needed in clinical pathology and diagnosis at amphibian disease labs

In general, there are big gaps of knowledge on some of the pathogens and their diagnostc tests above. Many EU countries never have used some of the diagnostic tests above. Therefore, it is important, first to start to use the available tests at EU level, than validate them, and than only decide which are the best methods to use.

4.3 Working towards standardization and validation

In the start of the project, a special column in each table on diagnostic methods was designated to standardisation, and validation, according to ISO 9001 or ISO17025. During the process of filling the tables, it appeared, that for many pathogens there was at maximum a standardized test, and for most of the pathogens there were no validated tests at all. Therefore, in this report, the status is only mentioned when given in the appropriate publication. Furthermore the status is mentioned as well established, or not known.

Many tests are there already, and are at least well established. This means, they are at least ISO 9001 (described, and used every time in the same way), with positive and negative controls. However, many methods still lack validation, i.e. according to the accreditation norms of ISO 17025. This means parallel testing in more than 1 test is recommended to make the test more reliable. For internal validation, participation in ring testing, and making a full validation report for Quality Assurance are needed.

As some of the pathogens of the WP2 list are notifiable for the OIE, and some of these also for the EU, positive results in screening or confirmation tests might have a high impact to the particular aquaculture site, and the health status of that region or member state. It is therefore of utmost importance to have these tests validated.

Quality Assurance is not new. There have been workshops on QA in Fish and shellfish diagnosis, and QA practicals and needs were published by Haenen et al., Bull. EAFP 19(6): 302-309 (1999). It is suggested to use the network of experts mentioned, and the CRL to organize QA improvement at NRL and regional laboratory level, possibly via workshops, on the most important pathogens of the WP2 list.

4.4 General remarks and links with other WPs

The development of new, more fast, more sensitive, and more specific tests is a continuous process. This means, that the "current diagnostic methods" is a dated term. However, old methods have their value for a long time, at least for validation of newer tests. The list of methods will need continuous updating the coming years, when PANDA would be maintained further.

WP4 was dependent on the list made by WP2. After they had made the list of hazards, WP4 started working. There is a link with WP3: the outcome of WP4 will be used in the data base of WP3 under the field of *diagnostic methods*. The lack of training related to WP4 was communicated with WP6, on training related to PANDA. The recommendations for training needs of WP4 can be found both in this report and in the one of WP6.

4.5 Recommendations to achieve harmonized implementation

The task force of WP4 has made the following recommendations for guidelines and policy/legislation to achieve the aim:

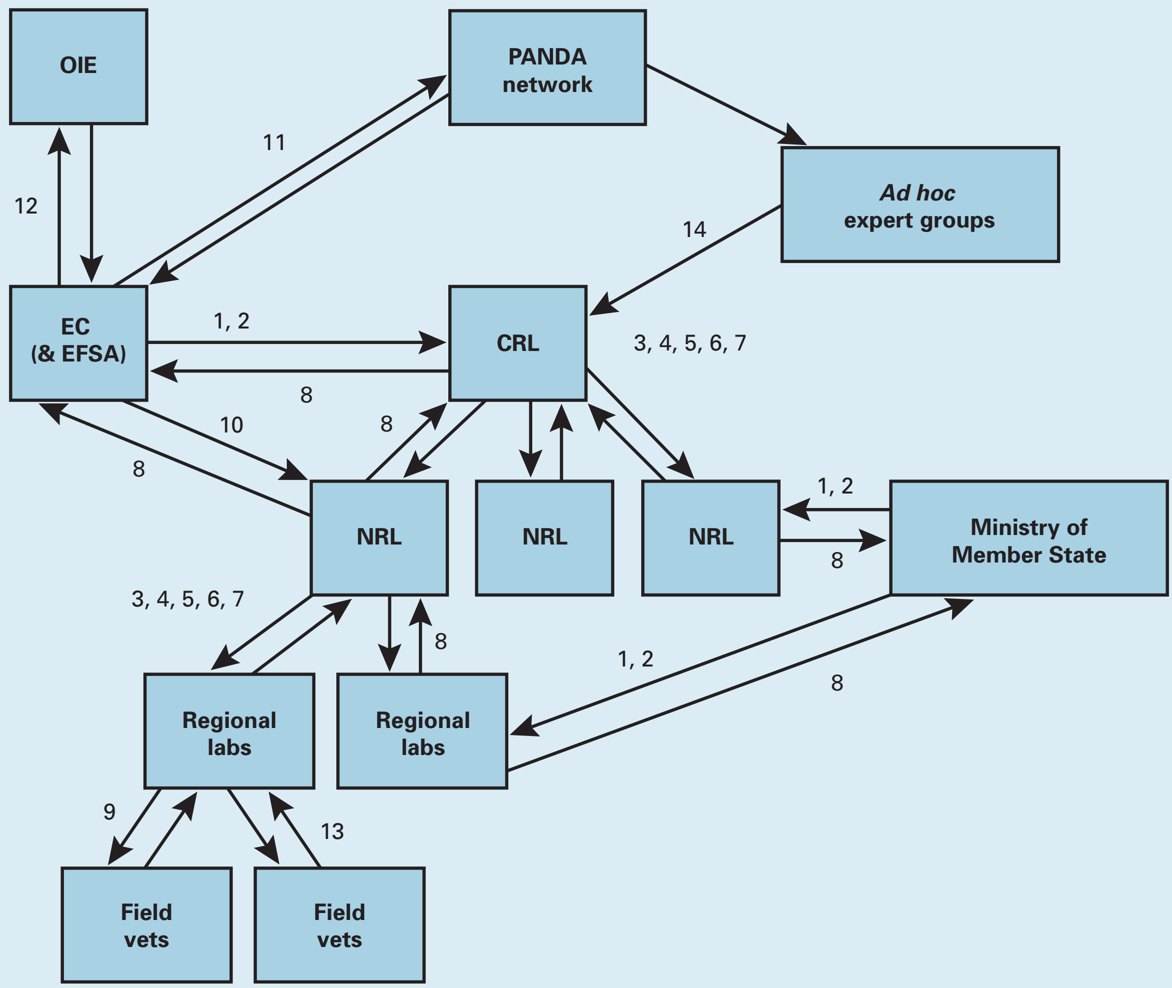
For current EC listed exotic hazards, like Infectious Salmon Anaemia (ISA) there is already much knowledge at the CRL and NRL's in Europe. Only for those laboratories, which have recently accessed the EC, workshops could be organized, to acquire knowledge and technical skills.

For EC non-exotic diseases/pathogens and non-exotic hazards identified by WP2, there is already much knowledge at the CRL and NRL's in Europe. Workshops could be organized for labs, which need it, to acquire knowledge and technical skills.

