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### Diseases of fish

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Diseases of bivalve molluscs

Diseases notifiable to the OIE*

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Diseases of crustaceans

Other significant diseases*

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List of OIE Reference Laboratories for fish, mollusc and crustacean diseases

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List of organisations with which the OIE has cooperation agreements

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* Diseases notifiable to the OIE are defined in the International Aquatic Animal Health Code. They are considered to be of socio-economic and/or public health importance within countries and also significant in the international trade of aquatic animals and aquatic animal products, and must be reported to the OIE as specified in the Code. These diseases were previously known as 'List B diseases'.

*Other significant diseases' are also defined in the International Aquatic Animal Health Code. They are of current or potential international significance in aquaculture but have not been included in the list of diseases notifiable to the OIE because of factors related to their importance or geographic distribution, or current knowledge about them.
FOREWORD

The Office International des Epizooties (OIE) is an intergovernmental organisation which was established in 1924 in order to promote world animal health, and its main activities are as follows:

1. To collect and disseminate to its Member Countries, information (including emergency information) on the occurrence, course and treatment of animal diseases

2. To provide guidelines and standards for health regulations applicable in the international trade of animals

3. To promote and co-ordinate research on the pathology, treatment and prevention of animal diseases when international collaboration in such research is desirable.

Aquatic animals are included in the concept of 'animals' above. Diagnostic procedures for some aquatic animal diseases used to be included in the OIE International Animal Health Code (1986 edition), but it became clear that a separate Code and Manual specific to aquatic animal health were needed. The reasons are that the conditions, problems and requirements in this field are different to those encountered in other animals, and that international trade in aquatic animals and their products is intensifying and increasing in importance.

The purpose of this Manual is to provide a uniform approach to the diagnosis of the diseases listed in the OIE International Aquatic Animal Health Code, so that the requirements for health certification in connection with trade in aquatic animals and aquatic animal products, can be met. The Manual is therefore a companion volume to the Code.

Although many publications exist on the diagnosis and control of aquatic animal diseases, the OIE Diagnostic Manual for Aquatic Animal Diseases will hopefully be a key document in describing methods that can be applied in aquatic animal health laboratories all over the world, thus increasing efficiency and promoting improvements in aquatic animal health worldwide.

The task of compiling the Manual was assigned to the OIE Fish Diseases Commission, and all the chapters were circulated to OIE Member Countries for comments and revision. The Manual will be continually revised and updated as new information on aquatic animal diseases in general, and new emerging diseases in particular, becomes available, and it is intended to publish a new edition approximately every four years.

Dr Jean Blancou
Director General, OIE

Prof. Tore Håstein
President, Fish Diseases Commission

1995
INTRODUCTION

The clinical signs in fish with the diseases listed in the *OIE International Aquatic Animal Health Code* are not pathognomonic. Moreover, these infections may take place as subclinical infections of asymptomatic pathogen carriers.

The only dependable approach for diagnosis of fish diseases therefore lies in the specific identification of the pathogens using laboratory methods. These methods, which are suitable for the diagnosis of isolated cases of disease as part of the operating of national aquatic animal health surveillance/control programmes, form the main contents of this *Manual*.

Basically such health surveillance programmes aim to infer, from the results provided by standardised laboratory procedures performed with samples collected according to defined rules, the health status of aquatic animal stocks from a particular production site and even a geographic zone or entire country. The satisfactory implementation of such aquatic animal health surveillance/control programmes, requires the existence of both adequate legislation and resources in each country interested in aquatic animal health.

The diagnostic methods presented in this *Manual* are all direct diagnostic methods. Due to insufficient development of the serological methodology, the detection of fish antibodies to viruses has not thus far been accepted as a routine diagnostic method for assessing the health status of fish populations. However, the validation of some serological techniques for diagnosis of certain fish virus infections could arise in the near future, rendering the use of serology more widely acceptable for diagnostic purposes. At present the only diagnostic methods which are accepted in those countries where aquatic animal health control programmes are implemented, are based either on isolation of the pathogen followed by its specific identification, or on demonstration of pathogen-specific antigens using an immunological detection method.

Mollusc diseases differ in some ways from fish diseases with respect to the considerations mentioned above. General information on diagnostic techniques for mollusc diseases is given in Chapter 15.

As explained in Part 3 of the *Code*, the list of notifiable diseases of aquatic animals includes only major diseases of proven aetiology and limited geographic range. The OIE Fish Diseases Commission therefore recommended the creation of a list entitled 'Other significant diseases'. The diseases on this list include:

- those which are serious, but have a broad geographic distribution;
- those causing significant mortality, transmissible, and of limited geographic range, but for which the aetiological agent has not yet been identified;
• those with the potential for causing large losses, but which are too new for the geographic range to be defined or for the essential epizootiological elements to be understood.

It is expected that the diseases on this list will either be elevated to notifiable status or dropped from the list as new information is obtained.

The *Manual* includes descriptions of diagnostic methods for these 'Other significant diseases' as well as for the notifiable diseases.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>AO</td>
<td>acridine orange</td>
</tr>
<tr>
<td>BF-2</td>
<td>bluegill fibroblast (cell line)</td>
</tr>
<tr>
<td>BKD</td>
<td>bacterial kidney disease</td>
</tr>
<tr>
<td>BMN(V)</td>
<td>baculoviral midgut gland necrosis virus</td>
</tr>
<tr>
<td>BP</td>
<td><em>Baculovirus penaei</em></td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BSS</td>
<td>balanced salt solution</td>
</tr>
<tr>
<td>CCO</td>
<td>channel catfish ovary (cell line)</td>
</tr>
<tr>
<td>CC(VD)</td>
<td>channel catfish (virus disease)</td>
</tr>
<tr>
<td>CFA</td>
<td>complete Freund's adjuvant</td>
</tr>
<tr>
<td>CHSE-214</td>
<td>chinook salmon embryo (cell line)</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathic effect</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNFP</td>
<td>deoxynucleotide</td>
</tr>
<tr>
<td>EHN(V)</td>
<td>epizootic haematopoietic necrosis (virus)</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPC</td>
<td><em>Epithelioma papulosum cyprini</em> (cell line)</td>
</tr>
<tr>
<td>ESC</td>
<td>enteric septicaemia of channel catfish</td>
</tr>
<tr>
<td>EUS</td>
<td>epizootic ulcerative syndrome</td>
</tr>
<tr>
<td>FAT</td>
<td>fluorescent antibody test</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's basal salt solution</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid</td>
</tr>
<tr>
<td>IF</td>
<td>immunofluorescence</td>
</tr>
<tr>
<td>IFA</td>
<td>incomplete Freund's adjuvant</td>
</tr>
<tr>
<td>IFAT</td>
<td>indirect fluorescent antibody test</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IHHN</td>
<td>infectious hypodermal and haematopoietic necrosis</td>
</tr>
<tr>
<td>IHN(V)</td>
<td>infectious haematopoietic necrosis (virus)</td>
</tr>
<tr>
<td>IPN(V)</td>
<td>infectious pancreatic necrosis (virus)</td>
</tr>
<tr>
<td>ISA</td>
<td>infectious salmon anaemia</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>KDM-2</td>
<td>kidney disease medium</td>
</tr>
<tr>
<td>MAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MBV</td>
<td><em>Penaeus monodon</em>-type baculovirus</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>NAb</td>
<td>neutralising antibody</td>
</tr>
<tr>
<td>NeVTA</td>
<td>nerka virus Towada Lake, Akita and Amori prefecture</td>
</tr>
<tr>
<td>OCT</td>
<td>embedding medium for frozen tissue specimens</td>
</tr>
<tr>
<td>OMV(D)</td>
<td><em>Oncorhynchus masou</em> virus (disease)</td>
</tr>
<tr>
<td>OPD</td>
<td>o-phenylenediamine</td>
</tr>
<tr>
<td>OVVD</td>
<td>oyster velar virus disease</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>phosphate buffered saline containing Tween</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PIB</td>
<td>polyhedral inclusion body</td>
</tr>
<tr>
<td>POB</td>
<td>polyhedral occlusion body</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RSD</td>
<td>red spot disease</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SJNNV</td>
<td>striped jack nervous necrosis virus</td>
</tr>
<tr>
<td>SKDM</td>
<td>selective kidney disease medium</td>
</tr>
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<td>SVC(V)</td>
<td>spring viraemia of carp (virus)</td>
</tr>
<tr>
<td>TRIS</td>
<td>tris(hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>VHS(V)</td>
<td>viral haemorrhagic septicemia (virus)</td>
</tr>
<tr>
<td>VN</td>
<td>virus neutralisation</td>
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<td>YHV</td>
<td>yellowhead virus</td>
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<tr>
<td>YTV</td>
<td>yamame tumour virus</td>
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DEFINITIONS

The *International Aquatic Animal Health Code* (companion volume to this *Manual*) contains a list of definitions which may be consulted for the meaning of terms used in this *Manual*. Some terms which are not used in the *Code* but which appear in the *Manual*, are defined below:

**Fry**

means newly hatched *fish* larvae.

**Sensitivity**

means the proportion of true positive tests given in a diagnostic test, i.e. the number of true positive results divided by the number of true positive and false negative results.

**Specificity**

means the probability that absence of infection will be correctly identified by a diagnostic test, i.e. the number of true negative results divided by the number of true negative and false positive results.
ACKNOWLEDGMENTS

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Miss G.S. Townshend is thanked for her excellent editorial work.

* Member of the OIE Fish Diseases Commission, 1991 to present
# Observer at OIE Fish Diseases Commission meetings, 1991 to present
DISEASES OF FISH

CHAPTER 1
GENERAL INFORMATION

A - GENERAL BASES OF
FISH HEALTH SURVEILLANCE/CONTROL PROGRAMMES

1. TARGET PATHOGENS AND DISEASES

Target pathogens and fish diseases are included in the Code according to the following basic considerations: they resist or respond poorly to therapy, have a restricted geographic range and are of high socio-economic importance, regardless of the host they infect. The list of fish diseases considered for notification and certification is currently restricted to five viral diseases. They are:

- Epizootic haematopoietic necrosis (EHN)
- Infectious haematopoietic necrosis (IHN)
- *Oncorhynchus masou* virus disease (OMVD)
- Spring viraemia of carp (SVC)
- Viral haemorrhagic septicaemia (VHS)

2. OVERALL APPROACH FOR ANIMAL HEALTH CONTROL IN FISH CULTURE

A comprehensive approach for animal health control in fish culture requires:

- Assessment of health status of animals in a production site based upon inspections and standardised sampling procedures followed by laboratory examinations conducted according to instructions given in this Manual.

- The constraint of restocking open waters and farming facilities only with products having a health status higher than or equal to that of animals already living in the considered areas.

- Eradication of disease when possible, by slaughtering of infected stocks, disinfection and restocking with pathogen-free fish.

- Notification by every Member Country of its particular requirements, beside those provided by the Code, for importation of aquatic animals and animal products.
If the above procedures are followed, it becomes possible to give adequate assurance of the health status of aquaculture products for specified diseases, according to their country, zone or production site of origin.

The issue of a health certificate by the appropriate official, based on a health status report and examinations in aquatic animals, provides assurance that the aquaculture products in a defined consignment originate from a whole country, a zone or a farm/harvesting site free of one or more of the specified diseases listed in the Code and possibly of other specified diseases (see model of international certificate in the Code).

The assessment of the health status of fish stocks is based upon inspection of fish production sites and further laboratory examination of fish organ samples originating from fish specimens taken among the stock of a defined fish population. This endeavour requires the fish sample collecting to be done according to defined sampling size charts and the organ processing to be conducted according to accepted methods.

### B - SAMPLING PROCEDURES

#### 1. Collection of fish specimens

Two situations can be encountered when collecting fish during inspection of fish production sites:

- fish exhibit the clinical signs of one of the diseases listed in the Code or other diseases.

- fish appear clinically normal.

The goals of the inspection/sampling procedures can thus be different: they are conducted:

- either to demonstrate the health status of a fish production site,

- or to confirm that a certain status is being maintained once it has been achieved after a minimum period of two years of implementation of the fish surveillance programme enforced in the country.

#### 1.1. Clinically infected fish

A minimum number of ten moribund fish or ten fish exhibiting clinical signs of the diseases in question, must be selected and taken. Fish should be alive when collected. They should be sent to the laboratory alive or sacrificed and packed separately in sealed aseptic refrigerated containers or on ice. The freezing of collected fish must be strictly
avoided. However, it is highly preferable and recommended to collect organ samples from the fish immediately after they have been selected at the fish production site and to store and process the samples as described in Sections 2 and 3. A label of identification mentioning the place and time of sampling must be attached to the sample.

1.2. Asymptomatic fish (healthy fish)

Fish collection must encompass a statistically significant number of specimens, but it is obvious that failure to detect certain pathogens from the sample does not guarantee the absence of these agents in the specimen examined or in the stock. This is particularly true of free-ranging or feral stocks from which it is difficult to collect a representative and random sample. However, the risk of a pathogen escaping the surveillance system is reduced in fish farms whose fish stocks have been inspected and checked for pathogens for several years (at least two), insofar as they are not exposed to possible recontamination by migratory fish.

When a given fish production site harbours a broodstock, it is essential for one of the sample collections made each year to be focused on the sexual products (sperm and ovarian fluid) released by broodfish at time of spawning (see below). If an adult broodstock includes fish of different ages, the older fish should be selected for sampling:

- Samples must comprise all susceptible species on the site, with each lot of a species being represented in the sample group. A lot is defined as a group of the same fish species that shares a common water supply and which originate from the same broodfish or spawning population. For closed-water fish farming such as pond culture of cyprinid fish, the fish population from a pond with no water connection with others, constitutes a lot. For closed-water fish production units which comprise the storage of fish in holding facilities after pond harvesting and fish grading and sorting, the fish stock harboured in such a facility can be considered as a lot providing fish sub-samples are taken from populations of all the holding tanks.

The geographic origin of samples should be defined by the name of the sampling site associated with either its geographic co-ordinates or its localisation along a river course expressed as a kilometric point.

- If any moribund fish are present in the fish population to be sampled, they should be selected first for sample collection and the remainder of the sample is to be made with randomly selected live fish from all containers which represent the lot being examined.
• The minimum sample size for each lot must be in accordance with a plan which provides 95% confidence that infected specimens will be included in the fish sampled, assuming a minimum prevalence of infection equal to or greater than 2%, 5% or 10%. The minimum sample size for lots varying from 50 to infinity in size, for each inspection is given in Table I (23).

• As in the case of clinically infected fish, organ and fluid samples must be taken and processed as soon as possible after fish specimen collection. Sample freezing must be avoided.

### Table I
Sample size based on assumed pathogen prevalence in lot

<table>
<thead>
<tr>
<th>Lot size</th>
<th>At 2% prevalence size of sample</th>
<th>At 5% prevalence size of sample</th>
<th>At 10% prevalence size of sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>50</td>
<td>35</td>
<td>20</td>
</tr>
<tr>
<td>100</td>
<td>75</td>
<td>45</td>
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<td>250</td>
<td>110</td>
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<td>500</td>
<td>130</td>
<td>55</td>
<td>26</td>
</tr>
<tr>
<td>1,000</td>
<td>140</td>
<td>55</td>
<td>27</td>
</tr>
<tr>
<td>1,500</td>
<td>140</td>
<td>55</td>
<td>27</td>
</tr>
<tr>
<td>2,000</td>
<td>145</td>
<td>60</td>
<td>27</td>
</tr>
<tr>
<td>4,000</td>
<td>145</td>
<td>60</td>
<td>27</td>
</tr>
<tr>
<td>10,000</td>
<td>145</td>
<td>60</td>
<td>27</td>
</tr>
<tr>
<td>100,000 or more</td>
<td>150</td>
<td>60</td>
<td>30</td>
</tr>
</tbody>
</table>

(After Ossiander and Wedemeyer, 1973)

1.3. Sampling specifications according to the objectives of a given fish surveillance programme

a) *Achievement of the health status of a fish stock/population at a given inspection site*

• A fish culture unit must be inspected twice a year during two years at the appropriate lifestage of the fish and at times of year when temperature and season offer the best opportunity for observing clinical signs and isolating the pathogens if present. Each time, fish must be collected in order to detect a prevalence of infection equal to or higher than 2% at 95% confidence level. Most often, 150 fish will thus be collected on each occasion or, during one of the two inspections, 150 ovarian fluid samples will be taken from broodfish if present in the given fish culture unit.
• If fish health surveillance is focused on wild fish populations at a given site of inspection or on rearing ponds without holding facilities in which different fish crops may be pooled, collection of 150 fish specimens must be done once a year for two years. Insofar as it is possible specimens of the oldest fish and/or ovarian fluid must be collected as a priority.