For new exotic hazards (diseases/pathogens) from the exotic disease list of 2006/88/EC and from the WP2 list, there is very few or no knowledge yet within Europe: Therefore it is necessary, to first build capacity and training, than implementation, than harmonisation (with funding) through training again:

- The EC will appoint CRL's for Crustacean diseases and Amphibian diseases, according to 2006/88/EC
- World wide specialists should be selected from the specific literature per disease/pathogen, as presented in the report of Deliverable 8 of PANDA
- Each CRL should have a leading or coordinative function for notifiable and emerging EC or WP2 listed diseases/pathogens
- Selected world wide specialists should be invited by the CRL's, or specialists from the CRL's should visit these specialists to acquire knowledge on the exotic diseases/pathogens
- Then specialists from the (PANDA) network should be identified, invited and funded: They are proposed to form ad hoc working group on those pathogens, coordinated by the CRL
- Funding of such actions will be essential for success, as all scientists already have projects of their institutes, and are too busy to do this additional work in spare time
- The *ad hoc* working groups make a plan for harmonisation and potential risk mitigation in the EC. Thereby, the cost-benefit of implementation will be important
- Each CRL should also identify specialists for the non-(OIE/EU) listed other? WP2 diseases individually?.
- These specialists should be funded to be a representative within the EC, to implement diagnosis of these WP2 diseases/pathogens, and be ready to diagnose the disease if suspicion would occur within the EC. As example there are various fish parasites listed in WP2
- The CRL could send a yearly small questionnaire to all NRL's (per target group of aquatic animals) on gaps in knowledge, and training needs on screening and confirmative diagnostic tests of the EC/WP2 listed diseases. The results would then be discussed during each Annual meeting.
- Each CRL should coordinate the preparation of disease diagnosis leaflets, which are informative on the EC/OIE/WP2 listed diseases on diagnosis, and their standardisation and reference laboratories. These leaflets should be open accessible at the CRL and NRL websites, and as hard copy distributed to all NRL's and regional European specialized laboratories (depending on the target group of aquaculture animals), the PANDA and EAFP members, and other interested specialists in the field. The leaflets and their distribution should be paid by the EC.
- These disease diagnosis leaflets could cover the following fields:
  - Name of disease and pathogen (and year of publication)
  - Description of disease (including pictures of clinical signs)
  - Susceptible animal species
  - Description of pathogen
  - Confirmative techniques for the disease
  - Screening techniques for the pathogen
  - Comments on available techniques (including QA status, costs, gaps)
  - Ring tests available? Who organizes them for whom?
  - EU-listed: yes/no
  - OIE-listed: yes/no

Fig. 31: Proposed organization to achieve harmonized implementation of confirmation and screening methods throughout Europe.



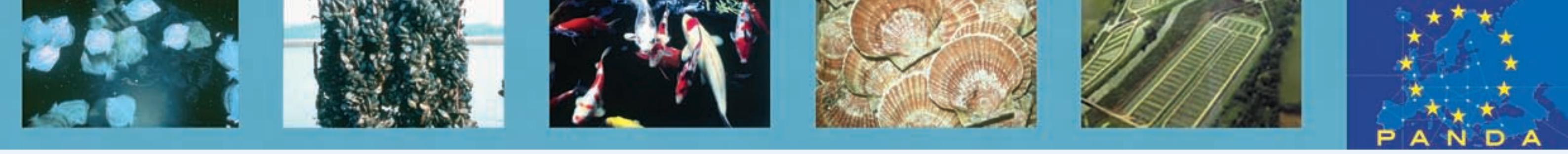
1) funding
2) responsibility and appointment
3) send yearly questionnaire on diagnostic methods
4) organize Annual meeting
5) ring test
6) provide biologics and standard operating procedures for tests
7) organize lab training workshops
8) provide data on test results, gaps in knowledge/diagnosis
9) organize training on sampling methods and diagnosis
10) invitation of experts & funding of Annual meetings
11) recruitment of experts for advisory panels
12) exchange of information/legislation
13) send diagnostic materials to the lab
14) make plans for harmonisation and potential risk mitigation in the EC

N.B. OIE = Office International des Epizooties, EC = European Commission, EFSA = European Food Safety Authority, CRL = Community Reference Laboratory, NRL = National Reference Laboratory.

- Reference laboratory (and expert with E-mail address, website)
- Literature

- The EC should make production, publication and distribution of the disease diagnosis leaflets possible, via coordination with the CRL's
- The EC could coordinate the education by direct contact with the CRL's, and participation in the Annual meetings of CRL & NRL's.
- It is important to use the right sampling procedure for new diseases/pathogens. This is not covered by WP4, but is an aspect of implementation of the new Directive 2006/88/EC. The NRL's would have an important task in this, educating their field vets in sampling procedures.

Extension of tasks of the CRL's is theoretically fine, and could be done in the new EC directive 2006/88/EC, but could give problems in reality. The number of diseases which should be covered by each CRL could go far over their limit. It would mean, tasks would need to be divided over more laboratories. Which other laboratories would be relevant to support the CRL function is not determined by the task force of PANDA. This needs a political discussion at EC-level, whereby the CRL can propose certain laboratories to be candidate for that support function. An independent *ad hoc* group of experts of the EC could judge the proposal, and appoint other laboratories accordingly.



section 5

General conclusions and recommendations

- In this report we made lists of the best diagnostic methods currently available for the most serious diseases, as identified by the risk analysis performed inWP2.
- There are several well established tests for diagnosis of most of the diseases given in the list provided by WP2.
- Some of the WP2 listed diseases or pathogens are not known by laboratories in the EC.
- For **fish diseases**, an import task would be to establish diagnostic tools and research platforms as well as training in detection of the pathogens causing EHNV, KHV and EUS, which partly is also true for ISAV. The CRL on Fish Diseases sofar organizes workshops and ring tests for important and current EC listed viruses (VHSV, IHNV and SVCV). Extension of the training and ring tests with the fish pathogens EHNV, KHV and EUS is advised, apart from ISAV. For the 3 mentioned fish bacteria, fast and specific additional tests are needed for confirmation. For the 4 fish parasites, expertise lacks in Europe, to screen for these parasites, and type them. However, as these parasites are not listed yet by EC or OIE, they have a lower priority.
- The CRL for **Mollusc Diseases** covers most of the exotic and non-exotic mollusc diseases by providing training and consultancy and by organizing periodical ring tests.
- So far the EC did not appoint a CRL on **Crustacean diseases**, which is strongly recommended. Training in clinical inspection and diagnosis of Yellowhead disease, White Spot Disease, and Taura syndrome is recommended. The task force furthermore recommended to enhance expertise and testing capability in EC of Crayfish plague caused by the fungi *Aphanomyces astaci*, as this disease is a serious threat for crustaceans all over Europe.
- The **amphibian diseases** caused by RANA virus and *Batrachochytrium dendrobatidis*, a fungus, are new to most laboratories. Appointment of a CRL by the EC is necessary, after which certain laboratories should get expertise and skills in testing amphibian diseases by training.
- Many of the internationally available tests are not properly validated, despite the fact that they are well establish in several laboratories by daily use. These tests need to go through a reliable validation and inter-laboratory proficiency testing, before being implemented as standards in European laboratories.
- For the exotic diseases/pathogens the knowledge is to be extracted from outside Europe, via invitation of experts or working visits to their lab, by the CRL
- According to the task force of WP4 of PANDA:
  - The EC needs to take responsibility in funding the process of acquiring knowledge and skills, and communication (leaflets) at CRL level
  - The CRL functions will expand, and possible division of tasks to support labs is suggested, and ad hoc expert groups to plan the process
  - The NRL functions will also expand, but to a limited extent
  - The NRL's or regional labs should organize training on sampling methods and diagnosis for field vets among others.
  - The PANDA network will be further consulted for this aim.





section 6

Acknowledgements

The PANDA project was funded by Directorate General Research of the European Commission. We thank them for their support, which made this project possible, including the travels and strengthening of our international networks.

Many volunteers contributed with their expertise for providing the tables, and this report.The task force members of WP4 are thanked for their valuable input and time, to the PANDA project. The additional scientists, mentioned in Annex 7.2, are thanked for their specific expertise and input in text and/or illustrations (Annex 7.6) for the disease/pathogen related sections of section 3. The PANDA consortium members (Annex 7.7) are acknowledged for their collegiality, support and good company during the PANDA project.

section 7

Annexes

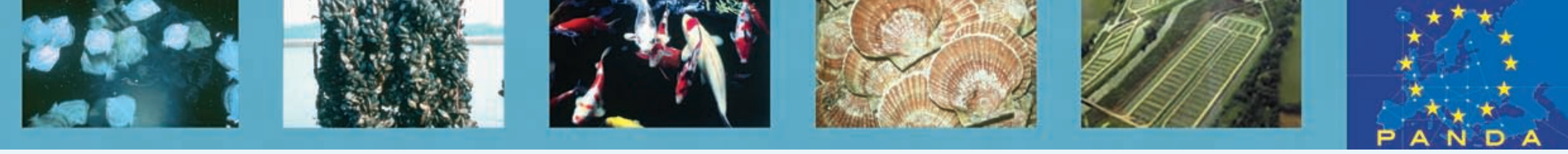
7.1 Task force

A task force was appointed summer 2004 by the WP leader, with specialists covering the various diseases listed. The task force of WP4 consisted first of 6 people. In 2006-2007, more experts were invited during the writing phase of the report:

Member	From institution	Country	Task/speciality
Olga Haenen	CIDC-Lelystad, NRL for Fish and Shellfish Diseases, Lelystad	Netherlands	WP4 leader, fish virology, parasitology, fish and amphibian fungi, QA
Inger Dalsgaard	Technical University of Denmark DTU, Danish Institute for Fisheries Research, Copenhagen	Denmark	Fish bacteriology
Niels Olesen	Technical University of Denmark DTU, National Veterinary Institute, CRL for Fish Diseases, Aarhus	Denmark	Fish virology
Jean-Robert Bonami	Pathogens and Immunity, ECOLAG, Université Montpellier	France	Crustacean diseases
Jean-Pierre Joly	IFREMER, CRL for Mollusc Diseases, La Tremblade	France	Mollusc diseases
Isabelle Arzul	IFREMER, CRL for Mollusc Diseases, La Tremblade	France	Mollusc diseases, steering group member

7.2 List of consulted experts per pathogen

Other contributors/ co-authors (intellectual input)	From institution	Country	Task/speciality
A. Hyatt	CSIRO, Australian Animal Health Laboratory,Geelong	Australia	EHNV
A. Bayley	CEFAS, Weymouth	UK	EHNV & ranavirus
T. Kurita	National Research Institute of Aquaculture, Mie	Japan	RSIV
T. Ito	National Research Institute of Aqua-culture, Tamaki	Japan	RSIV
K. Falk	National Veterinary Institute, Oslo	Norway	ISAV
T. Håstein	National Veterinary Institute, Oslo	Norway	ISAV
P.T.K. Woo	Dept. of Zoology, University of Guelph	Canada	*Trypanoplasma salmositica*
J. Bartholomew	Oregon State University, Dept Microbiology, Corvallis	USA	*Ceratomyxa shasta*
B. Nowak	Tasmanian Aquaculture and Fisheries Institute, Launceston	Australia	*Neoparamoeba pemaquidensis/ perurans*
C. Cunningham	FRS Marine Lab, Aberdeen	Scotland	*Gyrodactylus salaris*
J. Hawke	Dept of PBS, LSU School of Vet. Med., Baton Rouge, LA	USA	EUS/*Aphanomyces invadans*
T.Renault	IFREMER, La Tremblade	France	OsHV1
A.Villalba	Centro de Investiga-ciones Marinas, Valinova de Arousa	Spain	*Perkinsus*
S.Bower	Pacific Biological Station, Nanaimo	Canada	*Nocardia*
B. Hill	CEFAS, Weymouth	UK	ranavirus
Ellen Ariel	Danish Technical University, CVI	Denmark	Amphibian & fish virology, steering group member
Britt Bang Jensen	Danish Technical University, CVI	Denmark	Amphibian and fish virology
Laurence Miossec	IFREMER, LA Tremblade	France	Mollusc diseases, replacing steering group member



7.3 Table on methods described in literature for detection of ISAV and diagnosis of ISA

Question	Answer	Is the test used for: monitoring or confirmation of disease? *M or C*	Is the test standardized? *Yes/no*	Is the test validated? *Yes/no*	Accord -ing to ISO 17025 *Note no.*	Spec *	Sens **	References Numbers
Susceptible fish species	*Atlantic salmon*	M & C	Yes	No	No	?	?	(18)
TESTS:								
Clinical pathology	Gross pathology is essential for ISA diagnosis	Yes, M & C						(2, 8, 22, 28)
Haematology	Low haematocrit is a useful indicator of ISA	Yes, M & C			ISO 17025			(29)
Virus isolation	Cell lines: SHK-1, ASKII, CHSE-214, TO At temp. 15-16°C	M & C	Yes	No	ISO 17025	?	?	(1, 3, 4, 5, 7,13, 20, 26, 27, 31)
Immuno-fluorescence test	Antibodies used: MAb 3H6F8	Yes	Yes	Yes	ISO 17025	?	?	(9, 10, 21, 26)
Immuno-peroxidase test	Antibodies used: MAb 3H6F8.	No			ISO 9001 a.o.			(32)
ELISA for virus typing	Not in general use	No			ISO 9001 a.o.			
ELISA for serology	Yes under development		No					(14)
Serum Neutralization test	Antibodies used: No	No						-
Plaque Neutralization test	Antibodies used: No	No						-
Dot blot assay	Antibodies used: MAb 3H6F8	No						-

Continued

7.3 Table on methods described in literature for detection of ISAV and diagnosis of ISA (continued)

Question	**Answer**	**Is the test used for: monitoring or confirmation of disease?** *M or C*	**Is the test standardized?** *Yes/no*	**Is the test validated?** *Yes/no*	**Accord -ing to ISO 17025** *Note no.*	**Spec ***	**Sens ****	**References Numbers**
Susceptible fish species	*Atlantic salmon*	M & C	Yes	No	No	?	?	(18)
TESTS:								
PCR tests	Types of PCR tests: RT-PCR, real-time PCR, NASBA	M & C				(7, 21)	(19)	(7, 17, 18, 19, 24, 25, 26)
histo-pathology	Light microscopy is routinely used in diagnostic of ISA	Yes, M & C						(6)
Immuno-histo-chemistry	Antibodies used: Rabbit anti ISAV developed by K. Falk, Oslo. ISAV Mab-15 from Stirling University	Yes, M & C						(16, 19, 30)
In-situ hybridization	Probes used:	Yes, M & C						(12, 15)
Electron Microscopy	With special labelling? No	No						(11)
Haem-absorption test	Routinely in use in Marine Lab, Aberdeen	M						(23)

The numbers in the table refer to the following references

1. Bouchard, D., Keleher, W., Opitz, H. M., Blake, S., Edwards, K. C., and Nicholson, B. L. (1999). Isolation of infectious salmon anemia virus (ISAV) from Atlantic salmon in New Brunswick, Canada. Diseases of Aquatic Organisms 35, 131-137.