• During this two-year period, the fish production unit may only be restocked with fish from a unit whose health status has already been approved.

b) Maintenance of the health status

• Once a fish production unit including pond fish production units equipped with holding facilities, has been recognised to be free of all or certain diseases listed in the Code after two years of surveillance with laboratory tests and in the absence of any suspect clinical signs, twice-yearly inspections must continue. However, collection of fish specimens may be reduced to 30 fish, including broodfish when available. Moribund fish observed during inspection visits must, however, be collected for further laboratory examination.

• Maintenance of health status of wild fish populations relevant to diseases listed in the Code at a given site of inspection, can only be ascertained by annual collection of 150 individuals including as many broodfish as possible.

• The fish production unit may only be restocked with fish having a health status higher than or equal to that of those already present.

• If, during monitoring of samples, a cytopathic effect (CPE) appears in cell cultures inoculated with dilutions of the samples being tested, virus identification procedures have to be undertaken immediately (see the relevant chapters). Provisions have to be taken to suspend the approved health status of the production unit and/or the zone (if it was approved previously) from which the virus positive sample originated. The suspension of approved status will be maintained until it is demonstrated that the virus in question is not the one referred to in the granting of free status.

The above sampling specifications for the achievement and maintenance of the health status of fish at given fish production sites imply that all provisions given in Section A.2 (Overall approach for animal health control in fish culture) are in force.
2. **SAMPLE MATERIAL TO BE USED IN VIRAL AND BACTERIOLOGICAL TESTS**

Sample material depends both on the size of animals and the objective of testing i.e. diagnosis of overt disease or detection of fish that are asymptomatic pathogen carriers.

2.1. **Specifications according to fish size**

- **Alekin and sac fry**: sample entire fish but remove the yolk sac if present.
- **Fish 4-6 cm**: take the entire viscera including kidney. A piece of encephalon can be obtained after severing head at level of the rear edge of operculum and pressing it laterally.
- **Fish over 6 cm**: take the kidney, spleen and encephalon.
- **Broodfish**: take ovarian fluid and/or tissues as described in Chapters 2-6.

2.2. **Specifications according to clinical status**

In the case of clinical infection, beside whole alekin or entire viscera, organs to be sampled are anterior kidney, spleen and encephalon for virus screening and kidney and spleen for bacterial screening. Samples from 10 diseased fish will thus be taken and combined to form pools of a maximum of 5 fish each. The amount of material should not exceed 1.5 g/pool of material from 5 fish.

For detecting asymptomatic carriers, samples may be combined to form a pool (no more than 5 fish/pool), for a total weight of about 1.5 g. Pools of ovarian fluid from 5 broodfish must not exceed a total volume of 5 ml, i.e. 1 ml/broodfish. These ovarian fluid samples are to be taken individually from every sampled female and not after the pooling of ova.

Once aseptically removed from fish, organs and/or ovarian fluid sampled are each split into two parts, one intended for virological examination, the other for bacteriological examination. For bacteriological sampling, live fish, newly dead (chilled) fish or inoculated required medium are preferred.

3. **GENERAL PROCESSING OF ORGANS/FLUID SAMPLES FOR VIROLOGICAL EXAMINATION**

3.1. **Transportation and antibiotic treatment of samples**

Pools of organs or of ovarian fluids are placed in sterile vials and stored at 4°C until virus extraction is performed at the laboratory. Virus extraction should optimally be done within 24 h after fish
sampling but is still acceptable for up to 48 h.

Organ samples may also be transported to the laboratory by placing them in vials containing cell culture medium or Hanks' basal salt solution (HBSS) with added antibiotics to suppress the growth of bacterial contaminants (1 vol. of organ in at least 5 vol. of transportation fluid). Suitable antibiotic concentrations are: gentamycin 1,000 µg/ml or penicillin (800 IU/ml) and dihydrostreptomycin (800 µg/ml). The antifungal compounds Mycostatin® or Fungizone® may also be incorporated into the transport medium at a final concentration of 400 IU/ml. Serum or albumen 5-10% may be added to stabilise the virus if the transport time will exceed 12 hours.

3.2. Virus extraction

• Conduct this procedure below 15°C and preferably between 0°C and 4°C.
• Decant antibiotic-supplemented medium from organ sample.
• Homogenise organ pools with mortar and pestle or electric blender until a paste is obtained. This must be resuspended in transport medium to a final dilution of 1:10.
• If organ samples have not been treated with antibiotics prior to homogenisation, organ homogenates are to be resuspended in antibiotic-supplemented medium and incubated in this medium for 2-4 hours at 15°C or overnight at 4°C.
• Clarify diluted homogenates by centrifugation at 2,000 xg for 15 min. and collect supernatants.
• Ovarian fluid samples are to be centrifuged in the same way as organ homogenates and their supernatants used directly in subsequent steps.

3.3. Treatment to neutralise IPN viruses

In some countries, fish are often asymptomatic carriers of aquatic birnaviruses (such as infectious pancreatic necrosis virus, IPNV), which induce a cytopathic effect (CPE) in susceptible cell cultures and thus complicate and retard isolation and further identification of other viruses. In such situations, the infectivity of birnaviruses possibly present in sample homogenate supernatants must be neutralised before testing for the viruses listed in the Code. However, when it is important to determine whether IPNV is present, samples must be tested with and without neutralising antibodies present.

To neutralise birnaviruses, mix equal volumes (200 µl) of a solution of neutralising antibodies (NAb) to the indigenous birnavirus serotypes of
IPNV and of the supernatant to be tested. Allow the mixture to react for one hour at 15°C prior to inoculation onto susceptible cell monolayers. The titre of the NAb solution used (it may be a multivalent serum) should be at least 2,000 in a 50% plaque reduction test versus the IPNV serotypes present in the given geographic area.

When samples are from a country, region, fish population or production unit considered free from birnavirus infection, this treatment of the organ homogenate should be omitted.

4. **GENERAL PROCESSING OF SAMPLES INTENDED FOR BACTERIOLOGICAL EXAMINATION**

As in viral infections, internal organs may be used as a source of isolation whenever systemic infection is suspected. However, active proliferation of saprophytic associated microorganisms is such a disadvantage that live fish are preferred for bacteriological examination. The fact that no antibiotic substances may be added to the transport medium in which the samples are collected reinforces this preference.

*Renibacterium salmoninarum* is an instance of these cases in which biological products can be used with some advantage. Sexual products, namely the ovarian fluid, seem to represent quite a good source of pathogenic bacteria in mature salmonids.

**C - MATERIALS AND BIOLOGICAL PRODUCTS REQUIRED FOR THE ISOLATION AND IDENTIFICATION OF FISH PATHOGENS**

1. **FISH VIRUSES**

1.1. **Fish cell lines**

The following four fish cell lines will be required to test for the fish pathogens mentioned in the *Code*:

- Bluegill fibroblast (BF-2)
- Channel catfish ovary (CCO)
- Chinook salmon embryo (CHSE-214)
- *Epithelioma papulosum cyprini* (EPC)

Technical information on the use of these cells for the isolation of the fish pathogens listed in the *Code* is given in Table II.

1.2. **Culture media**

Traditional Eagle's minimum essential medium (MEM) with Earle's Salt supplemented with 10% fetal calf serum, antibiotics and 2 mM L-glutamine is the most widely used medium for fish cell culture.
Table II
Technical information on the most suitable fish cell lines for detection of the viral agents listed in the OIE Code

<table>
<thead>
<tr>
<th>Properties</th>
<th>BF-2</th>
<th>CCO</th>
<th>CHSE-214</th>
<th>EPC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell line nomenclature</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Culture characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell morphology</td>
<td>Fibroblastic</td>
<td>Fibro/epithelioid</td>
<td>Epithelioid</td>
<td>Epithelioid</td>
</tr>
<tr>
<td>Temperature range (°C)</td>
<td>15-28</td>
<td>15-35</td>
<td>4-25</td>
<td>10-33</td>
</tr>
<tr>
<td>Optimum growth temp. (°C)</td>
<td>20</td>
<td>30</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>Inoculum (No. cells x 10^4/cm^2)</td>
<td>(1)</td>
<td>(1)</td>
<td>(1)</td>
<td>(1)</td>
</tr>
<tr>
<td>Saturation density (No. cells x 10^4/cm^2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to saturation (days) (2)</td>
<td>8</td>
<td>5-6</td>
<td>10</td>
<td>8</td>
</tr>
</tbody>
</table>

(1) To achieve loose confluency within 24 hours. (2) At optimal growth temperature.

However, Stoker’s medium (Stoker and McPherson, 1961) which is a modified form of the above medium comprising a double strength concentration of certain amino acids and vitamins, is particularly recommended to enhance cell growth, using the same supplementations as above plus 10% tryptose phosphate.

These media are buffered with either sodium bicarbonate, 0.16 M trihydroxymethyl aminomethane (Tris) HCl, or, preferably, 0.02 M N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid (HEPES). The use of sodium bicarbonate alone is restricted to those cell cultures made in tightly closed cell culture vessels.

For cell growth, the fetal bovine serum content of the medium is usually 10%, whereas for virus isolation or virus production it may be reduced to 2%. Similarly the pH is 7.3-7.4 for cell growth and adjusted to 7.6 for virus production or virus assay.

The composition of the most frequently used antibiotic mixture is penicillin 100 IU/ml and dihydrostreptomycin 100 μg/ml. Mycostatin (50 IU/ml) may be used if a fungal contamination is expected. Other antibiotics or antibiotic concentrations may be used as convenient for the operator depending on the antibiotic sensitivity of the bacterial strains encountered.
1.3. Virus positive controls and antigen preparation

a) Virus nomenclature

• Epizootic haematopoietic necrosis virus (EHNV)
• Infectious haematopoietic necrosis virus (IHNV)
• *Oncorhynchus masou* virus (OMV)  
  (Syn. Salmonid herpesvirus type 2)
• Spring viraemia of carp virus (SVCV)
• Viral haemorrhagic septicaemia virus (VHSV)  
  (Syn. Egtved virus)

b) Virus production

For the production of most of these viruses the susceptible cell cultures must be inoculated with fairly low multiplicities of infection (MOI) i.e. $10^{-2}$ to $10^{-3}$ PFU per cell. However, the MOI for OMV has to be increased up to 0.1-1 PFU/cell. Furthermore, the best yields for OMV are obtained using inocula containing cell debris of the OMV-infected previous cell monolayer.

c) Virus preservation and storage

• Dilute virus-containing cell culture fluids in order to obtain virus titres averaging 1-2 x $10^6$ PFU/ml.
• Dispense the resulting viral suspensions into sterile vials at volumes of 0.3-0.5 ml each.
• Freeze and store each series of reference virus stocks at -80°C, and check the titre of each virus stock every two months if it has not been used during that time interval.

*Lyophilisation:* long term storage (decades) of the seeds of reference virus strains is achievable by lyophilisation. For this purpose, viral suspensions in cell culture medium supplemented with 10% fetal calf serum (FCS) are mixed vol./vol. with a 50% sodium glutamate solution in distilled water before processing. Seal or plug under vacuum and store at 4°C, in the dark.

2. FISH BACTERIA

2.1. Culture media

Few species of fish pathogenic bacteria require special media for cultivation, and most of usual isolation peptones (trypsin-soya, brain-heart, etc.) can conveniently be used. However, the low optimal temperatures of some species result in slow growth and mean that
small colonies are frequently obtained during isolation. In these cases it is usual to add enrichment factors such as serum or blood at 5-10% in order to improve the cultivation. Similarly, rapid diagnostic systems are convenient in theory, but in practice it is wise to question the relative value of negative characteristics, which may result merely from insufficient metabolic activity.

Conversely *Renibacterium salmoninarum* is a very fastidious organism and requires special media enriched with cysteine (No. A11B).

Bacteriology of fish is generally conducted at temperatures between 20 and 26°C. It is sometimes necessary or useful to have access to several incubators. *R. salmoninarum*, *Flexibacter psychrophilus* and others need 15°C for optimal growth and many of the bacteria isolated from warm water fish may be studied at 30 or 37°C to accelerate the diagnostic steps.

2.2. Conservation

Bacterial strains can be conserved in the short term on ordinary media, placing the slants or broths at 4°C. For most strains, the use of commercial conservation media or agar slants with mineral oil will extend viability to 1-2 years under the same conditions without further special requirements.

Freezing is probably the best way to preserve bacterial suspensions of high titre. However, it does not always prevent some phenotypical characteristics from changing. When stability of characteristics such as virulence is a major purpose it may be better to use lyophilisation, although the number of viable bacteria may be decreased dramatically.

Different kinds of supports have been proposed to improve the efficacy of freezing and lyophilisation, namely glycerol 5 to 15% in the first case, skim milk, lactose, dextran 5 to 10% in the second one. There is no general rule, and convenient conditions have to be determined in prior trials for all species. The addition to fresh cultures of one volume of support containing Bactopeptone 11% + Dextran 4% has provided excellent results for lyophilisation of certain fish bacteria, but other formulas would be worth testing in many cases.

3. Serology

3.1. Production of rabbit antisera and polyclonal antibodies to fish viruses

There are various ways in which antibodies against fish viruses can be raised in rabbits. However, titre and specificity are influenced by the inoculation programme used (see Hill et al., 1981). The following immunisation protocols may be used to produce antisera for use in the virus isolation and/or identification procedures described later.
a) Antisera to IPNV

Intravenous injection with 50-100 µg of purified virus on day 0 followed by an identical booster on day 21. Bleeding 5-7 days later. Rabbits may be reused if not bled completely.

b) Antisera to other viruses

The immunisation protocols alternate an intramuscular or intradermal injection with further intravenous boosters:

Day 0, primary injection: 500-1,000 µg of virus are mixed with adjuvant (Freund's complete or other\textsuperscript{1}) vol./vol. giving a total volume of 1.2 ml. This antigen is delivered to the rabbit as multipoint intradermal injections (20 points on each side) after the animal has been shaved.

Day 21: collect about 20 ml of blood and check for reactivity (neutralisation, fluorescence) and boost intravenously with the same amount of virus as in the primary injection.

Prior to the intravenous booster injection, the rabbit has to be treated with promethazine (12 mg IM) to prevent possible anaphylactic response.

Day 28: sample the blood, check the serum reactivity and bleed or boost according to the results.

3.2. Antisera to fish bacteria

It is still difficult to obtain antimicrobial sera in large amounts from commercial sources, and it will be often necessary to prepare such antisera. The general methods are the same as for viruses. Bacterial antigens are frequently used as crude preparations killed by heating or formalin 3.5 p.1000. Rabbit injection at increasing doses can be intramuscular, every week, with adjuvant the first time, or intravenous at 3-4 day intervals without adjuvant. A booster injection is often required after 15 days.

A special multipoint intradermal schedule has proven very efficient for anti-*Renibacterium* sera production, and is also valuable for other poorly antigenic bacteria.

The antigen is heat killed (60°C, 45 min) and adjusted to 2 mg/ml. The flanks of the animals are thoroughly shaved, and multipoint intradermal injections are performed using total amounts of 1 mg bacteria/animal, according to the following schedule:

\textsuperscript{1} Use of Freund's adjuvants may be restricted on animal welfare grounds. Alternative synthetic adjuvants include trehalose dimycolate (TDM) and monophosphate lipid A (MPL).
bacteria/animal, according to the following schedule:

<table>
<thead>
<tr>
<th>Day</th>
<th>Injection Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>1 mg + complete Freund adjuvant (CFA) v/v</td>
</tr>
<tr>
<td>Day 21</td>
<td>1 mg + incomplete Freund adjuvant (IFA) v/v</td>
</tr>
<tr>
<td>Day 42</td>
<td>1 mg + IFA</td>
</tr>
<tr>
<td>Day 63</td>
<td>1 mg antigen</td>
</tr>
<tr>
<td>Day 68</td>
<td>Collecting of blood samples (about 30 ml)</td>
</tr>
</tbody>
</table>

Withdrawal injections and bleeding for serum collection may be repeated at one month intervals.