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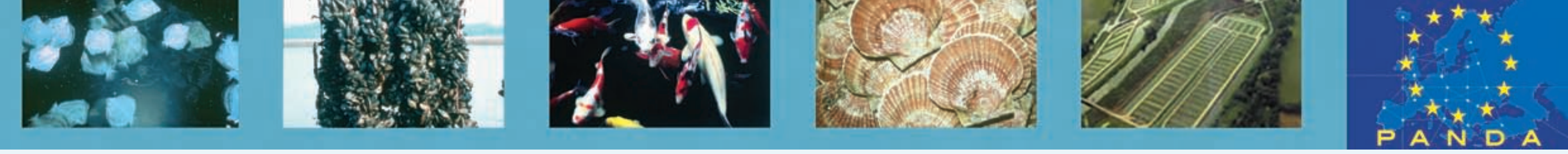

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7.4 Current available detection and diagnostic methods for some non WP2 listed diseases/pathogens of molluscs and crustaceans

7.4.1 Herpesvirus (oyster herpes-like virus disease, OsHV1)

OsHV-1 (Oyster Herpesvirus -1) infection causes mortality in the larvae and juveniles of several bivalve species including *Crassostreae gigas*, *Ostrea edulis*, *Ruditapes decussatus* and *R. philippinarum* as well as *Pecten maximus*. The virus can be found in adult bivalves (probably under a latent form) but without any mortality. Herpesviruses were also described in other mollusc species like *Crassostrea virginica*, *Ostrea angasi* and *O. chilensis* and more recently in abalones *Haliotis diversicolor*. However, molecular characterization could not be done or has not completely been performed yet (notably in the case of abalone herpesvirus).

Susceptible known species are: *Crassostrea gigas, Crassostrea angulata, Ostrea edulis, Ruditapes decussates, Ruditapes philippinarum,* and *Pecten maximus.*

Clinical pathology

Infected larvae show a reduction in feeding and swimming activities and mortality can reach 100% in few days. Affected spat show sudden and high mortalities mainly in summer time. The virus is associated with abnormal nuclei through connective tissues especially in mantle, labial palps, gills, and digestive gland.

Agent description

OsHV1 is a herpes-type virus or herpes-like virus. Ostreid Herpes Virus type 1 (OsHV-1) from *Crassostrea gigas* in France has been described. However, the apparent lack of host specificity and loss of several gene functions in OsHV-1 prompts speculation that this virus may have resulted from interspecies transmission in the context of introduction and intensive culture of non-native bivalve species (Arzul et al. 2001a, 2001b; ICES 2004). It is not known if the herpes-like viruses reported from various species of oysters and other bivalves are the same or different species of virus.

Confirmatory techniques for diagnosis

The different PCR protocols previously described in the section "screening techniques" can also be used as confirmatory techniques when suspicious lesions are observed by histology.

An *in situ* hybridization protocol has also been developed using dig-labelled A5/A6 and C1/C6 PCR products as probes (Lipart and Renault, 2002). Both probes were able to detect 50 pg of PCR amplified viral DNA by Southern Blot. No non-specific binding was observed when tests were performed on Human herpesvirus DNA. *In situ* hybridization is very convenient especially when infection level is low, like in adults. The test is performed on paraffin embedded tissues and requires 2 days before obtaining final results. The cost is estimated at 21 € for one individual (including personal cost).

Transmission electron microscopy is time consuming and can not be applied in routine but is recommended when herpesvirus is suspected in a new host species. Viral particles are typical of members of the family *Herpesviridae*. Capsids and nucleocapsids can be observed in the nucleus of infected cells while enveloped virions are present in the cytoplasm.

Sequencing is recommended as one of the final steps for confirmatory diagnostic. The genome of OsHV1 has been entirely sequenced and is available in Genbank (NC_005881 and AY509253). Obtained sequences should be compared with available ones in Genbank.

Screening techniques for the pathogen

Histology allows observing abnormalities but not specific to herpesviral infection. Cellular abnormalities are not associated with massive inflammatory reaction. Lesions are mainly observed in connective tissues in which fibroblastic –like cells exhibit enlarged nuclei with marginated chromatin and highly condensed nuclei in cells interpreted as hemocytes in spat.

A nested-PCR using primers A3-A4 and A5-A6 and targeting (after the second amplification) 940 bp of a gene coding an unknown protein was first developed to detect the virus in *Crassostrea gigas* larvae and spat (Renault et al. 2000). Up to 500 fg of viral DNA can be detected in samples and these primers could not amplify other herpesviruses.

A simple PCR using primers C1-C6 (Renault and Arzul 2001) has been then developed targeting 896 bp of a part of the viral genome located in an inverted repeat and coding fragments of unknown proteins. This protocol allows detecting up to 10 fg of viral DNA and these primers could not amplify other herpesviruses. This technique is often used for the detection of OsHV-1 especially in the context of abnormal mortalities. Larvae and spat are analysed by pool. This technique requires one day (from sample receipt to final results). Testing one pool of 5 juveniles costs about 6 € (including personal cost).

A competitive PCR method was also developed using previously designed primer pairs, C2-C6, amplifying a 710 fragment of the viral genome located in an inverted repeat and coding fragments of unknown proteins (Renault and Arzul 2001 and Renault et al. 2004). This technique is based on the use of oyster herpesvirus specific primers and an internal standard competitor that differs from the target DNA by a deletion of 76 bp. The assay allows detecting up 1 fg of viral DNA in 0.5 mg of oyster tissues. Moreover, this technique allows checking the presence of PCR inhibitors as well as performing a semi quantification of viral DNA.

Comments and recommendations on available techniques

Protocols for PCR and *in situ* hybridization are available in pre cited articles. However both techniques need to be validated and more specifically specificity and sensitivity values are lacking.

What should we do for diagnosis at suspicion?

In case of suspicion in larvae: all dead and moribund larvae should be collected for DNA extraction and PCR according to Renault et al. 2000.

In case of suspicion in juveniles: analyses should preferably be performed on moribund individuals. 30 individuals should be analysed in pools of five animals. DNA extraction and PCR are performed according to Renault et al. 2000.

In case of suspicion in adults: OsHV-1 was never associated with mortality of adults. However, adults might be asymptomatic carriers. In situ hybridization can be used to test the presence of OsHV-1 in connective tissues of adults.

EU-legislation related to techniques

Not listed by the EU legislation.

OIE recommendations related to techniques (& ref lab OIE)

Not listed by the OIE Manual of Diagnostic Tests for Aquatic Animals (2007 version) nor by the Aquatic Animal Health Code (2007 version).

Assessment

The tests are discussed at yearly CRL/NRL meetings. Use the methods according to Table 7.5 for screening, and confirmation respectively.

References

See the Full Mollusc Reference list 3.4.20.

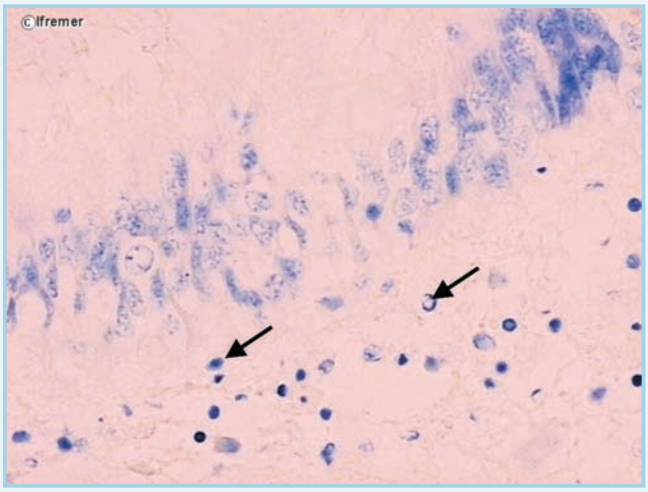

Fig. 32: Nucleus abnormalities (arrows) associated with OsHV-1 infection in mantle connective tissue in *Crassostrea gigas* spat (Unna blue staining)(IFREMER acknowl.).

7.4.2 *Bonamia ostreae*

Bonamia ostreae is a protistan parasite responsible for bonamiosis also named microcell disease or haemocyte disease of flat oysters, *Ostrea edulis*. Although the life cycle outside the host is unknown, it has been possible to transmit the disease experimentally in the laboratory by cohabitation or inoculation of purified parasites.

The parasite may occur throughout the year but prevalence and intensity of infection tend to increase during the warm season. There is a seasonal variation in infection by *B. ostreae* with the highest prevalence occurring in September. A prepatent period of at least 3 months is observed.

Bonamia ostreae naturally occurs in *Ostrea edulis* and when moved in endemic zones in *O. puelchana, O. angasi, Ostrea chilensis (= Tiostrea chilensis = Tiostrea lutaria),* and probably *Crassostrea ariakensis (= Crassostrea rivularis*). *Ostrea conchaphila* (= *Ostrea lurida*), *Crassostrea angulata* and *Crassostrea ariakensis* (= *Crassostrea rivularis*) have been speculated to be susceptible to *Bonamia ostreae* but infections with *B. ostreae* have not been actually demonstrated.

Bonamia ostreae has been reported in Europe, North America and recently in Morocco. Susceptible known species: *Ostrea edulis, Ostrea puelchana, Ostrea angasi, Ostrea chilensis.*

Clinical pathology

Bonamiosis is a lethal infection of the haemocytes of flat oysters sometimes accompanied by yellow discoloration and extensive lesions on the gills and mantle. However, most of the infected oysters appear normal. Histologically, lesions occur in the connective tissue of the gills, mantle, and digestive gland. This intrahaemocytic protozoan quickly becomes systemic with overwhelming numbers of parasites coinciding with the death of the oysters.

Agent description

Bonamia ostreae is a protistan parasite of the phylum Haplosporidia responsible for bonamiosis also named microcell disease or haemocyte disease of flat oysters, *Ostrea edulis*.

Confirmatory techniques for diagnosis

Both PCR protocols previously described in the section "screening techniques" can also be used as confirmatory techniques. However, both assays are not species specific. A protocol of RFLP applied on PCR products obtained using Cochennec et al. PCR technique has been developed and allows to differentiate *Bonamia ostreae*, *B. exitiosa* and *B. roughleyi* (Cochennec et al. 2003; Hine et al. 2001). This technique needs to be validated.

Two *in situ* hybridization protocols have been developed. The first one (Cochennec et al. 2000) uses a 300 bp digoxigenin-labeled probe produced by PCR and using primer pairs Bo-Boas and the second one (Carnegie et al. 2003) uses three fluorescein-labeled oligonucleotide probes (UME-BO-1, UME-BO-2 and UME-BO- 3). The probe Bo-Boas is able to detect *Haplosporidium nelsoni* in *Crassostrea virginica, Bonamia exitiosa* in *Ostrea chilensis* but not *Mikrocytos mackini* in *C. gigas*. The specificity of the oligoprobe cocktail UME-BO-1, 2 and 3 has been tested and proved against *H. nelsoni* but this ISH assay probably detects other microcells including at least *B. exitiosa*.

In situ hybridization can help to detect early infection which is more difficult to detect in traditional histological sections.

Transmission electron microscopy is time consuming and can not be applied in routine but is recommended when *Bonamia* like parasites are described in a new host species. Moreover, transmission electron microscopy can help to differentiate *B. ostreae* from other members of this genus like *B. exitiosa*.

Different stages including uninucleate, diplocaryotic and plasmodial stages have been reported. Intracellular structures include mitochondria, haplosporosomes, Golgi apparatus and persistent intranuclear microtubules.

Dense forms of *Bonamia ostreae* are more dense, slightly smaller in size in comparison to *Bonamia exitiosa*, have less haplosporosomes, mitochondrial profiles and lipoid bodies per ultrastructure section, and have larger tubulo-vesicular mitochondria than *B. exitiosa*. In addition, dense forms of *Bonamia ostreae* lack nuclear membrane-bound Golgi/nuclear cup complexes and a vacuolated stage (Hine et al. 2001).

Sequencing is recommended as one of the final steps for confirmatory diagnostic. Targeted regions are SSU rDNA and ITS1. Obtained sequences should be compared with available ones in gene banks.

Screening techniques for the pathogen

Tissue imprints can be realised using oyster spat or heart ventricle or gills from live adult hosts. *Bonamia ostreae* appears as small spherical or ovoid organisms (2-5 µm wide) inside haemocytes. However, the parasite might also occur extracellularly. Using Wright, Wright-Giemsa or equivalent stain (e.g., Hemacolor, Merck; Diff-QuiK, Baxter) these parasites show a basophilic cytoplasm and an eosinophilic nucleus. Multinucleated cells can be observed.

Histopathology should be realised on tissue sections that include gills, digestive gland, mantle, and gonad and stained with hematoxylin and eosin. Infected oysters, parasites can be observed as very small cells of 2-5 µm wide, within the haemocytes or freely in connective tissue or sinuses of gill, gut and mantle epithelium, often associated with intense inflammatory reaction.

Tissue imprints appear less reliable than histopathology for the detection of the parasite in case of low level of infections. However, Tissue imprints are more rapid and less expensive than histopathology (cost for one individual is estimated at about 5 € and 20 € -including personal cost- respectively).

Two PCR protocols with two different primer pairs targeting the SSU rDNA have been developed for *Bonamia ostreae*: the first one uses the primer pair Bo-Boas (Cochennec et al. 2000) and the second one the primer pair C_F-C_R (Carnegie et al. 2000). Based on target DNA sequence similarity, the first assay should amplify all microcell haplosporidians and the second one at least *Bonamia ostreae* and *B. exitiosa*. These assays has not bee validated directly against one another but they appear to be roughly equivalent in sensitivity. PCR developed by Cochennec et al. (2000) has been compared to histopathology and cytology together (Balseiro et al. 2006). Sensitivity of PCR was 92% (between 64 and 69% for histological methods together) and specificity of PCR was estimated between 85 and 90% (97% for histological methods together).

Comments and recommendations on available techniques

Protocols for PCR and *in situ* hybridization are available in pre cited articles. PCR technique developed by Cochennec et al (2000) has been submitted to several validation tests against histological methods. However, validation is still required for *in situ* hybridization methods.

What should we do for diagnosis at suspicion?

In some cases, highly infected oysters might present some gill indentations. When suspected, *Bonamia ostreae* can be detected by heart or gill imprints. In parallel, piece of gills can be fixed in ethanol for PCR analysis and a section of oysters should be fixed in Davidson's fixative for histological examination.

EU-legislation related to techniques

Bonamia ostreae is listed by the EU legislation (91/67/EEC Annex A), and also in the new EU Directive 2006/88/EC, as non exotic pathogen.

OIE recommendations related to techniques (& ref lab OIE)

Bonamia ostreae is listed by the OIE Manual of Diagnostic Tests for Aquatic Animals (2007 version) and by the Aquatic Animal Health Code (2007 version).

The OIE recommends:

- Tissue imprints and histopathology for surveillance
- Tissue imprints and histopathology for **presumptive** diagnostic
- PCR-RFLP and transmission electron microscopy for **confirmatory** diagnostic

Assessment

The tests are discussed at yearly CRL/NRL meetings. Use the methods according to Table 7.5 for screening, and confirmation respectively.