### 3.3. Processing and storage of immune sera

After blood clotting, collect and centrifuge the serum at 20°C and heat it for 30 min at 56°C. Filter the resulting heat-inactivated serum through a 450 nm membrane and temporarily store it at +4°C during the time necessary for the screening of its reactivity and specificity and for checking that these properties are not affected by preservation conditions (e.g. freezing or lyophilisation). Sterile rabbit sera can be kept for at least two months at 4°C without any change in their properties.

Dispense (usually as small volumes) and freeze at -20°C or lyophilise.

Immunoglobulins may be extracted from antisera using conventional methods suitable for Ig purification. Selective attachment to protein A constitutes a reliable and effective method. The concentration of Ig solutions is adjusted to the values required for further conjugate preparation or storage.

*Preservation of Ig:* Mix vol./vol. a solution of Ig of titre 2 mg/l with sterile pure glycerol and keep at -20°C. Ig of solutions of higher titre may also be prepared in glycerol.

### 3.4. Mouse monoclonal antibodies to fish viruses and bacteria

Monoclonal antibodies to several fish viruses (IPNV, VHSV, IHNV, CCV) have been raised during the past years. Some of them, singly or as two or three associated MAbs, have given rise to biological reagents suitable for the identification of virus groups (IPN, VHS, IHN). Other MAbs, taken individually or as components of Ab panels, allow accurate typing of VHSV and IHNV. These MAbs can be obtained from the Reference Laboratories listed at the end of the Manual.

The production of monoclonal antibodies to bacteria has also been described. It has resulted in the development of commercial diagnostic kits for Renibacterium in 1992, but in most cases remains limited to specialised laboratories.
may be impaired by processes such as enzymatic or radio-labelling or lyophilisation. It is thus mandatory to test the stability of every MAb to processing and preservation conditions.

BIBLIOGRAPHY


DISEASES OF FISH
DISEASES NOTIFIABLE TO THE OIE
CHAPTER 2
EPIZOOTIC HAEMATOPOIETIC NECROSIS
(B413)

SUMMARY

Epizootic haematopoietic necrosis (EHN) is an iridovirus infection of redfin perch (Perca fluviatilis) and rainbow trout (Oncorhynchus mykiss). The geographic range of the infection is currently restricted to Australia. For a recent and more detailed review of the condition, see Wolf (10).

The infection of fish is most often lethal in perch and much less so in rainbow trout, in a clinical context of haemorrhages and oedema accompanied by necrotic lesions of the vascular walls, liver, spleen and haematopoietic tissue of the kidney. Necrotising hepatitis seems to be a consistent sign of the condition.

The antigenicity of EHNV is currently under investigation using rabbit antibodies (Ab). This virus has not so far generated synthesis of NAb but can be identified by immunofluorescence or ELISA tests. However, indirect fluorescent antibody tests (IFA) and Western-blotting indicate that EHNV shares common antigenic domains with two other fish iridoviruses isolated from the sheatfish (Silurus glanis) and the catfish (Ictalurus melas) in Europe and also with frog virus 3 and Bohle iridovirus.

The epidemiology of EHNV in rainbow trout is incompletely understood. Infection may recur annually at a production site but it is uncertain whether this is due to persistence of infection on the site or reintroduction of infection from wild fish in the catchment. A carrier state in naturally infected rainbow trout appears to be very uncommon. Neither EHNV antigen nor anti-EHNV antibody are detected in rainbow trout surviving an outbreak of EHNV.

The factors modulating the susceptibility of fish to EHNV infection are poorly understood. Clinical disease is associated with poor water quality. In rainbow trout infection occurs naturally from 11-17°C and experimentally from 8-21°C. Infection in redfin perch is non-permissive at temperatures below 12°C. The following fish species were found to be susceptible to EHNV following bath exposure: redfin perch, rainbow trout, Macquarie perch (Macquaria australasica), mosquito fish (Gambusa affinis), silver perch
Both juvenile and adult redfin perch may be affected in outbreaks, but juveniles may be more susceptible to the disease.

The diagnostic procedures for EHN are based upon isolation of virus in cell culture, ELISA, IFA, and electron microscopy. Antigen capture ELISA has a sensitivity of 80% and a specificity of 99% when used to test tissues from both rainbow trout and redfin perch. Antigen capture ELISA is the method of choice for confirming the cause of CPE in cell culture; however, IFA and electron microscopy are also useful. IFA or immunoperoxidase staining may be used also for diagnosis on formalin fixed tissues.

**DIAGNOSTIC PROCEDURES**

The diagnosis of EHN is based upon direct methods which are either the isolation of EHN virus (EHNV) in cell culture followed by its immunological identification (conventional approach), or the immunological demonstration of EHNV antigen (Ag) in infected fish tissues.

Due to insufficient knowledge of the fish serology of virus infections, the detection of fish antibodies to viruses has not thus far been recognised as a valuable diagnostic method for assessing the virus status of fish populations. However, the validation of some serological techniques for diagnosis of certain fish virus infections could arise in the near future, rendering the use of fish serology more widely acceptable for diagnostic purposes.

Infected fish material suitable for virological examination is:

- **during overt infection**: whole alevin (body length ≤ 4 cm), viscera including kidney (4 cm ≤ body length ≤ 6 cm) or, for larger size fish, kidney, spleen and liver.

- **during dormant infection** (detection of asymptomatic virus carrier fish): encephalon (any size fish) and/or ovarian fluid from broodfish at time of spawning.

**Sampling procedures**: see Chapter 1 Part B.

1. **STANDARD MONITORING METHODS FOR EHN**

1.1. **Isolation of EHN in cell culture**

   Cell line(s) to be used: BF-2.

   a) **Inoculation of cell monolayers**

      i) Make two additional tenfold dilutions of the 1:10 organ
homogenate supernatants and transfer an appropriate volume of each of the three dilutions onto 24-hour-old BF-2 cell monolayers. Inoculate at least 2 cm\(^2\) of cell monolayer with 100 µl of each dilution.

ii) Allow to adsorb for 0.5-1 hour at 10-15°C and, without withdrawing inoculate, add cell culture medium buffered at pH 7.6 and supplemented with 2% FCS (1 ml/well for 24 well cell culture plates) and incubate at 20-22°C.

b) Monitoring incubation

i) Follow the course of infection in positive controls and other inoculated cell cultures, by daily microscopic examination at magnification 40x to 100x, during 14 days. The use of a phase contrast microscope is recommended.

ii) Maintain the pH of the cell culture medium between 7.3 and 7.6 over the whole incubation phase. This can be achieved by addition to the inoculated cell culture medium of sterile bicarbonate buffer (for tightly closed cell culture flasks) or 2 M Tris buffer solution (for cell culture plates) or, even better, by using HEPES-buffered media.

iii) If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of the tested homogenate supernatants, identification procedures have to be undertaken immediately (see below). If a fish health surveillance/control programme is being implemented, provisions have to be taken to suspend the approved health status of the production unit and/or the zone (if it was approved previously) from which the virus positive sample originated. The suspension of approved status will be maintained until it is demonstrated that the virus in question is not EHNV.

iv) If no CPE occurs, except in positive control cell cultures, subcultivation steps have to be made even after 7 days of incubation, in certain of the infected cell cultures. However, if no CPE is observed even in positive controls, another series of virological examinations have to be undertaken, using susceptible cells and new batches of organ samples.

c) Subcultivation procedures

i) Collect aliquots of cell culture medium from all monolayers inoculated with dilutions of each supernatant of organ homogenates.
ii) Centrifuge at 2,000 x g for 15 min at 4°C and collect supernatant.

iii) Repeat optional neutralisation test to IPNV if needed, with dilution of the above supernatant (1:1 to 1:100).

iv) Inoculate BF-2 cell monolayers as described above (1.1.a).

v) Incubate and monitor as in 1.1.b.

1.2. Virus identification

a) Neutralisation test

EHNV cannot be identified by neutralisation as the antisera generated by the immunisation of rabbits have few neutralising antibodies.

b) Indirect fluorescent antibody test

This virus identification test is to be conducted either directly after virus isolation in cell culture, or as a confirmatory test following the neutralisation test described above.

i) Prepare monolayers of BF-2 cells in 2 cm² wells of cell culture plastic plates or on coverglasses in order to reach around 80% confluency, which is usually achieved within 4 hours of incubation at 25°C (seed 6 cell monolayers per virus isolate to be identified, plus 2 for positive and 2 for negative controls). The FCS content of the cell culture medium can be reduced to 2-4%. If numerous virus isolates have to be identified, the use of Terasaki plates is strongly recommended.

ii) When the cell monolayers are ready for infection, i.e. on the same day or on the day after seeding, inoculate the virus suspensions to be identified by making ten-fold dilution steps directly in the cell culture wells or flasks.

iii) Dilute the control virus suspension of EHNV in a similar way, in order to obtain a virus titre of about 5,000-10,000 PFU/ml in the cell culture medium.

iv) Incubate at 20°C for 24 hours.

v) When the incubation time is over, aspirate the cell culture medium, rinse once with PBS 0.01 M pH 7.2, then 3 times briefly with cold fixative. This fixative will be acetone (stored at -20°C) for coverglasses or a mixture of acetone 30%-ethanol 70% (vol./vol.), also stored at -20°C.
vi) Afterwards, let the fixative act for 15 min. A volume of 0.5 ml is adequate for 2 cm² of cell monolayer.

vii) Allow the cell monolayers to air dry for at least 30 min and process immediately or freeze at -20°C.

viii) Prepare a solution of purified antibody or serum to EHNV in PBS 0.01 M, pH 7.2 containing 0.05% Tween 80, at the appropriate dilution (which has been established previously or is given by the reagent supplier).

ix) Rehydrate the dried cell monolayers by 4 rinsing steps with the above PBS and eliminate this buffer thoroughly after the last rinsing.

x) Treat the cell monolayers with the antibody solution for 1 hour at 37°C in a moist chamber. The volume of solution to be used is 0.25 ml/2 cm² well.

xi) Rinse 4 times with PBS-Tween as above.

xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate-conjugated (FITC) antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.

xiii) Rinse 4 times with PBS-Tween.

xiv) Observe the treated cell monolayers on plastic plates immediately, or mount the coverglasses using glycerol saline at pH 8.5 prior to microscopic observation.

xv) Conduct this observation under incident UV light using a microscope with x10 eye pieces and x20 to x40 objective lens having numerical aperture >0.65 and >1.3 respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

c) Enzyme-linked immunosorbent assay (ELISA)

i) Coat the wells of microplates designed for ELISA tests, with appropriate dilutions of purified immunoglobulins (Ig) or serum specific for EHNV, in PBS 0.01 M pH 7.2 (200 µl/well). Ig may be polyclonal or monoclonal Ig originating most often from rabbit or mouse, respectively.

ii) Incubate overnight at 4°C.
iii) Rinse 4 times with PBS 0.01 M containing 0.05 % Tween 20 (PBST).

iv) Block with skim milk (5% in PBST) or other blocking solution for 1 hour at 37°C (200 µl/well).

v) Add 2% of Triton X 100 to the virus suspension to be identified.

vi) Rinse 4 times with PBST.

vii) Dispense 100 µl/well of a 2 or 4 step dilution of the virus to be identified and of EHNV control virus, and allow to react with the coated antibody to EHNV for 1 hour at 20°C.

viii) Rinse 4 times with PBST.

ix) Add to the wells, biotinylated polyclonal antibody to EHNV.

x) Incubate 1 hour at 37°C.

xi) Rinse 4 times with PBST.

xii) Add streptavidin-conjugated horse radish peroxidase to those wells which have received the biotin-conjugated antibody and incubate for 1 hour at 20°C.

xiii) Rinse 4 times.

xiv) Add the substrate (H₂O₂) and chromogen (O-phenylenediamide, OPD or other approved chromogen). Stop the course of the test when positive controls react, and read the results.

xv) Alternatively: add H₂O₂ + chromogen to those wells containing the peroxidase conjugated antibody and proceed as above.

2. Diagnostic Procedures for Confirmation of EHN in Suspected Outbreaks

Confirmation of EHNV can be achieved by any of the following methods.

2.1. Conventional virus isolation with subsequent serological identification as in Section 1.

2.2. Virus isolation with simultaneous identification

Not possible; see 1.2.a.
2.3. Indirect fluorescent antibody test

i) Bleed the fish thoroughly.

ii) Make kidney imprints on cleaned glass slides or at the bottom of the wells of a plastic cell culture plate.

iii) Store the kidney pieces (as indicated in B.3.1. in Chapter 1 [General Information]) together with the other organs required for virus isolation in case this later becomes necessary.

iv) Allow the imprint to air-dry for 20 min.

v) Fix with acetone or ethanol-acetone and dry as indicated in 1.2.b. points v-vii.

vi) Rehydrate the above preparations (see 1.2.b. point ix) and block with 5% skim milk or 1% bovine serum albumin (BSA), in PBST for 30 min at 37°C.

vii) Rinse 4 times with PBST.

viii) Treat the imprints with the solution of antibody to EHNV and rinse as indicated in 1.2.b.

ix) Block and rinse as formerly.

x) Reveal the reaction with suitable FITC, rinse and observe as indicated in 1.2.b. points xii-xv.

If the immunofluorescence test is negative, process the organ samples stored at 4°C, for virus isolation in cell culture as in 1.1.

2.4. ELISA

a) Microplate processing

As 1.2.c of this chapter up to point iv (inclusive).

b) Sampling procedures

See the following sections in Chapter 1 (General Information):
B.1.1. for the selection of fish specimens
B.2.1. & 2.2. for the selection of materials sampled.

c) Processing of organ samples

See the following sections in Chapter 1 (General Information):
B.3.1. for transportation
B.3.2. for virus extraction and obtaining of organ homogenates.
d) Carrying out the ELISA

i) Set aside an aliquot of 1/4 of each homogenate in case further virus isolation in cell culture is required.

ii) Treat the remaining part of homogenate with 2% Triton X 100 (vol./vol.) as 1.2.c point v and 2 mM of phenyl methyl sulfonide fluoride (PMSF); mix gently.

iii) Complete the other steps of procedure 1.2.c.

REFERENCES


CHAPTER 3
INFECTIONOUS HAEMATOPOIETIC NECROSIS (B405)

SUMMARY

Infectious haematopoietic necrosis (IHN) is a rhabdovirus infection of rainbow trout (Onchorhyncus mykiss) including steelhead, several Pacific salmon i.e. sockeye (O. nerka), chinook (O. tshawystcha), chum (O. keta), yamame (O. masou), amago (O. rhodurus), and more recently coho (O. kisutch) and Atlantic salmon (Salmo salar). Until 1987, the geographic range of IHN was limited to the North Pacific Rim (North America and the Far East) but it has recently spread to continental Europe. For a recent and more detailed review of the condition, see Wolf (7).

IHN has become a real matter of concern because of its clinical and economic consequences in trout and salmon farming and in fisheries. Infection is often lethal, due to the impairment of the salt-water balance, which occurs in a clinical context of oedema and haemorrhages. Virus multiplication in endothelial cells of blood capillaries, haematopoietic tissues and nephron cells, underlies the clinical signs.

In survivors, IHN virus (IHNV) infection results in a strong protective immunity, synthesis of circulating antibodies to IHNV as well as, in certain individuals, an asymptomatic carrier state. This carrier state frequently leads to virus shedding via the sexual products at times of spawning.

On the basis of antigenic studies conducted with neutralising polyclonal antibodies from rabbit (rabbit antisera), IHNV isolates appear to form a homogeneous virus group. However, mouse monoclonal antibodies have revealed at least three neutralising virus-types (and more likely five) as well as the existence of an IHNV-group non-neutralising epitope borne by the nucleocapsid (N protein). Variations in the virulence of IHNV strains have been recorded during both natural cases of disease and in experimental infections.

The reservoirs of IHNV are clinically infected fish and asymptomatic carriers from either cultured, feral or wild fish. Virus is shed via faeces, urine, sexual fluids and external mucus whereas kidney, spleen, encephalon and digestive tract are the sites in which virus is the most abundant during the course of overt infection. The transmission of IHNV is horizontal and possibly vertical or rather, "egg-associated". Horizontal transmission may be direct or through
a vector, water being the major abiotic vector. Animate vectors and fomites also act in IHNV transmission. Egg-associated transmission seems to be an infrequent event but is the only mechanism substantiating the onset of IHN in alevins originating from disinfected eggs which had been incubated and hatched in virus-free water. Once IHNV is established in a farmed stock or in a watershed due to either spawning of infected migratory fish or from river restocking for recreational purposes, the disease becomes endemic because of carrier fish.