References

See the Full Mollusc Reference list 3.4.20.

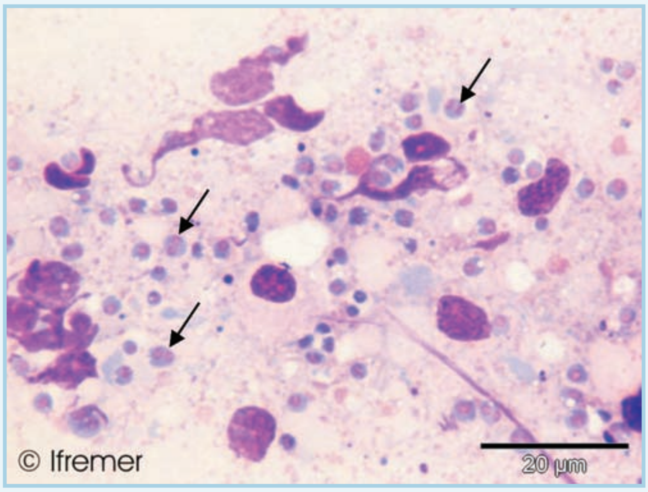

Fig. 33: Heart imprint of European flat oyster *Ostrea edulis* showing free cells and some multinucleate cells (arrows) of *Bonamia ostreae* (Hemacolor staining)(IFREMER acknowl.).

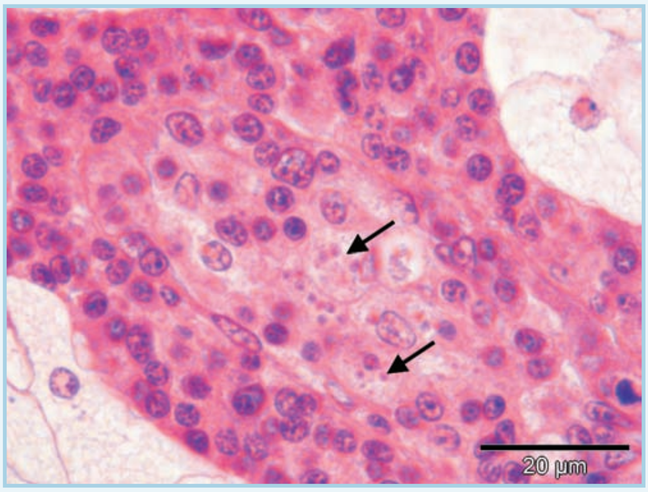

Fig. 34: *Bonamia ostreae* present in haemocytes of flat oyster *Ostrea edulis* (H&E staining). (IFREMER acknowl.).

7.4.3 *Marteilia refringens*

Marteilia refringens and *M. maurini* are protistan parasites which are responsible for marteiliosis in flat oysters *Ostrea edulis* and mussels *Mytilus edulis* and *M. galloprovincialis* respectively. Infection with *Marteilia refringens* is a lethal disease of oysters. Mussels are usually not adversely affected by marteilioisis.

The parasite can survive in the environment from several days up to 2–3 weeks depending on the environmental conditions. Transmission of the parasite from oyster to oyster is not possible directly. A copepod, *Paracartia grani*, seems to be involved in the parasite life cycle and could act as an intermediate host.

Marteilia refringens naturally occurs in *Ostrea edulis* and when moved in endemic zones in *O. puelchana, O. angasi, Ostrea chilensis*. *M. maurini* naturally infects *Mytilus edulis* and *M. galloprovincialis*. *Marteilia refringens* and *M. maurini* have been reported in Southern Europe and in Morocco. Susceptible known species are: *Ostrea edulis, Ostrea puelchana, Ostrea angasi, Ostrea chilensis, Mytilus edulis,* and*Mytilus galloprovincialis.*

Remark: Because taxonomic relationships between *Marteilia refringens* and *M. maurini* are still not clear, we consider in this section that *Marteilia refringens* infects flat oysters *Ostrea edulis* and *Marteilia maurini* infects mussels *Mytilus edulis* and *M. galloprovincialis.*

Clinical pathology

Infection with *Marteilia refringens* is a lethal disease of oysters. Death occurs during the second year after initial infection. Different stages of the parasite can be observed in infected oysters and mussels. Young plasmodia are mainly found in the epithelium of labial palps and the stomach. Sporulation takes place in the digestive gland tubules and ducts. Propagules are released into the lumen of the digestive tract and shed into the environment in faeces.

Agent description

Marteilia refringens and *M. maurini* are protistan parasites belonging to the phylum Paramyxea and which are responsible for marteiliosis in flat oysters *Ostrea edulis* and mussels *Mytilus edulis* and *M. galloprovincialis* respectively. Because taxonomic relationships between *Marteilia refringens* and *M. maurini* are still not clear, we consider in this section that *Marteilia refringens* infects flat oysters *Ostrea edulis* and *Marteilia maurini* infects mussels *Mytilus edulis* and *M. galloprovincialis.*

Confirmatory techniques for diagnosis

The PCR protocol previously described in the section "screening techniques" can also be used as confirmatory technique. However, this assay can not differentiate *Marteilia refringens* and *M. maurini.* A protocol of RFLP applied on PCR products obtained using ITS-1 primers has been developed and allows differentiating *Marteilia refringens* and *M. maurini.* (Le Roux et al. 2001). This technique needs to be validated.

An *in situ* hybridization protocol has been developed and is based on the use of Smart2, a 266 bp digoxy-

genin-labelled probe targeting the SSU rDNA (Le Roux et al. 1999). Smart 2 is able to detect *Marteilia* species including *Marteilia refringens*, *M. maurini* and *M. Sydneyi* (Le Roux et al. 1999; Kleeman et al. 2002). Values of specificity and sensitivity for *in situ* hybridization were estimated at 0.9 and 0.99 respectively when co validated with histology (Thébault et al. 2004). *In situ* hybridization can help to detect early infection which is more difficult to detect in traditional histological sections.

Transmission electron microscopy is time consuming and can not be applied in routine but can be recommended when *Marteilia* like parasites are described in a new host species. Ultrastructural criteria are not enough discriminant to differentiate *Marteilia refringens* and *M. maurini*. Haplosporosomes in mature *Marteilia* from oysters and mussels appear similar in shape, although those from mussels seem to be marginally smaller in size, and spore wall morphology vary depending on the state of maturity of the parasite (Longshaw et al. 2001).

Sequencing is recommended as one of the final steps for confirmatory diagnostic. Targeted regions are SSU rDNA and ITS1. Obtained sequences should be compared with available ones in gene banks.

Screening techniques for the pathogen

Tissue imprints can be realised using digestive gland from live or gapping bivalves. *Marteilia refringens* and *M. maurini* appears as cells ranging in size up to 30–40 µm. Using Wright, Wright-Giemsa or equivalent stain (e.g., Hemacolor, Merck; Diff-QuiK, Baxter) these parasites show a basophilic cytoplasm and an eosinophilic nucleus. Pale halo around large, strongly stained (refringent) granules and in larger cells, cell within cell arrangements are observed.

Histopathology should be realised on tissue sections that include digestive gland, gills and palps and stained with hematoxylin and eosin or equivalent staining. *Marteilia* cells have a size comprised between 4 up to 40 µm. Young plasmodia (uninucleate) are mainly found in the epithelium of labial palps and stomach. Sporulation involves divisions of cells within cells and takes place in the digestive gland tubules and ducts. Refringent granules appear in the course of sporulation, but are not observed in early stages. In late phases of infection, sporangia are observed free in the lumen of the digestive tract.