Beside the salmonid fish species susceptible to natural IHNV infections, pike fry (Esox lucius) can be easily infected via the water route under experimental conditions. As usual, among individuals of each fish species, there is a high degree of variability in susceptibility to IHNV. The age of fish appears to be extremely important: the younger the fish, the more susceptible to overt infection. As with VHS, a good overall fish health condition seems to decrease the susceptibility to overt IHNV, while handling stress and other types of stress frequently cause subclinical infection to become overt.

The most prominent environmental factor affecting IHNV is water temperature. Clinical disease does not occur above 18°C under natural conditions, but certain virus strains are only pathogenic below 14-15°C.

The diagnostic procedures for IHNV are all based upon direct methods. The conventional approach is the most widely used and involves isolation of the virus in cell culture followed by immunological identification by neutralisation, immunofluorescence or ELISA.

Control methods currently rely on the implementation of control policy rules and of hygiene practices in the operating of salmonid husbandry. The thorough disinfection of fertilised eggs and the incubation of eggs and further rearing of fry and alevins in premises completely separated from those harbouring possible virus carriers and free from possible contact with fomites, are critical for preventing the occurrence of IHNV in a defined fish production site. Vaccination is only at an experimental stage at present.

**DIAGNOSTIC PROCEDURES**

The monitoring for and diagnosis of IHN is based upon direct methods which are either the isolation of IHN virus (IHNV) in cell culture followed by its immunological identification (conventional approach), or the immunological demonstration of IHNV antigen (Ag) in infected fish tissues.
Due to insufficient knowledge on the fish serology of virus infections, the detection of fish antibodies to viruses has not thus far been accepted as a routine diagnostic method for assessing the virus status of fish populations. However, the validation of some serological techniques for diagnosis of certain fish virus infections could arise in the near future, rendering the use of fish serology more widely acceptable for diagnostic purposes.

Infected fish material suitable for virological examination is:

- **during overt infection**: whole alevin (body length ≤ 4 cm), viscera including kidney (4 cm ≤ body length ≤ 6 cm) or, for larger size fish, kidney, spleen and encephalon.

- **during dormant infection** (asymptomatic virus carrier fish): encephalon (any size fish) and/or ovarian fluid from broodfish at time of spawning.

**Sampling procedures**: see Chapter 1 Part B.

1. **STANDARD MONITORING METHODS FOR IHN**

1.1. **Isolation of IHNV in cell culture**

Cell line(s) to be used: EPC.

*a) Inoculation of cell monolayers*

i) Make two additional tenfold dilutions of the 1:10 organ homogenate supernatants and transfer an appropriate volume of each of the three dilutions onto 24-hour-old EPC cell monolayers. Inoculate at least 2 cm² of cell monolayer with 100 µl of each dilution.

ii) Allow to adsorb for 0.5-1 hour at 10-15°C and, without withdrawing inoculate, add cell culture medium buffered at pH 7.6 and supplemented with 2% FCS (1 ml/well for 24 well cell culture plates) and incubate at 15°C.

*b) Monitoring incubation*

i) Follow the course of infection in positive controls and other inoculated cell cultures, by daily microscopic examination at magnification 40x to 100x, for 7 days. The use of a phase contrast microscope is recommended.

ii) Maintain the pH of the cell culture medium between 7.3 and 7.6 over the whole incubation period. This can be achieved by addition to the inoculated cell culture medium of sterile bicarbonate buffer (for tightly closed cell culture flasks) or 2 M
Tris buffer solution (for cell culture plates) or, even better, by using HEPES-buffered media.

iii) If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of the homogenate, identification procedures must be undertaken immediately (see below). *If a fish health surveillance/control programme is being implemented, steps have to be taken to suspend the approved health status of the production unit and/or the zone (if it was approved previously) from which the suspected virus positive sample originated. The suspension of approved status will be maintained until it is demonstrated that the virus in question is not IHNV.*

iv) If no CPE occurs after 7 days of incubation (except in positive control cell cultures), subcultivation of the inoculated cell cultures must be performed. However, if no CPE is observed in positive controls, another series of virological examinations must be undertaken, using susceptible cells and new batches of organ samples.

c) *Subcultivation procedures*

i) Collect aliquots of cell culture medium from all monolayers inoculated with dilutions of each supernatant of organ homogenates.

ii) Centrifuge at 2,000 x g for 15 min at 4°C and collect supernatant.

iii) If required, repeat neutralisation test to IPNV as previously described, with dilution of the above supernatant (1:1 to 1:100).

iv) Inoculate EPC cell monolayers as described above (1.1.a).

v) Incubate and monitor as in 1.1.b.

vi) If no CPE occurs the test may be declared negative.

1.2. Virus identification

a) *Neutralisation test*

i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge it at 2,000 x g for 15 min at 4°C, or filter through a 450 nm pore membrane to remove cell debris.
ii) Dilute virus-containing medium 10^{-2} and 10^{-4}.

iii) Mix aliquots (for example 200 μl) of each dilution with equal volumes of an antibody solution for IHNV, and similarly treat aliquots of each virus dilution with cell culture medium.

(The titre of neutralising antibody (NAb) solution must be at least 2,000 for 50% plaque reduction.)

iv) In parallel, a neutralisation test must be performed against homologous IHNV (positive neutralisation test).

v) If required, do a similar neutralisation test with antibodies to IPN, to ensure that no IPNV contaminant might have escaped the first anti-IPNV test.

vi) Incubate all the mixtures at 15°C for 1 hour.

vii) Transfer aliquots of each of the above mixtures onto EPC cell monolayers (inoculate 2 cell cultures per dilution) and allow adsorption to occur for 0.5 to 1 hour at 15°C. Twenty-four or 12 well cell culture plates are suitable for this purpose, using a 50 μl inoculum.

viii) When adsorption is completed, add cell culture medium supplemented with 2% FCS and buffered at pH 7.4-7.6 into each well and incubate at 15°C.

ix) Check the cell cultures for the onset of CPE and read the results as soon as it occurs in non-neutralised controls (cell monolayers being protected in positive neutralisation controls). Results are recorded either after a simple microscopic examination (phase contrast preferable) or after discarding cell culture medium and staining cell monolayers with a solution of 1% crystal violet in 20% ethanol.

x) The tested virus is identified as IHNV when CPE is prevented or noticeably delayed in the cell cultures which received the virus suspension treated with the IHNV-specific antibody, whereas CPE is evident in all other cell cultures.

xi) In the absence of any neutralisation by NAb to IHNV, it is mandatory to conduct an indirect fluorescent antibody test with the suspect sample. Indeed some cases of antigenic drift of surface antigen have been observed, resulting in occasional failure of the neutralisation test using NAb to IHNV.
b) *Indirect fluorescent antibody test*

This virus identification test is to be conducted either directly after virus isolation in cell culture, or as a confirmatory test following the neutralisation test described above.

i) Prepare monolayers of EPC cells in 2 cm² wells of cell culture plastic plates or on coverglasses in order to reach around 80% confluency within 24 hours of incubation at 22°C (seed 6 cell monolayers per virus isolate to be identified, plus 2 for positive and 2 for negative controls). The FCS content of the cell culture medium can be reduced to 2-4%. If numerous virus isolates have to be identified, the use of Terasaki plates is strongly recommended.

ii) When the cell monolayers are ready for infection, i.e. on the same day or on the day after seeding, inoculate the virus suspensions to be identified by making ten-fold dilution steps directly in the cell culture wells or flasks.

iii) Dilute the control virus suspension of IHNV in a similar way, in order to obtain a virus titre of about 5,000-10,000 PFU/ml in the cell culture medium.

iv) Incubate at 15°C for 24 hours.

v) Remove the cell culture medium, rinse once with PBS 0.01 M pH 7.2, then 3 times briefly with cold acetone (stored at -20°C) for coverglasses or a mixture of acetone 30%-ethanol 70% (vol./vol.), also at -20°C, for plastic wells.

vi) Let the fixative act for 15 min. A volume of 0.5 ml is adequate for 2 cm² of cell monolayer.

vii) Allow the cell monolayers to air dry for at least 30 min and process immediately or freeze at -20°C.

viii) Prepare a solution of purified antibody or serum to IHNV in PBS 0.01 M, pH 7.2 containing 0.05% Tween 80, at the appropriate dilution (which has been established previously or is given by the reagent supplier).

ix) Rehydrate the dried cell monolayers by 4 rinsing steps with the above PBS-Tween and eliminate this buffer thoroughly after the last rinsing.

x) Treat the cell monolayers with the antibody solution for 1 hour at 37°C in a moist chamber and do not allow evaporation to occur. The volume of solution to be used is 0.25 ml/2 cm² well.
xi) Rinse 4 times with PBS-Tween as above.

xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate-conjugated (FITC) antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.

xiii) Rinse 4 times with PBS-Tween.

xiv) Examine the treated cell monolayers on plastic plates immediately, or mount the coverglasses using glycerol saline at pH 8.5 prior to microscopic observation.

xv) Examine under incident UV light using a microscope with x10 eye pieces and x20 to x40 objective lens having numerical aperture >0.65 and >1.3 respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

c) Enzyme-linked immunosorbent assay (ELISA)

i) Coat the wells of microplates designed for ELISA tests, with appropriate dilutions of purified immunoglobulins (Ig) or serum specific for IHNV. in PBS 0.01 M pH 7.2 (200 μl/well). Ig may be polyclonal or monoclonal Ig originating most often from rabbit or mouse, respectively. For the identification of IHNV, monoclonal antibodies specific for certain domains of the nucleocapsid protein (N) are suitable.

ii) Incubate overnight at 4°C.

iii) Rinse 4 times with PBS 0.01 M containing 0.05 % Tween 20 (PBST).

iv) Block with skim milk (5% in PBST) or other blocking solution for 1 hour at 37°C (200 μl/well).

v) Rinse 4 times with PBST.

vi) Add 2% of Triton X 100 to the virus suspension to be identified.

vii) Dispense 100 μl/well of 2 or 4 step dilutions of the virus to be identified and of IHNV control virus, and allow to react with the coated antibody to IHNV for 1 hour at 20°C.

viii) Rinse 4 times with PBST.
ix) Add to the wells, biotinylated polyclonal antibody to IHNV; or MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin.

x) Incubate 1 hour at 37°C.

xi) Rinse 4 times with PBST.

xii) Add streptavidin-conjugated horse radish peroxidase to those wells which have received the biotin-conjugated antibody and incubate for 1 hour at 20°C.

xiii) Rinse 4 times with PBST.

xiv) Add the substrate (H$_2$O$_2$) and chromogen (O-phenylenediamide, OPD or other approved chromogen). Stop the course of the test when positive controls react, and read the results.

xv) Alternatively: add H$_2$O$_2$ + chromogen to those wells containing the peroxidase conjugated antibody and proceed as above.

2. Diagnostic procedures for confirmation of IHN in suspected outbreaks

Confirmation of IHNV can be achieved by any of the following methods:

2.1. Conventional virus isolation with subsequent serological identification

As Section 1.

2.2. Virus isolation with simultaneous serological identification

a) Sampling procedures

As B.1.1. in Chapter 1 (General Information) for the selection of fish specimens.

As B.2.1. & 2.2. in Chapter 1 (General Information) for the selection of materials sampled.

b) Processing of organ samples

See the following sections in Chapter 1 (General Information):

B.3.1. for transportation

B.3.2. for virus extraction and obtaining of organ homogenates

B.3.3. for treatment to neutralise birnaviruses (if required).
c) *Virus identification by neutralisation test*

i) Dilute organ homogenates 1:100, 1:1,000 and 1:10,000 in cell culture medium.

ii) Mix with equal volume of a solution of antibody to IHNV as in 1.2.a, inoculate the cell monolayers, incubate at 15°C and monitor the fate of cell infection as in 1.2.a.

iii) Subcultivation: if no CPE appears after one week subcultivate the cell culture fluids of non antibody-treated controls as in 1.1.c.

2.3. **Indirect fluorescent antibody test**

i) Bleed the fish thoroughly.

ii) Make kidney imprints on cleaned glass slides or at the bottom of the wells of a plastic cell culture plate.

iii) Store the kidney pieces (as indicated in B.3.1. in Chapter 1 [General Information]) together with the other organs required for virus isolation in case this later becomes necessary.

iv) Allow the imprint to air-dry for 20 min.

v) Fix with acetone or ethanol-acetone and dry as indicated in 1.2.b. points v-vii.

vi) Rehydrate the above preparations (see 1.2.b. point ix) and block with 5% skim milk or 1% bovine serum albumin (BSA), in PBST for 30 min at 37°C.

vii) Rinse 4 times with PBST.

viii) Treat the imprints with the solution of antibody to IHNV and rinse as indicated in 1.2.b.

ix) Block and rinse as formerly.

x) Reveal the reaction with suitable FITC, rinse and observe as indicated in 1.2.b. points xii-xv.

If the immunofluorescence test is negative, process the organ samples stored at 4°C, for virus isolation in cell culture as in 1.1.
2.4. ELISA

a) Microplate processing

As 1.2.c of this chapter up to point iv (inclusive).

b) Sampling procedures

See the following sections in Chapter 1 (General Information):
B.1.1. for the selection of fish specimens
B.2.1. & 2.2. for the selection of materials sampled.

c) Processing of organ samples

See the following sections in Chapter 1 (General Information):
B.3.1. for transportation
B.3.2. for virus extraction and obtaining of organ homogenates.

d) Carrying out the ELISA

i) Set aside an aliquot of 1/4 of each homogenate in case further virus isolation in cell culture is required.

ii) Treat the remaining part of homogenate with 2% Triton X 100 (vol./vol.) as 1.2.c point v and 2 mM of phenyl methyl sulphonide fluoride (PMSF); mix gently.

iii) Complete the other steps of procedure 1.2.c.

REFERENCES


ONCORHYNCHUS MASOU VIRUS DISEASE (B406)

SUMMARY

Oncorhynchus masou virus disease (OMVD) is an oncogenic condition among salmonid fish in Japan and probably of the coastal rivers of Eastern Asia which harbour Pacific salmon. The causative virus (OMV) is also known as Yamame tumor virus (YTV), or Nerka virus Towada Lake, Akita and Amori prefecture (NeVTA). For a recent and more detailed review of the condition, see Wolf (5).

The susceptible fish species are kokanee salmon (Oncorhynchus nerka), masou salmon (O. masou), chum salmon (O. keta), coho salmon (O. kisutch) and rainbow trout (O. mykiss).

Clinically, the initial infection by OMV appears as a systemic and frequently lethal infection which occurs in a context of oedema and haemorrhages. Virus multiplication in endothelial cells of blood capillaries, haematopoietic tissue and hepatocytes underlies the clinical signs. Four months after this first clinical condition, a varying number of surviving fish exhibit epithelioma occurring mainly around the mouth (upper and lower jaw), and to a lesser extent, on the caudal fin operculum and body surface. These neoplasia may persist for up to one year post-infection.

Following the septicaemia phase of OMV infection, an immune response takes place and results in the synthesis of neutralising antibodies to OMV. A carrier state frequently occurs which leads to virus shedding via the sexual products at times of spawning.

On the basis of antigenic studies conducted with neutralising polyclonal rabbit antisera, OMV differs from the herpesvirus of Salmonidace type 1 which is present in the western USA and only weakly pathogenic.

The reservoirs of OMV are clinically infected fish and asymptomatic carriers among groups of cultured, feral or wild fish. Infectious virus is shed via faeces, urine, sexual products and probably skin mucus, whereas the kidney, spleen, liver and tumors are the sites in which virus is the most abundant during the course of overt infection. The transmission of OMV is horizontal and possibly vertical or rather "egg-associated". Horizontal transmission may be direct or vectorial, water being the major abiotic factor. Animate vectors and fomites also act in OMV transmission. Egg-associated transmission, even if infrequent, is the only mechanism
The disease in alevins originating from disinfectected eggs which had been incubated and hatched in virus-free water.

Salmonids are the only fish species susceptible to OMV infection. The grouping of the fish species from the most to the least susceptible is kokanee, masou salmon, chum salmon, coho salmon and rainbow trout. The age of the fish is critical and one month-old alevins offer the most susceptible targets for the virus infection. The main environmental factor favoring OMV infection is low water temperature (below 14°C).