Values of sensitivity and specificity for histopathology were estimated at 0.7 and 0.99, respectively when co validated with *in situ* hybridization (Thébault et al. 2004). Tissue imprints appear less reliable than histopathology for the detection of the parasite in case of low level of infections. However, tissue imprints are more rapid and less expensive than histopathology (cost for one individual is estimated at about 5 € and 20 € -including personal cost- respectively).

A PCR protocol targeting the ITS1 has been developed for the detection of *Marteilia refringens* (Le Roux et al. 2001). No cross-reaction has occurred with tested samples and specificity is considered very high. This PCR is expected to detect both *Marteilia refringens* and *Marteilia maurini*. Because infection may be focal and also because infection targets different tissues in the early and late stages, the sensitivity of PCR detection may be lower than expected theoretical PCR performances However, this technique has not be validated against histology.

Comments and recommendations on available techniques

Protocols for PCR and *in situ* hybridization are available in pre cited articles. *In situ* hybridization developed by Le Roux et al (1999) has been co validated with histology (Thébault et al. 2004). However, validation is still required for PCR.

What should we do for diagnosis at suspicion?

When suspected, *Marteilia refringens* can be detected by digestive gland imprints. In parallel, pieces of digestive gland can be fixed in ethanol for PCR analysis and a section of oysters should be fixed in Davidson's fixative for histological examination.

EU-legislation related to techniques

Marteilia refringens is listed by the EU legislation (91/67/EEC Annex A).

OIE recommendations related to techniques (& ref lab OIE)

Marteilia refringens is listed by the OIE Manual of Diagnostic Tests for Aquatic Animals (2007 version) and by the Aquatic Animal Health Code (2007 version).

- Histopathology for **surveillance**
- Tissue imprints, histopathology and *in situ* hybridization for **presumptive** diagnostic
- Histopathology, PCR, ISH and sequencing for **confirmatory** diagnostic

Assessment

The tests are discussed at yearly CRL/NRL meetings. Use the methods according to Table 8.5 for screening, and confirmation respectively.

References

See the Full Mollusc Reference list 3.4.20.

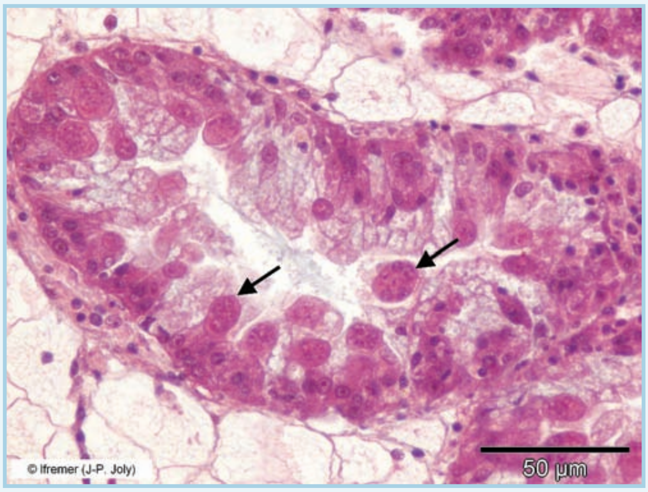

Fig. 35: *Marteilia refringens* sporangia present in diverticule epithelium of the digestive gland in flat oysters *Ostrea edulis* (Masson trichrome staining)(J.-P. Joly acknowl.).

7.4.4 Gaffkemia (*Aerococcus viridans*)

Gaffkemia is a fatal disease of both American and European lobsters *Homarus americanus* and *H. gammarus* due to a bacterium *Gaffkya homari*, renamed Aerococcus *viridans* var. *homari* (Stewart & Zwicker, 1974 a).

Clinical pathology

In lobster the bacterium develops mainly in the circulatory system and affects blood composition.

Agent description

Aerococcus viridans is a non motile, Gram + coccus, forming tetrads (0.8 -1.1 µm). The bacteria growths on a large variety of media and is non encapsulated.

Confirmatory techniques for diagnosis

Bio-chemicals characterization of the isolated and cultivated cocciform bacteria constitute a good confirmatory technique.

Screening techniques for the pathogen

Observation of small tetrads in smears of haemolymph, of diseased lobster. The tetrats must be Gram positive. The culture, isolation and characterization of the pathogen appear necessary in the diagnostic.

Comments and recommendations on available techniques

For confirmation, isolation and biochemical characterization are good. For screening, smears of haemolymph are, apart from culture recommended to use, like described above.

What should we do for diagnosis at suspicion?

At suspicion, isolate the bacterium at standard agar plates (sheep blood for instance) from the lobster, and type it biochemically.

EU-legislation related to techniques

Aerococcus viridans is not listed by the EU. Therefore, no recommendations are made by the EU.

OIE recommendations related to techniques (& ref lab OIE)

Aerococcus viridans is not listed by the OIE. Therefore, no recommendations are made by the OIE.

Assessment

The isolation of the bacterium is a good method, followed by biochemical typing, according to standard methods.

References

See the Full Crustacean Reference list 3.4.27.

7.4.5 Crayfish plague (*Aphanomyces astaci*)

Aphanomyces astaci is a pathogenic oomycete of crayfish. All stages of the European crayfish (*Astacus astacus, A. leptodactylus, Austrapotamobius pallipes* and *Au. torrentium*) are highly susceptible to the disease. At the opposite, all North American crayfish (*Pacifastacus leniusculus, Procambarus clarkii* and *Orconectes sp.*) can carry and consequently disseminate the agent without noticeable mortalities.

Clinical pathology

The vegetative hyphae of the parasite, developing in host tissues (mainly connective tissue and blood vessels), produce sporangia releasing primary spores; after germination they give biflagellate zoospores which attach and germinate to produce invasive vegetative hyphae in a new host after penetrating the cuticle (Alderman & Polglase, 1986; Alderman *et al.*, 1987).

Agent description

The etiological agent is *Aphanomyces astaci*. Regarded during several years as a fungus, the Oomycetida are now classified as diatoms and brown algae.

Confirmatory techniques for diagnosis

The PCR detection method and isolation and culture are the two confirmatory techniques for the diagnosis of this disease.

Screening techniques for the pathogen

Using wet mounts of small pieces of soft cuticle from joints of pereiopods or ventral intersternal cuticle of the tail, the disease is characterized by the presence of aseptate hyphae of the fungus, 7-9 µm in diameter. These structures are often associated with hemocytes infiltration and melanisation.

The best method is detection of the agent and identification. Isolation and culture methods were described by Alderman & Polglase (1986) on agar medium containing yeast extract, glucose and anti-microbial agents (Isolation medium). Growing colonies are colourless containing vegetative aseptate hyphae. When these colonies are transferred in natural river water, sporangia form in about 24 hours. The full developing cycle of the fungus can be observed by this way.

More recently a PCR method was described by Oidtmann *et al*. (2002). The OIE Manual suggests slight modifications of this method using the following primers:

- P525: 5'-AAG AAG GCT AAA TTG CGG TA-3'

- P640: 5'-CTA TCC GAC TCC GCA TTC TG-3'

Positive results give a 115 bp amplicons.

Comments and recommendations on available techniques

Isolation and culture of the fungus is a time consuming method which should be replaced by the PCR technique.