The diagnostic procedures for OMV are based upon direct isolation of the virus in cell culture followed by immunological identification by neutralization or immunofluorescence.

Control methods currently rely on the implementation of avoidance and hygiene practices in the operating of salmonid husbandry. The thorough disinfection of fertilised eggs and the incubation of these eggs and rearing of fry and alevins, in premises completely separated from those harbouring virus carriers and free from contact with fomites, are key measures to decrease contamination of OMV in a defined fish production site.

DIAGNOSTIC PROCEDURES

The diagnosis of Oncorhynchus masou virus disease is based upon direct methods which are either the isolation of the virus (OMV) in cell culture followed by its immunological identification (conventional approach), or the immunological demonstration of OMV antigen (Ag) in infected fish tissues.

Due to insufficient knowledge of the fish serology of virus infections, the detection of fish antibodies to viruses has not thus far been recognised as a valuable diagnostic method for assessing the virus status of fish populations. However, the validation of some serological techniques for diagnosis of certain fish virus infections could arise in the near future, rendering the use of fish serology more widely acceptable for diagnostic purposes.

Infected fish material suitable for virological examination is:

- **During overt infection**: whole alevin (body length ≤ 4 cm), viscera including kidney (4 cm ≤ body length ≤ 6 cm) or, for larger size fish, kidney, spleen and encephalon.

- **During dormant infection** (detection of asymptomatic virus carrier fish): kidney, spleen and encephalon (any size fish) and/or ovarian fluid from broodfish at time of spawning.
Sampling procedures: see Chapter 1 Part B.

1. STANDARD MONITORING METHODS FOR OMVD

1.1. Isolation of OMV in cell culture

Cell line(s) to be used: CHSE-214.

a) Inoculation of cell monolayers

i) Make two additional tenfold dilutions of the 1:10 organ homogenate supernatants and transfer an appropriate volume of each of the three dilutions onto 24-hour-old CHSE-214 cell monolayers. Inoculate at least 2 cm² of cell monolayer with 100 µl of each dilution.

ii) Allow to adsorb for 0.5-1 hour at 10-15°C and, without withdrawing inoculate, add cell culture medium buffered at pH 7.6 and supplemented with 2% FCS (1 ml/well for 24 well cell culture plates) and incubate at 10-15°C.

b) Monitoring incubation

i) Follow the course of infection in positive controls and other inoculated cell cultures, by daily microscopic examination at magnification 40x to 100x, during 14 days. The use of a phase contrast microscope is recommended.

ii) Maintain the pH of the cell culture medium between 7.3 and 7.6 over the whole incubation phase. This can be achieved by addition to the inoculated cell culture medium of sterile bicarbonate buffer (for tightly closed cell culture flasks) or 2 M Tris buffer solution (for cell culture plates) or, even better, by using HEPES-buffered media.

iii) If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of the tested homogenate supernatants, identification procedures have to be undertaken immediately (see below). If a fish health surveillance/control programme is being implemented, provisions have to be taken to suspend the approved health status of the production unit and/or the zone (if it was approved previously) from which the virus positive sample originated. The suspension of approved status will be maintained until it is demonstrated that the virus in question is not OMV.

iv) If no CPE occurs, except in positive control cell cultures, subcultivation steps have to be made even after 7 days of
incubation, in certain of the infected cell cultures. However, if no CPE is observed even in positive controls, another series of virological examinations have to be undertaken, using susceptible cells and new batches of organ samples.

c) *Subcultivation procedures*

i) Collect aliquots of cell culture medium from all monolayers inoculated with dilutions of each supernatant of organ homogenates.

ii) Centrifuge at $2,000 \times g$ for 15 min at $4^\circ C$ and collect supernatant.

iii) Repeat optional neutralisation test to IPNV if needed, with dilution of the above supernatant (1:1 to 1:100).

iv) Inoculate CHSE-214 cell monolayers as described above (1.1.a).

v) Incubate and monitor as in 1.1.b.

1.2. Virus identification

a) *Neutralisation test*

i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge it at $2,000 \times g$ for 15 min at $4^\circ C$ to remove cell debris, or

ii) Dilute virus-containing medium from $10^{-2}$ to $10^{-4}$.

iii) Mix aliquots (for example 200 μl) of each virus dilution with equal volumes of an antibody solution specific for OMV, and similarly treat aliquots of each virus dilution with cell culture medium.

The titre of neutralising antibody (NAb) solution must be around 2,000 in the 50% plaque reduction assay.

iv) In parallel, other neutralisation tests must be performed against:

- a homologous virus strain (positive neutralisation test)
- a heterologous virus strain (negative neutralisation test).

v) Incubate all the mixtures at $15^\circ C$ for 1 hour.
vi) After incubation, transfer aliquots of each of the above mixtures onto CHSE-214 cell monolayers (inoculate 2 cell cultures per dilution) and allow adsorption to occur for 0.5 to 1 hour at 15°C. Twenty-four or 12 well cell culture plates are suitable for this purpose, using a 50 μl inoculum.

vii) When adsorption is completed, add cell culture medium supplemented with 2% FCS and buffered at pH 7.4-7.6 into each well and incubate at 10-15°C.

viii) Check the cell cultures for the onset of CPE and read the results as soon as it occurs in non-neutralised controls (cell monolayers being protected in positive neutralisation controls). Results are recorded either after a simple microscopic examination (phase contrast preferable) or after discarding cell culture medium and staining cell monolayers with a solution of 1% crystal violet in ethanol 20%.

ix) The tested virus is identified as OMV when CPE is abolished or noticeably delayed in the cell cultures which had received the virus suspension treated with the OMV-specific antibody, whereas CPE is evident in all other cell cultures.

x) In the absence of any neutralisation by NAb to OMV, it is mandatory to conduct an indirect fluorescent antibody test with the suspect sample.

b) *Indirect fluorescent antibody test*

This virus identification test is to be conducted either directly after virus isolation in cell culture, or as a confirmatory test following the neutralisation test described above.

i) Prepare monolayers of CHSE-214 cells in 2 cm² wells of cell culture plastic plates or on coverglasses in order to reach around 80% confluence, which is usually achieved within 4 hours of incubation at 22°C (seed 6 cell monolayers per virus isolate to be identified, plus 2 for positive and 2 for negative controls). The FCS content of the cell culture medium can be reduced to 2-4%. If numerous virus isolates have to be identified, the use of Terasaki plates is strongly recommended.

ii) When the cell monolayers are ready for infection, i.e. on the same day or on the day after seeding, inoculate the virus suspensions to be identified by making ten-fold dilution steps directly in the cell culture wells or flasks.

iii) Dilute the control virus suspension of OMV in a similar way,
in order to obtain a virus titre of about 5,000-10,000 PFU/ml in the cell culture medium.

iv) Incubate at 15°C for 48 hours.

v) When the incubation time is over, aspirate the cell culture medium, rinse once with PBS 0.01 M pH 7.2, then 3 times briefly with cold fixative. This fixative will be acetone (stored at -20°C) for coverglasses or a mixture of acetone 30%-ethanol 70% (vol./vol.), also stored at -20°C.

vi) Afterwards, let the fixative act for 15 min. A volume of 0.5 ml is adequate for 2 cm² of cell monolayer.

vii) Allow the cell monolayers to air dry for at least 30 min and process immediately or freeze at -20°C.

viii) Prepare a solution of purified antibody or serum to OMV in PBS 0.01 M, pH 7.2 containing 0.05% Tween 80, at the appropriate dilution (which has been established previously or is given by the reagent supplier).

ix) Rehydrate the cell monolayers by 4 rinsing steps with the above PBS and eliminate this buffer thoroughly after the last rinsing.

x) Treat the cell monolayers with the antibody solution for 1 hour at 37°C in a moist chamber and do not allow evaporation to occur. The volume of solution to be used is 0.25 ml/2 cm² well.

xi) Rinse 4 times with PBS-Tween as above.

xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate-conjugated (FITC) antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.

xiii) Rinse 4 times with PBS-Tween.

xiv) Observe the treated cell monolayers on plastic plates immediately, or mount the coverglasses using glycerol saline at pH 8.5 prior to microscopic observation.

xv) Conduct this observation under incident UV light using a microscope with x10 eye pieces and x20 to x40 objective lens having numerical aperture >0.65 and >1.3 respectively.
Positive and negative controls must be found to give the expected results prior to any other observation.

c) *Enzyme-linked immunosorbent assay (ELISA)*

i) Coat the wells of microplates designed for ELISA tests, with appropriate dilutions of purified immunoglobulins (Ig) or serum specific for OMV, in PBS 0.01 M pH 7.2 (200 µl/well).

ii) Incubate overnight at 4°C.

iii) Rinse 4 times with PBS 0.01 M containing 0.05 % Tween 20 (PBST).

iv) Block with skim milk (5% in PBST) or other blocking solution for 1 hour at 37°C (200 µl/well).

v) Add 2% of Triton X 100 to the virus suspension to be identified.

vi) Rinse 4 times with PBST.

vii) Dispense 100 µl/well of a 2 or 4 step dilution of the virus to be identified and of OMV control virus, and allow to react with the coated antibody to OMV for 1 hour at 20°C.

viii) Rinse 4 times with PBST.

ix) Add to the wells, biotinylated polyclonal antibody to OMV.

x) Incubate 1 hour at 37°C.

xi) Rinse 4 times with PBST.

xii) Add streptavidin-conjugated horse radish peroxidase to those wells which have received the biotin-conjugated antibody and incubate for 1 hour at 20°C.

xiii) Rinse 4 times.

xiv) Add the substrate (H₂O₂) and chromogen (O-phenylenediamide, OPD or other approved chromogen). Stop the course of the test when positive controls react, and monitor the results.

xv) Alternatively: add H₂O₂ + chromogen to those wells containing the peroxidase conjugated antibody and proceed as above.
2. **DIAGNOSTIC PROCEDURES FOR CONFIRMATION OF OMVD IN SUSPECTED OUTBREAKS**

Confirmation of *Oncorhynchus masou* virus can be achieved by any of the following methods.

2.1. **Conventional virus isolation with subsequent serological identification**

As Section 1.

2.2. **Virus isolation with simultaneous identification**

   a) *Sampling procedures*

   As B.1.1. in Chapter 1 (General Information) for the selection of fish specimens.
   As B.2.1. & 2.2. in Chapter 1 (General Information) for the selection of materials sampled.

   b) *Processing of organ samples*

   See the following sections in Chapter 1 (General Information):

   B.3.1. for transportation
   B.3.2. for virus extraction and obtaining of organ homogenates
   B.3.3. for treatment to neutralise birnaviruses (if required).

   c) *Virus identification by neutralisation test*

   i) Dilute the anti-IPN-treated organ homogenates 1:100, 1:1,000 and 1:10,000 in cell culture medium.

   ii) Mix with equal volume of a solution of antibody to OMV as in 1.2.a, inoculate the cell monolayers, incubate at 10-15°C and monitor the fate of cell infection as in 1.2.a.

   iii) Subcultivation: if no CPE appears after one week subcultivate the cell culture fluids of non antibody-treated controls as in 1.1.c.

2.3. **Indirect fluorescent antibody test**

   i) Bleed the fish thoroughly.

   ii) Make kidney imprints on cleaned glass slides or at the bottom of the wells of a plastic cell culture plate.
iii) Store the kidney pieces (as indicated in B.3.1. in Chapter 1 [General Information]) together with the other organs required for virus isolation in case this later becomes necessary.

iv) Allow the imprint to air-dry for 20 min.

v) Fix with acetone or ethanol-acetone and dry as indicated in 1.2.b. points v-vii.

vi) Rehydrate the above preparations (see 1.2.b. point ix) and block with 5% skim milk or 1% bovine serum albumin (BSA), in PBST for 30 min at 37°C.

vii) Rinse 4 times with PBST.

viii) Treat the imprints with the solution of antibody to OMV and rinse as indicated in 1.2.b.

ix) Block and rinse as formerly.

x) Reveal the reaction with suitable FITC, rinse and observe as indicated in 1.2.b. points xii-xv.

If the immunofluorescence test is negative, process the organ samples stored at 4°C, for virus isolation in cell culture as in 1.1.

2.4. ELISA

a) Microplate processing

As 1.2.c of this chapter up to point iv (inclusive).

b) Sampling procedures

See the following sections in Chapter 1 (General Information):

B.1.1. for the selection of fish specimens
B.2.1. & 2.2. for the selection of materials sampled.

c) Processing of organ samples

See the following sections in Chapter 1 (General Information):

B.3.1. for transportation
B.3.2. for virus extraction and obtaining of organ homogenates.
d) Carrying out the ELISA

i) Set aside an aliquot of 1/4 of each homogenate in case further virus isolation in cell culture is required.

ii) Treat the remaining part of homogenate with 2% Triton X 100 (vol./vol.) as 1.2.c point v and 2 mM of phenyl methyl sulfonide fluoride (PMSF); mix gently.

iii) Complete the other steps of procedure 1.2.c.

REFERENCES


CHAPTER 5  
SPRING VIRAEMIA OF CARP  
(B404)  

SUMMARY  

Spring viraemia of carp (SVC) is a rhabdovirus infection of several carp species and of some other cyprinid fish species. Overt infections have been recognised in common carp (Cyprinus carpio), grass carp (Ctenopharyngodon idellus), silver carp (Hypophthalmichthys molitrix), bighead carp (Aristichthys nobilis), crucian carp (Carassius carassius), goldfish (C. auratus), tench (Tinca tinca) and sheatfish (Silurus glanis). The geographic range of SVC is currently limited to countries of the European continent which experience low water temperatures during winter. For a recent and more detailed review of the condition, see Wolf (4).

Infection by SVC virus (SVCV) can be lethal, due to, as in other rhabdoviruses of fish, the impairment of the salt-water balance, which occurs in a clinical context of oedema and haemorrhages. Virus multiplication, especially in endothelial cells of blood capillaries, haematopoietic tissue and nephron cells, underlies the clinical signs.

Overcoming SVCV infection results in a strong protective immunity associated with the presence of circulating antibodies (Ab) detectable by methods such as virus neutralisation (VN), immunofluorescence (IF) or ELISA. This health status also results, in certain individuals, in an asymptomatic virus carrier state.

On the basis of antigenic studies conducted with rabbit polyclonal neutralising antibodies, SVCV was found to present only one serotype using the VN test, but both IFAT and ELISA tests have revealed that SVCV shares common antigenic domain(s) with the pike-fry rhabdovirus. Variations in virulence of virus strains have been recorded during both natural cases of disease and experimental infections.

The reservoirs of SVC virus are clinically infected fish and asymptomatic virus carriers from either cultured, feral or wild fish. Virulent virus is shed via faeces, urine, sexual fluids and probably gill and skin epithelia, whereas kidney, spleen, gill and encephalon are the organs in which SVCV is most abundant during the course of overt infection.

The mode of transmission for SVCV is horizontal but an "egg-associated" transmission (usually called "vertical") cannot be ruled
Horizontal transmission may be direct or vectorial, water being the major abiotic vector. Animate vectors and fomites are also involved in transmission of SVCV. Among animate vectors, the parasitic invertebrates *Argulus foliaceus* (Crustacea, Branchiura) and *Piscicola piscicola* (Annelida, Hirudinea) are able to transfer SVCV from diseased to healthy fish. Once SVCV is established in pond stock or pond farm stock, it may be very difficult to eradicate without destroying all kinds of life on the fish production site.

Apart from the formerly cited cyprinid species susceptible to SVCV infection, it seems that very young fish from various pond fish species are susceptible to SVC, regardless of water temperature. The most striking example is that of the pike (*Esox lucius*) which can be easily infected via the water route. There is a high variability in the degree of susceptibility to SVC among individuals of the same fish species. Apart from the physiological state of the fish, the role of which is poorly understood, age appears to be extremely important: the younger the fish, the more susceptible to overt infection. It remains certain that the environmental factor which is critical for SVC infection is water temperature: overt infection is not often observed above 15°C.

The diagnostic procedures for SVCV are based upon direct methods. The most widely used is isolation of the virus in cell culture followed by immunological identification using VN, immunofluorescence or ELISA tests.

The implementation of hygiene measures and control policy rules are the only control methods currently feasible. Vaccination is only experimental.

**DIAGNOSTIC PROCEDURES**

The monitoring for and diagnosis of SVC is based upon direct methods which are either the isolation of SVC virus (SVCV) in cell culture followed by its immunological identification (conventional approach), or the immunological demonstration of SVCV antigen (Ag) in infected fish tissues.

Due to insufficient knowledge on the fish serology of virus infections, the detection of fish antibodies to viruses has not thus far been accepted as a routine diagnostic method for assessing the virus status of fish populations. However, the validation of some serological techniques for diagnosis of certain fish virus infections could arise in the near future, rendering the use of fish serology more widely acceptable for diagnostic purposes.

Infected fish material suitable for virological examination is:

- **during overt infection**: whole alevin (body length ≤ 4 cm), viscera
including kidney (4 cm ≤ body length ≤ 6 cm) or, for larger size fish, kidney, spleen and encephalon.

- during dormant infection (asymptomatic virus carrier fish): encephalon (any size fish) and/or ovarian fluid from broodfish at time of spawning.

**Sampling procedures:** see Chapter 1 Part B.

1. **STANDARD MONITORING METHODS FOR SVC**

1.1. **Isolation of SVCV in cell culture**

Cell line(s) to be used: EPC.

\[a\) Inoculation of cell monolayers\]

i) Make two additional tenfold dilutions of the 1:10 organ homogenate supernatants and transfer an appropriate volume of each of the three dilutions onto 24-hour-old EPC cell monolayers. Inoculate at least 2 cm\(^2\) of cell monolayer with 100 µl of each dilution.

ii) Allow to adsorb for 0.5-1 hour at 10-15°C and, without withdrawing inoculate, add cell culture medium buffered at pH 7.6 and supplemented with 2% FCS (1 ml/well for 24 well cell culture plates) and incubate at 15°C.

\[b\) Monitoring incubation\]

i) Follow the course of infection in positive controls and other inoculated cell cultures, by daily microscopic examination at magnification 40x to 100x, for 7 days. The use of a phase contrast microscope is recommended.

ii) Maintain the pH of the cell culture medium between 7.3 and 7.6 over the whole incubation period. This can be achieved by addition to the inoculated cell culture medium of sterile bicarbonate buffer (for tightly closed cell culture flasks) or 2 M Tris buffer solution (for cell culture plates) or, even better, by using HEPES-buffered media.

iii) If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of the homogenate, identification procedures must be undertaken immediately (see below). **If a fish health surveillance/control programme is being implemented, steps have to be taken to suspend the approved health status of the production unit and/or the zone (if it was approved previously) from which the suspected virus positive**
sample originated. The suspension of approved status will be maintained until it is demonstrated that the virus in question is not SVCV.

iv) If no CPE occurs after 7 days of incubation (except in positive control cell cultures), subcultivation of the inoculated cell cultures must be performed. However, if no CPE is observed in positive controls, another series of virological examinations must be undertaken, using susceptible cells and new batches of organ samples.

c) Subcultivation procedures

i) Collect aliquots of cell culture medium from all monolayers inoculated with dilutions of each supernatant of organ homogenates.

ii) Centrifuge at 2,000 x g for 15 min at 4°C and collect supernatant.

iii) If required, repeat neutralisation test to IPNV as previously described, with dilution of the above supernatant (1:1 to 1:100).

iv) Inoculate EPC cell monolayers as described above (1.1.a).

v) Incubate and monitor as in 1.1.b.

vi) If no CPE occurs the test may be declared negative.

1.2. Virus identification

a) Neutralisation test

i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge it at 2,000 x g for 15 min at 4°C, or filter through a 450 nm pore membrane to remove cell debris.

ii) Dilute virus-containing medium 10⁻² and 10⁻⁴.

iii) Mix aliquots (for example 200 µl) of each dilution with equal volumes of an antibody solution for SVCV, and similarly treat aliquots of each virus dilution with cell culture medium.

(The titre of neutralising antibody (NAb) solution must be at least 2,000 for 50% plaque reduction.)

iv) In parallel, a neutralisation test must be performed against homologous SVCV (positive neutralisation test).
v) If required, do a similar neutralisation test with antibodies to IPN, to ensure that no IPNV contaminant might have escaped the first anti-IPNV test.

vi) Incubate all the mixtures at 20°C for 1 hour.

vii) Transfer aliquots of each of the above mixtures onto EPC cell monolayers (inoculate 2 cell cultures per dilution) and allow adsorption to occur for 0.5 to 1 hour at 15-20°C. Twenty-four or 12 well cell culture plates are suitable for this purpose, using a 50 µl inoculum.

viii) When adsorption is completed, add cell culture medium supplemented with 2% FCS and buffered at pH 7.4-7.6 into each well and incubate at 15-20°C.

ix) Check the cell cultures for the onset of CPE and read the results as soon as it occurs in non-neutralised controls (cell monolayers being protected in positive neutralisation controls). Results are recorded either after a simple microscopic examination (phase contrast preferable) or after discarding cell culture medium and staining cell monolayers with a solution of 1% crystal violet in 20% ethanol.

x) The tested virus is identified as SVCV when CPE is prevented or noticeably delayed in the cell cultures which received the virus suspension treated with the SVCV-specific antibody, whereas CPE is evident in all other cell cultures.

xi) In the absence of any neutralisation by NAb to SVCV, it is mandatory to conduct an indirect fluorescent antibody test with the suspect sample.

b) Indirect fluorescent antibody test

This virus identification test is to be conducted either directly after virus isolation in cell culture, or as a confirmatory test following the neutralisation test described above.

i) Prepare monolayers of EPC cells in 2 cm² wells of cell culture plastic plates or on coverglasses in order to reach around 80% confluency within 24 hours of incubation at 30°C (seed 6 cell monolayers per virus isolate to be identified, plus 2 for positive and 2 for negative controls). The FCS content of the cell culture medium can be reduced to 2-4%. If numerous virus isolates have to be identified, the use of Terasaki plates is strongly recommended.
ii) When the cell monolayers are ready for infection, i.e. on the same day or on the day after seeding, inoculate the virus suspensions to be identified by making ten-fold dilution steps directly in the cell culture wells or flasks.

iii) Dilute the control virus suspension of SVCV in a similar way, in order to obtain a virus titre of about 5,000-10,000 PFU/ml in the cell culture medium.

iv) Incubate at 20°C for 24 hours.

v) Remove the cell culture medium, rinse once with PBS 0.01 M pH 7.2, then 3 times briefly with cold acetone (stored at -20°C) for coverglasses or a mixture of acetone 30%-ethanol 70% (vol./vol.), also at -20°C, for plastic wells.

vi) Let the fixative act for 15 min. A volume of 0.5 ml is adequate for 2 cm² of cell monolayer.

vii) Allow the cell monolayers to air dry for at least 30 min and process immediately or freeze at -20°C.

viii) Prepare a solution of purified antibody or serum to SVCV in PBS 0.01 M, pH 7.2 containing 0.05% Tween 80, at the appropriate dilution (which has been established previously or is given by the reagent supplier).

ix) Réhydrate the dried cell monolayers by 4 rinsing steps with the above PBS-Tween and eliminate this buffer thoroughly after the last rinsing.

x) Treat the cell monolayers with the antibody solution for 1 hour at 37°C in a moist chamber and do not allow evaporation to occur. The volume of solution to be used is 0.25 ml/2 cm² well.

xi) Rinse 4 times with PBS-Tween as above.

xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate-conjugated (FITC) antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.

xiii) Rinse 4 times with PBS-Tween.

xiv) Examine the treated cell monolayers on plastic plates immediately, or mount the coverglasses using glycerol saline at pH 8.5 prior to microscopic observation.
xv) Examine under incident UV light using a microscope with x10 eye pieces and x20 to x40 objective lens having numerical aperture >0.65 and >1.3 respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

c) Enzyme-linked immunosorbent assay (ELISA)

i) Coat the wells of microplates designed for ELISA tests, with appropriate dilutions of purified immunoglobulins (Ig) or serum specific for SVCV, in PBS 0.01 M pH 7.2 (200 µl/well). Ig may be polyclonal or monoclonal Ig originating most often from rabbit or mouse, respectively.

ii) Incubate overnight at 4°C.

iii) Rinse 4 times with PBS 0.01 M containing 0.05 % Tween 20 (PBST).

iv) Block with skim milk (5% in PBST) or other blocking solution for 1 hour at 37°C (200 µl/well).

v) Rinse 4 times with PBST.

vi) Add 2% of Triton X 100 to the virus suspension to be identified.

vii) Dispense 100 µl/well of 2 or 4 step dilutions of the virus to be identified and of SVCV control virus, and allow to react with the coated antibody to SVCV for 1 hour at 20°C.

viii) Rinse 4 times with PBST.

ix) Add to the wells, biotinylated polyclonal antibody to SVCV; or MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin.

x) Incubate 1 hour at 37°C.

xi) Rinse 4 times with PBST.

xii) Add streptavidin-conjugated horse radish peroxidase to those wells which have received the biotin-conjugated antibody and incubate for 1 hour at 20°C.

xiii) Rinse 4 times with PBST.

xiv) Add the substrate (H₂O₂) and chromogen (O-phenylenediamide, OPD or other approved chromogen). Stop
the course of the test when positive controls react, and read the results.

xv) Alternatively: add $\text{H}_2\text{O}_2 + \text{chromogen}$ to those wells containing the peroxidase conjugated antibody and proceed as above.

2. **Diagnostic Procedures for Confirmation of SVCV in Suspected Outbreaks**

Confirmation of SVCV can be achieved by any of the following methods:

2.1. **Conventional virus isolation with subsequent serological identification**

As Section 1.

2.2. **Virus isolation with simultaneous serological identification**

a) *Sampling procedures*

As B.1.1. in Chapter 1 (General Information) for the selection of fish specimens.
As B.2.1. & 2.2. in Chapter 1 (General Information) for the selection of materials sampled.

b) *Processing of organ samples*

See the following sections in Chapter 1 (General Information):
  B.3.1. for transportation
  B.3.2. for virus extraction and obtaining of organ homogenates
  B.3.3. for treatment to neutralise birnaviruses (if required).

c) *Virus identification by neutralisation test*

i) Dilute organ homogenates 1:100, 1:1,000 and 1:10,000 in cell culture medium.

ii) Mix with equal volume of a solution of antibody to SVCV as in 1.2.a, inoculate the cell monolayers, incubate at 15-20°C and monitor the fate of cell infection as in 1.2.a.

iii) Subcultivation: if no CPE appears after one week subcultivate the cell culture fluids of non antibody-treated controls as in 1.1.c.
2.3. **Indirect fluorescent antibody test**

i) Bleed the fish thoroughly.

ii) Make kidney imprints on cleaned glass slides or at the bottom of the wells of a plastic cell culture plate.

iii) Store the kidney pieces (as indicated in B.3.1. in Chapter 1 [General Information]) together with the other organs required for virus isolation in case this later becomes necessary.

iv) Allow the imprint to air-dry for 20 min.

v) Fix with acetone or ethanol-acetone and dry as indicated in 1.2.b. points v-vii.

vi) Rehydrate the above preparations (see 1.2.b. point ix) and block with 5% skim milk or 1% bovine serum albumin (BSA), in PBST for 30 min at 37°C.

vii) Rinse 4 times with PBST.

viii) Treat the imprints with the solution of antibody to SVCV and rinse as indicated in 1.2.b.

ix) Block and rinse as formerly.

x) Reveal the reaction with suitable FITC, rinse and observe as indicated in 1.2.b. points xii-xv.

If the immunofluorescence test is negative, process the organ samples stored at 4°C, for virus isolation in cell culture as in 1.1.

2.4. **ELISA**

a) **Microplate processing**

As 1.2.c of this chapter up to point iv (inclusive).

b) **Sampling procedures**

See the following sections in Chapter 1 (General Information):

B.1.1. for the selection of fish specimens  
B.2.1. & 2.2. for the selection of materials sampled.

c) **Processing of organ samples**

See the following sections in Chapter 1 (General Information):

B.3.1. for transportation  
B.3.2. for virus extraction and obtaining of organ homogenates.
d) Carrying out the ELISA

i) Set aside an aliquot of 1/4 of each homogenate in case further virus isolation in cell culture is required.

ii) Treat the remaining part of homogenate with 2% Triton X 100 (vol./vol.) as 1.2.c point v and 2 mM of phenyl methyl sulfonide fluoride (PMSF); mix gently.

iii) Complete the other steps of procedure 1.2.c.

REFERENCES


CHAPTER 6
VIRAL HAEMORRHAGIC SEPTICAEMIA
(B401)

SUMMARY

Viral haemorrhagic septicaemia (VHS) is a coldwater rhabdovirus infection of rainbow trout (Oncorhynchus mykiss), brown trout (Salmo trutta), grayling (Thymallus thymallus), white fish (Coregonus sp.), pike (Esox lucius) and turbot (Scophthalmus maximus). Infections with VHS virus in Pacific salmon, Pacific cod and Pacific herring have always been associated with genetically characteristic virus strains, which appear to be of low pathogenicity to rainbow trout.

It occurs in continental Europe but is mainly a matter of concern because of its clinical and economic consequences in rainbow trout farming. For a recent and more detailed review of the condition, see Wolf (8).

The infection of fish is often lethal, due to the impairment of the salt-water balance, which occurs in a clinical context of oedema and haemorrhages. Virus multiplication in endothelial cells of blood capillaries, leucocytes, haematopoietic tissues and nephron cells, underlies the clinical signs.

In survivors, VHS virus (VHSV) infection results in strong protective immunity, synthesis of circulating antibodies to VHSV as well as, in certain individuals, an asymptomatic carrier state. This carrier state frequently turns to high virus excretion at times of spawning.

Three neutralising subtypes of VHSV have been recognised using a panel of polyclonal and monoclonal antibody preparations. Apart from the above variation, VHSV seem to share a VHS-group neutralising epitope, and several non-neutralising epitopes located on the viral glycoprotein (G protein). Variations in virus strain virulence have been recorded in both natural cases of disease and infection trials.

The reservoirs of VHSV are clinically infected fish and asymptomatic carriers among cultured, feral or wild fish. Virulent virus is shed in the faeces, urine, and sexual fluids whereas kidney, spleen, brain and digestive tract are the sites in which virus is the most abundant. Once VHSV is established in a farm stock and therefore in the water catchment system, the disease becomes endemic because of the virus carrier fish.
Several factors influence susceptibility to VHS. Apart from the six fish species found to be susceptible to VHSV under natural conditions three others, namely sea bass (Dicentrarchus labrax), sea bream (Chrysopterus aurata) and lake trout (Salvelinus namaycush), were demonstrated to be susceptible to water-borne experimental infection. Among each fish species, there is a high degree of individual variability in susceptibility to VHSV. The age of fish appears to be highly important - the younger the fish the higher the susceptibility to overt infection. Similarly, a good overall fish health condition seems to decrease the susceptibility to overt VHS, while handling stress and other types of stress frequently cause subclinical infection to become overt.

An important environmental factor is water temperature. Clinical disease may occur between 14-18°C, although new infections are rarely established above 15°C under natural conditions.

The diagnostic procedures for VHS are based mainly on direct methods and the classical approach involving virus isolation in cell culture followed by immunological virus identification (neutralisation, immunofluorescence, ELISA), is the most widely used. However, more rapid diagnostic methods evidencing viral antigen in infected organ imprints or homogenates (fluorescence, ELISA) may be suitable for fish with overt disease. Fish serology (neutralisation, ELISA) could be of high interest for detecting the carrier state among fish stocks, but still has to be validated.

Control methods for VHS currently lie in official health surveillance schemes coupled with control policy measures and a genetic approach (selection and intergeneric hybridisation). Vaccination is only at an experimental stage at present.

**DIAGNOSTIC PROCEDURES**

The monitoring for and diagnosis of VHS is based upon direct methods which are either the isolation of VHS virus (VHSV) in cell culture followed by its immunological identification (conventional approach), or the immunological demonstration of VHSV antigen (Ag) in infected fish tissues.

Due to insufficient knowledge on the fish serology of virus infections, the detection of fish antibodies to viruses has not thus far been accepted as a routine diagnostic method for assessing the virus status of fish populations. However, the validation of some serological techniques for diagnosis of certain fish virus infections could arise in the near future, rendering the use of fish serology more widely acceptable for diagnostic purposes.
Infected fish material suitable for virological examination is:

- **during overt infection**: whole alevin (body length ≤ 4 cm), viscera including kidney (4 cm ≤ body length ≤ 6 cm) or, for larger size fish, kidney, spleen and encephalon.