What should we do for diagnosis at suspicion?

Isolate and culture the pathogen as described above. The PCR detection method can be used as confirmatory technique.

EU-legislation related to techniques

Aphanomyces astaci is not listed by the EU. Therefore, no recommendations are made by the EU.

OIE recommendations related to techniques (& ref lab OIE)

Aphanomyces astaci is listed by the OIE.

The OIE (Manual of Diagnostics Tests for Aquatic Animals, 2006) rates the tests against purpose of use:

The methods currently available for surveillance, detection, and diagnosis of crayfish plague are listed below. The designations used indicate:

A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity;

B = requires experience and diagnostic expertise that may not be readily available

These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility.

The OIE (2006) recommends for:

- Surveillance of susceptible species:
  - Gross and microscopic signs (B)
  - Isolation and culture (A)
  - PCR (A)
- Surveillance of resistant species:
  - PCR (A)
- Presumptive diagnosis of infection or disease (detection):
  - Gross and microscopic signs (A)
  - Isolation and culture (A)
  - PCR (A)
- Confirmatory diagnosis of infection or disease (diagnosis):
  - Gross and microscopic signs (B)
  - Isolation and culture (A)
  - PCR (A)
  - Histopathology is not recommended for screening or confirmation.

Confirmation of a site free of crayfish plague must be done by a cohabitation assay: caging a few susceptible crayfish and observing them for several months.

OIE Reference Laboratories for ***Aphanomyces astaci***:

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- Institute of Zoology, University of Munich, Prof.dr. R. Hoffmann, E-mail: r.hoffmann@zoofisch.vetmed.uni-muenchen.de

Assessment

Follow the recommendations of the OIE.

References

See the Full Crustacean Reference list 3.4.27.

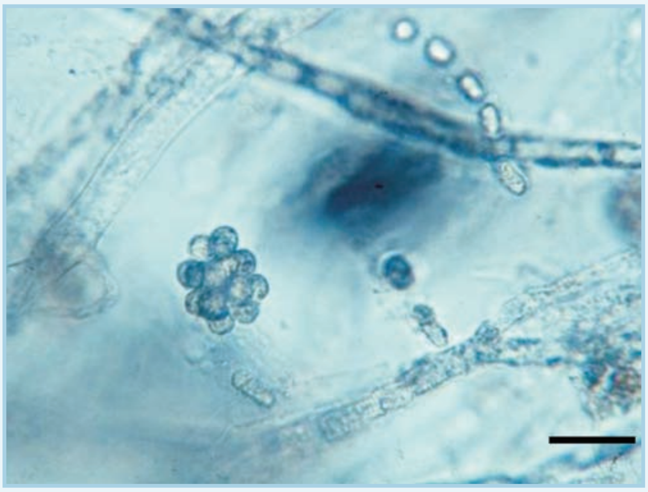


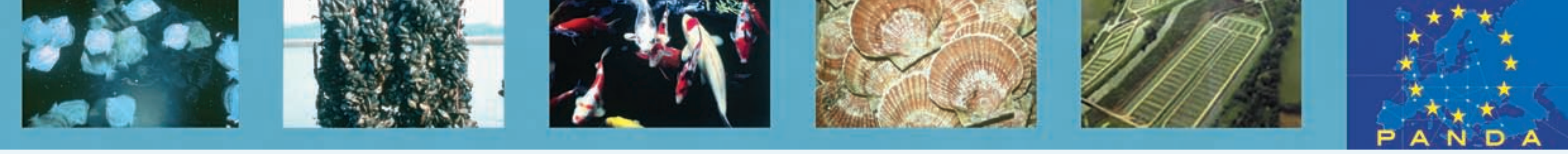
Fig. 36: *Aphanomyces astaci* in crayfish cuticle with encysted primary spores in typical clusters. Bar = 100µm (D. Alderman acknowl.)

7.5 Table on the evaluation of available methods for non WP2 listed mollusc and crustacean diseases/pathogen

Summary of WP4 results on **non-WP2 listed** diseases/pathogens of molluscs and crustaceans.: current screening/diagnostic methods and their evaluation.

Disease/ pathogen	Confirmatory technique (well established)	Screening technique (well established)	Evaluation
OsHV-1	PCR, DNA sequencing, ISH, TEM	Histo, PCR	• No special further test needs • Apart from NRL meeting no training needs
Bonamia ostreae	PCR, DNA sequencing, ISH, TEM	Histo, cytology, PCR	• PCR and ISH only genus specific • Apart from NRL meeting no training needs
Marteilia refringens	PCR, DNA sequencing, ISH, TEM	Histo, cytology, PCR	• ISH only genus specific • PCR needed for sequencing • Apart from NRL meeting no training needs
Gaffkemia *Aerococcus viridans*	Morphology; biochemical typing; serological grouping; DNA sequencing; IHC	Smears of haemolymph, isolation, biochemistry	• Methods are o.k. • No training needed
Crayfish plague *Aphano-myces astaci*	Isolation; morphology (staining and colony type); PCR	Clin.pathol, isolation; morphology (staining and colony type); PCR	• Pathogen with high impact to Europe • Urgently training needed on clinics, and detection and diagnostic methods

IHC = immunohistochemistry; ISH = in situ hybridization; TEM = transmission electron microscopy



7.6 List of illustrations and author

Photo no.	Pathogen/disease	Author(s) are acknowledged
1	RSIV	M.Sano, J. Kurita and T. Ito
2-3	ISAV	N.J. Olesen
4	KHV	M.Engelsma & O.Haenen
5	*Strept. agalactiae (3 pictures)*	Joyce Evans
6	*Strept. iniae*	Joyce Evans
7	*Lactococcus garv.*	A.Manfrin
8-9	*Trypanosoma salmositica*	P.T.K. Woo
10	*Ceratomyxa shasta*	J.Bartholomew
11-12	*Neoparamoeba*	B.Nowak
13	*Gyrodactylus salaris*	O'Dowd, Copyright Cefas Photo Library
14-16	*Aphanomyces invadans*	John Hawke and Al Camus
17-23	Mollusc diseases	IFREMER
24	Crust. yellowhead	D.V. Lightner
25-26	Crust. White spot	J.R. Bonami & D.V. Lightner
27	Crust. Taura	D.V. Lightner
28	Crust. IHHNV	J.R. Bonami
29-30	*Batrachochytrium dendrobatidis*	F. Mutschmann
32	Oyster herpes-like virus (OsHV-1)	IFREMER
33-34	*Bonamia ostreae*	IFREMER
35	*Marteilia refringens*	IFREMER
36	Crust. *Aphanomyces astaci*	D.Alderman





7.7 The PANDA consortium

Partner	Representative	Role
1. Cefas, Weymouth, UK	Barry Hill	Project coordinator
2. Danish Veterinary Institute, Aarhus, Denmark	Ellen Ariel	Steering group member
3. IFREMER, La Tremblade, France	Isabelle Arzul	Steering group member
4. CIDC, Lelystad, The Netherlands	Olga Haenen	Diagnostic methods workpackage leader
5. National Veterinary Institute, Oslo, Norway	Edgar Brun	Epidemiology workpackage leader
6. Federation of European Aquaculture Producers, Belgium	Panos Christofilogiannis	Environmentally safe disease control workpackage leader
7. National University of Ireland, Galway, Ireland	Maura Hiney	Training needs and opportunities workpackage leader
8. IRTA, Tarragona, Spain	Chris Rodgers	Risk analysis workpackage leader