- **during dormant infection** (asymptomatic virus carrier fish): encephalon (any size fish) and/or ovarian fluid from broodfish at time of spawning.

**Sampling procedures**: see Chapter 1 Part B.

1. **STANDARD MONITORING METHODS FOR VHSV**

1.1. **Isolation of VHSV in cell culture**

   Cell line(s) to be used: BF-2 or EPC.

   **a) Inoculation of cell monolayers**

   i) Make two additional tenfold dilutions of the 1:10 organ homogenate supernatants and transfer an appropriate volume of each of the three dilutions onto 24-hour-old BF-2 or EPC cell monolayers. Inoculate at least 2 cm² of cell monolayer with 100 µl of each dilution.

   ii) Allow to adsorb for 0.5-1 hour at 10-15°C and, without withdrawing inoculate, add cell culture medium buffered at pH 7.6 and supplemented with 2% FCS (1 ml/well for 24 well cell culture plates) and incubate at 15°C.

   **b) Monitoring incubation**

   i) Follow the course of infection in positive controls and other inoculated cell cultures, by daily microscopic examination at magnification 40x to 100x, for 7 days. The use of a phase contrast microscope is recommended.

   ii) Maintain the pH of the cell culture medium between 7.3 and 7.6 over the whole incubation period. This can be achieved by addition to the inoculated cell culture medium of sterile bicarbonate buffer (for tightly closed cell culture flasks) or 2 M Tris buffer solution (for cell culture plates) or, even better, by using HEPES-buffered media.

   iii) If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of the homogenate, identification procedures must be undertaken immediately (see below). If a fish health surveillance/control programme is being imple-
mented, steps have to be taken to suspend the approved health status of the production unit and/or the zone (if it was approved previously) from which the suspected virus positive sample originated. The suspension of approved status will be maintained until it is demonstrated that the virus in question is not VHSV.

iv) If no CPE occurs after 7 days of incubation (except in positive control cell cultures), subcultivation of the inoculated cell cultures must be performed. However, if no CPE is observed in positive controls, another series of virological examinations must be undertaken, using susceptible cells and new batches of organ samples.

c) Subcultivation procedures

i) Collect aliquots of cell culture medium from all monolayers inoculated with dilutions of each supernatant of organ homogenates.

ii) Centrifuge at 2,000 x g for 15 min at 4°C and collect supernatant.

iii) If required, repeat neutralisation test to IPNV as previously described, with dilution of the above supernatant (1:1 to 1:100).

iv) Inoculate BF-2 or EPC cell monolayers as described above (1.1.a).

v) Incubate and monitor as in 1.1.b.

vi) If no CPE occurs the test may be declared negative.

1.2. Virus identification

a) Neutralisation test

i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge it at 2,000 x g for 15 min at 4°C, or filter through a 450 nm pore membrane to remove cell debris.

ii) Dilute virus-containing medium 10^{-2} and 10^{-4}.

iii) Mix aliquots (for example 200 μl) of each dilution with equal volumes of an antibody solution for VHSV, and similarly treat aliquots of each virus dilution with cell culture medium.
(The titre of neutralising antibody (NAb) solution must be at least 2,000 for 50% plaque reduction.)

iv) In parallel, a neutralisation test must be performed against homologous VHSV (positive neutralisation test).

v) If required, do a similar neutralisation test with antibodies to IPNV, to ensure that no IPNV contaminant might have escaped the first anti-IPNV test.

vi) Incubate all the mixtures at 15°C for 1 hour.

vii) Transfer aliquots of each of the above mixtures onto BF-2 or EPC cell monolayers (inoculate 2 cell cultures per dilution) and allow adsorption to occur for 0.5 to 1 hour at 15°C. Twenty-four or 12 well cell culture plates are suitable for this purpose, using a 50 µl inoculum.

viii) When adsorption is completed, add cell culture medium supplemented with 2% FCS and buffered at pH 7.4-7.6 into each well and incubate at 15°C.

ix) Check the cell cultures for the onset of CPE and read the results as soon as it occurs in non-neutralised controls (cell monolayers being protected in positive neutralisation controls). Results are recorded either after a simple microscopic examination (phase contrast preferable) or after discarding cell culture medium and staining cell monolayers with a solution of 1% crystal violet in 20% ethanol.

x) The tested virus is identified as VHSV when CPE is prevented or noticeably delayed in the cell cultures which received the virus suspension treated with the VHSV-specific antibody, whereas CPE is evident in all other cell cultures.

xi) In the absence of any neutralisation by NAb to VHSV, it is mandatory to conduct an indirect fluorescent antibody test with the suspect sample. Indeed some cases of antigenic drift of surface antigen have been observed, resulting in occasional failure of the neutralisation test using NAb to VHSV.

b) *Indirect fluorescent antibody test*

This virus identification test is to be conducted either directly after virus isolation in cell culture, or as a confirmatory test following the neutralisation test described above.
i) Prepare monolayers of BF-2 or EPC cells in 2 cm\(^2\) wells of cell culture plastic plates or on coverglasses in order to reach around 80% confluency within 24 hours of incubation at 22°C (seed 6 cell monolayers per virus isolate to be identified, plus 2 for positive and 2 for negative controls). The FCS content of the cell culture medium can be reduced to 2-4%. If numerous virus isolates have to be identified, the use of Terasaki plates is strongly recommended.

ii) When the cell monolayers are ready for infection, i.e. on the same day or on the day after seeding, inoculate the virus suspensions to be identified by making ten-fold dilution steps directly in the cell culture wells or flasks.

iii) Dilute the control virus suspension of VHSV in a similar way, in order to obtain a virus titre of about 5,000-10,000 PFU/ml in the cell culture medium.

iv) Incubate at 15°C for 24 hours.

v) Remove the cell culture medium, rinse once with PBS 0.01 M pH 7.2, then 3 times briefly with cold acetone (stored at -20°C) for coverglasses or a mixture of acetone 30%-ethanol 70% (vol./vol.), also at -20°C, for plastic wells.

vi) Let the fixative act for 15 min. A volume of 0.5 ml is adequate for 2 cm\(^2\) of cell monolayer.

vii) Allow the cell monolayers to air dry for at least 30 min and process immediately or freeze at -20°C.

viii) Prepare a solution of purified antibody or serum to VHSV in PBS 0.01 M, pH 7.2 containing 0.05% Tween 80, at the appropriate dilution (which has been established previously or is given by the reagent supplier).

ix) Réhydrate the dried cell monolayers by 4 rinsing steps with the above PBS-Tween and eliminate this buffer thoroughly after the last rinsing.

x) Treat the cell monolayers with the antibody solution for 1 hour at 37°C in a moist chamber and do not allow evaporation to occur. The volume of solution to be used is 0.25 ml/2 cm\(^2\) well.

xi) Rinse 4 times with PBS-Tween as above.

xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate-conjugated (FITC) antibody to the
immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.

xiii) Rinse 4 times with PBS-Tween.

xiv) Examine the treated cell monolayers on plastic plates immediately, or mount the coverglasses using glycerol saline at pH 8.5 prior to microscopic observation.

xv) Examine under incident UV light using a microscope with x10 eye pieces and x20 to x40 objective lens having numerical aperture >0.65 and >1.3 respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

c) Enzyme-linked immunosorbent assay (ELISA)

i) Coat the wells of microplates designed for ELISA tests, with appropriate dilutions of purified immunoglobulins (Ig) or serum specific for VHSV, in PBS 0.01 M pH 7.2 (200 µl/well). Ig may be polyclonal or monoclonal Ig originating most often from rabbit or mouse, respectively. For the identification of VHSV, monoclonal antibodies specific for certain domains of the nucleocapsid protein (N) are suitable.

ii) Incubate overnight at 4°C.

iii) Rinse 4 times with PBS 0.01 M containing 0.05 % Tween 20 (PBST).

iv) Block with skim milk (5% in PBST) or other blocking solution for 1 hour at 37°C (200 µl/well).

v) Rinse 4 times with PBST.

vi) Add 2% of Triton X 100 to the virus suspension to be identified.

vii) Dispense 100 µl/well of 2 or 4 step dilutions of the virus to be identified and of VHSV control virus, and allow to react with the coated antibody to VHSV for 1 hour at 20°C.

viii) Rinse 4 times with PBST.

ix) Add to the wells, biotinylated polyclonal antibody to VHSV; or MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin.
x) Incubate 1 hour at 37°C.

xi) Rinse 4 times with PBST.

xii) Add streptavidin-conjugated horse radish peroxidase to those wells which have received the biotin-conjugated antibody and incubate for 1 hour at 20°C.

xiii) Rinse 4 times with PBST.

xiv) Add the substrate (H₂O₂) and chromogen (O-phenylenediamide, OPD or other approved chromogen). Stop the course of the test when positive controls react, and read the results.

xv) Alternatively: add H₂O₂ + chromogen to those wells containing the peroxidase conjugated antibody and proceed as above.

2. Diagnostic Procedures for Confirmation of VHS in Suspected Outbreaks

Confirmation of VHS can be achieved by any of the following methods:

2.1. Conventional virus isolation with subsequent serological identification

   As Section 1.

2.2. Virus isolation with simultaneous serological identification

   a) Sampling procedures

   As B.1.1. in Chapter 1 (General Information) for the selection of fish specimens. As B.2.1. & 2.2. in Chapter 1 (General Information) for the selection of materials sampled.

   b) Processing of organ samples

   See the following sections in Chapter 1 (General Information):
   B.3.1. for transportation
   B.3.2. for virus extraction and obtaining of organ homogenates
   B.3.3. for treatment to neutralise birnaviruses (if required).

   c) Virus identification by neutralisation test

   i) Dilute organ homogenates 1:100, 1:1,000 and 1:10,000 in cell culture medium.
ii) Mix with equal volume of a solution of antibody to VHSV as in 1.2.a, inoculate the cell monolayers, incubate at 15°C and monitor the fate of cell infection as in 1.2.a.

iii) Subcultivation: if no CPE appears after one week subcultivate the cell culture fluids of non antibody-treated controls as in 1.1.c.

2.3. Indirect fluorescent antibody test

i) Bleed the fish thoroughly.

ii) Make kidney imprints on cleaned glass slides or at the bottom of the wells of a plastic cell culture plate.

iii) Store the kidney pieces (as indicated in B.3.1. in Chapter 1 [General Information]) together with the other organs required for virus isolation in case this later becomes necessary.

iv) Allow the imprint to air-dry for 20 min.

v) Fix with acetone or ethanol-acetone and dry as indicated in 1.2.b. points v-vii.

vi) Rehydrate the above preparations (see 1.2.b. point ix) and block with 5% skim milk or 1% bovine serum albumin (BSA), in PBST for 30 min at 37°C.

vii) Rinse 4 times with PBST.

viii) Treat the imprints with the solution of antibody to VHSV and rinse as indicated in 1.2.b.

ix) Block and rinse as formerly.

x) Reveal the reaction with suitable FITC, rinse and observe as indicated in 1.2.b. points xii-xv.

If the immunofluorescence test is negative, process the organ samples stored at 4°C, for virus isolation in cell culture as in 1.1.

2.4. ELISA

a) Microplate processing

As 1.2.c of this chapter up to point iv (inclusive).

b) Sampling procedures

See the following sections in Chapter 1 (General Information):
B.1.1. for the selection of fish specimens
B.2.1. & 2.2. for the selection of materials sampled.
c) Processing of organ samples

See the following sections in Chapter 1 (General Information):
B.3.1. for transportation
B.3.2. for virus extraction and obtaining of organ homogenates.

d) Carrying out the ELISA

i) Set aside an aliquot of 1/4 of each homogenate in case further virus isolation in cell culture is required.

ii) Treat the remaining part of homogenate with 2% Triton X 100 (vol./vol.) as 1.2.c point v and 2 mM of phenyl methyl sulfonide fluoride (PMSF); mix gently.

iii) Complete the other steps of procedure 1.2.c.

REFERENCES


DISEASES OF FISH

OTHER SIGNIFICANT DISEASES

CHAPTER 7

CHANNEL CATFISH VIRUS DISEASE
(B411)

SUMMARY

Channel catfish virus disease (CCVD) is caused by a herpesvirus and affects channel catfish (Ictalurus punctatus) in the USA. For a recent and more detailed review of the condition, see Wolf (5).

CCVD is of importance because of its clinical and economic consequences in channel catfish farming. CCVD, which affects mostly the juveniles, is often lethal, due to the impairment of osmotic balance, which occurs in a clinical context of oedema and haemorrhages. Virus multiplication in endothelial cells of blood capillaries, haematopoietic tissue and hepatocytes underlies the clinical signs.

In survivors, CCV infection results in a strong protective immunity, synthesis of circulating antibodies to the virus, and, in certain individuals, an asymptomatic virus carrier state. This carrier state frequently leads to virus shedding via the sexual products at times of spawning.

On the basis of antigenic studies conducted with polyclonal rabbit antibodies, CCV isolates appear to form a homogeneous group by neutralisation, fluorescence and ELISA tests. Some variation in the virulence of CCV strains has been recorded during natural outbreaks of disease and demonstrated experimentally.

The reservoirs of CCV are clinically infected fish and asymptomatic carriers. Infectious CCV is shed via faeces, urine and the sexual products whereas kidney, spleen, intestine and encephalon are the sites in which virus is the most abundant during the course of overt infection. The transmission of CCV is horizontal and possibly vertical or, rather, "egg-associated". Horizontal transmission may be direct or vectorial, water being the major abiotic vector. Animate vectors and fomites also act in CCV transmission. Egg-associated transmission seems to be an infrequent event but is the only route substantiating the onset of CCVD in alevins originating from thoroughly disinfected eggs which had been incubated and hatched in virus-free water. Once CCVD is established in a fish stock, it becomes endemic because of carrier fish.
Channel catfish is the only fish species susceptible to CCV and variations in susceptibility to CCV have been recorded depending on fish strains. The age of fish is extremely important for overt infection: the younger the fish, the more susceptible to CCVD. Water temperature is the critical environmental factor: the mortality rate is high at 25°-30°C but readily decreases and ceases below 18°C.

The diagnostic procedures for CCV are all based upon direct methods. The conventional approach is the most widely used and involves isolation of the virus in cell culture followed by immunological identification by neutralisation, immunofluorescence or ELISA tests. Rapid techniques by immunofluorescence or ELISA test are suitable mainly for diagnosis in clinically infected fish, but it is likely that ELISAs using certain monoclonal antibodies specific for the virus nucleocapsid antigen will become a recommended method for virus carrier screening, by detection of the viral antigen in the encephalon.

Control methods currently rely on the implementation of control policy rules and of hygiene practices in the operating of catfish husbandry. The thorough disinfection of fertilised eggs and the incubation of eggs and further rearing of fry and alevins in premises completely separated from those harbouring possible virus carriers and free from possible contact with fomites, are critical for preventing the occurrence of CCV in a defined fish production site. Vaccination, although experimentally promising, is not used in practice.

**DIAGNOSTIC PROCEDURES**

The diagnosis of CCVD is based upon direct methods which are either the isolation of CC virus (CCV) in cell culture followed by its immunological identification (conventional approach), or the immunological demonstration of CCV antigen (Ag) in infected fish tissues.

Due to insufficient knowledge of the fish serology of virus infections, the detection of fish antibodies to viruses has not thus far been recognised as a valuable diagnostic method for assessing the virus status of fish populations. However, the validation of some serological techniques for diagnosis of certain fish virus infections could arise in the near future, rendering the use of fish serology more widely acceptable for diagnostic purposes.

Infected fish material suitable for virological examination is:

- **during overt infection**: whole alevin (body length ≤ 4 cm), viscera including kidney (4 cm ≤ body length ≤ 6 cm) or, for larger size fish,
kidney, spleen and encephalon.

- **during dormant infection** (detection of asymptomatic virus carrier fish): encephalon (any size fish) and/or ovarian fluid from broodfish at time of spawning.

**Sampling procedures:** see Chapter 1 Part B.

1. **STANDARD MONITORING METHODS FOR CCVD**

1.1. **Isolation of CCV in cell culture**

Cell line(s) to be used: CCO.

   **a) Inoculation of cell monolayers**

   i) Make two additional tenfold dilutions of the 1:10 organ homogenate supernatants and transfer an appropriate volume of each of the three dilutions onto 24-hour-old CCO cell monolayers. Inoculate at least 2 cm² of cell monolayer with 100 µl of each dilution.

   ii) Allow to adsorb for 0.5-1 hour at 25-30°C and, without withdrawing inoculate, add cell culture medium buffered at pH 7.6 and supplemented with 2% FCS (1 ml/well for 24 well cell culture plates) and incubate at 25-30°C.

   **b) Monitoring incubation**

   i) Follow the course of infection in positive controls and other inoculated cell cultures, by daily microscopic examination at magnification 40x to 100x, during 14 days. The use of a phase contrast microscope is recommended.

   ii) Maintain the pH of the cell culture medium between 7.3 and 7.6 over the whole incubation phase. This can be achieved by addition to the inoculated cell culture medium of sterile bicarbonate buffer (for tightly closed cell culture flasks) or 2 M Tris buffer solution (for cell culture plates) or, even better, by using HEPES-buffered media.

   iii) If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of the tested homogenate supernatants, identification procedures have to be undertaken immediately (see below). If a fish health surveillance/control programme is being implemented, provisions have to be taken to suspend the approved health status of the production unit and/or the zone (if it was approved previously) from which the virus positive sample originated. The suspension of approved
status will be maintained until it is demonstrated that the virus in question is not CCV.

iv) If no CPE occurs, except in positive control cell cultures, subcultivation steps have to be made even after 7 days of incubation, in certain of the infected cell cultures. However, if no CPE is observed even in positive controls, another series of virological examinations have to be undertaken, using susceptible cells and new batches of organ samples.

c) Subcultivation procedures

i) Collect aliquots of cell culture medium from all monolayers inoculated with dilutions of each supernatant of organ homogenates.

ii) Centrifuge at 2,000 x g for 15 min at 4°C and collect supernatant.

iii) Repeat optional neutralisation test to IPNV if needed, with dilution of the above supernatant (1:1 to 1:100).

iv) Inoculate CCO cell monolayers as described above (1.1.a).

v) Incubate and monitor as in 1.1.b.

vi) Make a second (and last) subcultivation step, if the first one remains virus-negative.

1.2. Virus identification

a) Neutralisation test

i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge it at 2,000 x g for 15 min at 4°C to remove cell debris, or

ii) Dilute virus-containing medium from 10^-2 to 10^-4.

iii) Mix aliquots (for example 200 µl) of each virus dilution with equal volumes of an antibody solution specific for CCV, and similarly treat aliquots of each virus dilution with cell culture medium.

The titre of neutralising antibody (NAb) solution must be around 2,000 in the 50% plaque reduction assay.
iv) In parallel, other neutralisation tests must be performed against:

- a homologous virus strain (positive neutralisation test)
- a heterologous virus strain (negative neutralisation test).

v) Incubate all the mixtures at 25°C for 1 hour.

vi) Transfer aliquots of each of the above mixtures onto CCO cell monolayers (inoculate 2 cell cultures per dilution) and allow adsorption to occur for 0.5 to 1 hour at 25°C. Twenty-four or 12 well cell culture plates are suitable for this purpose, using a 50 µl inoculum.

vii) When adsorption is completed, add cell culture medium supplemented with 2% FCS and buffered at pH 7.3-7.6 into each well and incubate at 25-30°C.

viii) Check the cell cultures for the onset of CPE and read the results as soon as it occurs in non-neutralised controls (cell monolayers being protected in positive neutralisation controls). Results are recorded either after a simple microscopic examination (phase contrast preferable) or after discarding cell culture medium and staining cell monolayers with a solution of 1% crystal violet in ethanol 20%.

ix) The tested virus is identified as CCV when CPE is abolished or noticeably delayed in the cell cultures which had received the virus suspension treated with the CCV-specific antibody, whereas CPE is evident in all other cell cultures.

x) In the absence of any neutralisation by NAb to CCV, it is mandatory to conduct an indirect fluorescent antibody test with the suspect sample.

b) Indirect fluorescent antibody test

This virus identification test is to be conducted either directly after virus isolation in cell culture, or as a confirmatory test following the neutralisation test described above.

i) Prepare monolayers of CCO cells in 2 cm² wells of cell culture plastic plates or on coverglasses in order to reach around 80% confluency, which is usually achieved within 4 hours of incubation at 30°C (seed 6 cell monolayers per virus isolate to be identified, plus 2 for positive and 2 for negative controls). The FCS content of the cell culture medium can be reduced to 2-4%. If numerous virus isolates have to be identified, the use
of Terasaki plates is strongly recommended.

ii) When the cell monolayers are ready for infection, i.e. on the same day or on the day after seeding, inoculate the virus suspensions to be identified by making ten-fold dilution steps directly in the cell culture wells or flasks.

iii) Dilute the control virus suspension of CCV in a similar way, in order to obtain a virus titre of about 5,000-10,000 PFU/ml in the cell culture medium.

iv) Incubate at 25°C for 18 hours.

v) When the incubation time is over, aspirate the cell culture medium, rinse once with PBS 0.01 M pH 7.2, then 3 times briefly with cold fixative. This fixative will be acetone (stored at -20°C) for coverglasses or a mixture of acetone 30%-ethanol 70% (vol./vol.), also stored at -20°C.

vi) Afterwards, let the fixative act for 15 min. A volume of 0.5 ml is adequate for 2 cm² of cell monolayer.

vii) Allow the cell monolayers to air dry for at least 30 min and process immediately or freeze at -20°C.

viii) Prepare a solution of purified antibody or serum to CCV in PBS 0.01 M, pH 7.2 containing 0.05% Tween 80, at the appropriate dilution (which has been established previously or is given by the reagent supplier).

ix) Rehydrate the dried cell monolayers by 4 rinsing steps with the above PBS and eliminate this buffer thoroughly after the last rinsing.

x) Treat the cell monolayers with the antibody solution for 1 hour at 37°C in a moist chamber. The volume of solution to be used is 0.25 ml/2 cm² well.

xi) Rinse 4 times with PBS-Tween as above.

xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate-conjugated (FITC) antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.

xiii) Rinse 4 times with PBS-Tween.

xiv) Observe the treated cell monolayers on plastic plates
immediately, or mount the coverglasses using glycerol saline at pH 8.5 prior to microscopic observation.

xv) Conduct this observation under incident UV light using a microscope with x10 eye pieces and x20 to x40 objective lens having numerical aperture >0.65 and >1.3 respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

c) Enzyme-linked immunosorbent assay (ELISA)

i) Coat the wells of microplates designed for ELISA tests, with appropriate dilutions of purified immunoglobulins (Ig) or serum specific for CCV, in PBS 0.01 M pH 7.2 (200 µl/well). Ig may be polyclonal or monoclonal Ig originating most often from rabbit or mouse, respectively.

ii) Incubate overnight at 4°C.

iii) Rinse 4 times with PBS 0.01 M containing 0.05 % Tween 20 (PBST).

iv) Block with skim milk (5% in PBST) or other blocking solution for 1 hour at 37°C (200 µl/well).

v) Add 2% of Triton X 100 to the virus suspension to be identified.

vi) Rinse 4 times with PBST.

vii) Dispense 100 µl/well of a 2 or 4 step dilution of the virus to be identified and of CC control virus, and allow to react with the coated antibody to CCV for 1 hour at 20°C.

viii) Rinse 4 times with PBST.

ix) Add to the wells, biotinylated polyclonal antibody to CCV.

x) Incubate 1 hour at 37°C.

xi) Rinse 4 times with PBST.

xii) Add streptavidin-conjugated horse radish peroxidase to those wells which have received the biotin-conjugated antibody and incubate for 1 hour at 20°C.

xiii) Rinse 4 times.

xiv) Add the substrate (H₂O₂) and chromogen (O-phenylenediamide, OPD or other approved chromogen). Stop the course of the test when positive controls react, and monitor the results.
2. Diagnostic procedures for confirmation of CCVD in suspected outbreaks

Confirmation of CCV can be achieved by any of the following methods.

2.1. Conventional virus isolation with subsequent serological identification as in Section 1.

2.2. Virus isolation with simultaneous identification

   a) Sampling procedures

   As B.1.1. in Chapter 1 (General Information) for the selection of fish specimens.
   As B.2.1. & 2.2. in Chapter 1 (General Information) for the selection of materials sampled.

   b) Processing of organ samples

   See the following sections in Chapter 1 (General Information):
   B.3.1. for transportation
   B.3.2. for virus extraction and obtaining of organ homogenates
   B.3.3. for treatment to neutralise birnaviruses.

   c) Virus identification by neutralisation test

   i) Dilute the anti-IPN-treated organ homogenates 1:100, 1:1,000 and 1:10,000 in cell culture medium.
   ii) Mix with equal volume of a solution of antibody to CCV as in 1.2.a, inoculate the cell monolayers, incubate at 25-30°C and monitor the fate of cell infection as in 1.2.a.
   iii) Subcultivation: if no CPE appears after one week subcultivate the cell culture fluids of non antibody-treated controls as in 1.1.c.

2.3. Indirect fluorescent antibody test

   i) Bleed the fish thoroughly.
   ii) Make kidney imprints on cleaned glass slides or at the bottom of the wells of a plastic cell culture plate.
iii) Store the kidney pieces (as indicated in B.3.1. in Chapter 1 [General Information]) together with the other organs required for virus isolation in case this later becomes necessary.

iv) Allow the imprint to air-dry for 20 min.

v) Fix with acetone or ethanol-acetone and dry as indicated in 1.2.b. points v-vii.

vi) Rehydrate the above preparations (see 1.2.b. point ix) and block with 5% skim milk or 1% bovine serum albumin (BSA), in PBST for 30 min at 37°C.

vii) Rinse 4 times with PBST.

viii) Treat the imprints with the solution of antibody to CCV and rinse as indicated in 1.2.b.

ix) Block and rinse as formerly.

x) Reveal the reaction with suitable FITC, rinse and observe as indicated in 1.2.b. points xii-xv.

If the immunofluorescence test is negative, process the organ samples stored at 4°C, for virus isolation in cell culture as in 1.1.

2.4. ELISA

a) Microplate processing

As 1.2.c of this chapter up to point iv (inclusive).

b) Sampling procedures

See the following sections in Chapter 1 (General Information):
B.1.1. for the selection of fish specimens
B.2.1. & 2.2. for the selection of materials sampled.

c) Processing of organ samples

See the following sections in Chapter 1 (General Information):
B.3.1. for transportation
B.3.2. for virus extraction and obtaining of organ homogenates.

d) Carrying out the ELISA

i) Set aside an aliquot of 1/4 of each homogenate in case further virus isolation in cell culture is required.
ii) Treat the remaining part of homogenate with 2% Triton X 100 (vol./vol.) as 1.2.c point v and 2 mM of phenyl methyl sulfonide fluoride (PMSF); mix gently.

iii) Complete the other steps of procedure 1.2.c.

REFERENCES


CHAPTER 8

VIRAL ENCEPHALOPATHY AND RETINOPATHY
(No OIE number)

SUMMARY

A distinctive syndrome of vacuolating encephalopathy and retinopathy, or viral nervous necrosis (VNN), occurs in larval and, sometimes, juvenile sea bass (Lates calcarifer and Dicentrarchus labrax), turbot (Scophthalmus maximus), Japanese parrotfish (Oplegnathus fasciatus), redspotted grouper (Epinephelus akaara), and striped jack (Pseudocaranx dentex). Typically there is massive (often 100%) mortality in affected stocks.

The causative agents are icosahedral, non-enveloped viruses about 25-30 nm in diameter and, until recently, described as picornavirus-like. The agent (SJNNV) of VNN in striped jack has been identified as a nodavirus and serological relationships have been shown between this virus and the agents causing vacuolating encephalopathy and retinopathy in all the other species except turbot (in this instance no tests have been made). Diagnosis depends on microscopy (light and electron) and a range of recently developed immunological and molecular procedures.

Control measures are based on improved hatchery hygiene and reduced stocking rates. In the case of VNN in striped jack, where vertical transmission unequivocally occurs, identification and culling of carrier broodfish is desirable.

INTRODUCTION

Vacuolating encephalopathy and retinopathy, or viral nervous necrosis (VNN), of larval marine fish has been described in Australasian sea bass (Lates calcarifer), European sea bass (Dicentrarchus labrax), turbot (Scophthalmus maximus), Japanese parrotfish (Oplegnathus fasciatus), redspotted grouper (Epinephelus akaara), and striped jack (Pseudocaranx dentex). Except for turbot, there have been confirmed and unconfirmed reports of these syndromes from most places where the above species are intensively cultured. Recently, apparently identical disease outbreaks have been reported in tiger puffer (Taligiugu rubripes), Japanese flounder (Paralichthys olivaceus), kelp grouper (Epinephelus moara) and rock porgy (Oplegnathus punctatus).

Virus particles of about 25-30 nm in diameter have been visualised in affected fish and the agent (SJNNV) associated with the disease (VNN) in striped jack has been characterised and placed in the family Nodaviridae. Immunological studies have shown a relationship between SJNNV and the agents of the two sea bass diseases and VNN of Japanese parrotfish and redspotted grouper.
Clinical signs and macroscopic lesions

There is great commonality of clinical signs with 'mass mortality' and a variety of neurological abnormalities, as follows:

L. calcarifer  
uncoordinated darting, corkscrew swimming, pale colour, anorexia, wasting

D. labrax  
whirling swim pattern, swimbladder hyperinflation, anorexia

O. fasciatus  
spiral swimming, dark colour

E. akaara  
whirling swim pattern

P. dentex  
abnormal swimming behaviour, swimbladder hyperinflation

S. maximus  
spiral and/or looping swim pattern, belly-up at rest, dark colour

Apart from colour changes and wasting there are no consistent macroscopic findings.

Interesting differences in relation to the occurrence and severity of the diseases are shown in Table 1.

Epidemiology

The incubation period for the L. calcarifer disease is four days whereas the earliest known occurrence of natural disease is nine days after hatch (Table 1), suggesting that vertical transmission of virus is unlikely in this species.

In contrast, it has been demonstrated that vertical transmission of the causative agent occurs in P. dentex and this fact is reflected by early occurrence of clinical disease.

The mode of transmission of the viruses, other than in gametes, has not been demonstrated but the possibilities include influent water, juvenile fish held on the same site, and carriage on utensils, vehicles, etc. It is possible that these small viruses are quite resistant to environmental conditions and therefore readily translocated by commercial activities.

Control

Control of VNN in striped jack is difficult because of its vertical transmission. The newly developed PCR method should permit screening of potential carrier broodfish. Also, there is some evidence that reduced stress at spawning can ameliorate the condition.
Table 1. Important features of viral encephalopathies of larval and juvenile fish

<table>
<thead>
<tr>
<th>Species</th>
<th>Earliest occurrence of disease</th>
<th>Usual onset of disease</th>
<th>Latest occurrence of new outbreaks</th>
<th>Usual mortality rate</th>
<th>Highest mortality rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. calcarifer</td>
<td>9 d.p.h.</td>
<td>15-18 d.p.h.</td>
<td>≥24 d.p.h.</td>
<td>50-100%/month</td>
<td>100% in &lt;1 month</td>
</tr>
<tr>
<td>D. labrax</td>
<td>10 d.p.h.</td>
<td>25-40 d.p.h.</td>
<td>Bodyweight 5 g</td>
<td>10%/month</td>
<td>Up to 100%</td>
</tr>
<tr>
<td>O. fasciatus</td>
<td>6-25 mm t.l.</td>
<td>9-10 mm t.l.</td>
<td>&lt;40 mm t.l.</td>
<td>80%</td>
<td>Up to 100%</td>
</tr>
<tr>
<td>E. akaara</td>
<td>14 d.p.h.</td>
<td>9-10 mm t.l.</td>
<td>&lt;40 mm t.l.</td>
<td>80%</td>
<td>Up to 100%</td>
</tr>
<tr>
<td>P. dentex</td>
<td>1 d.p.h.</td>
<td>1-4 d.p.h.</td>
<td>&lt;20 d.p.h.</td>
<td>100%</td>
<td>Up to 100%</td>
</tr>
<tr>
<td>S. maximus</td>
<td>&lt;21 d.p.h.</td>
<td></td>
<td>Bodyweight 50-100 mg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

d.p.h. = days post-hatch; t.l. = total length

In contrast, control of clinical disease in L. calcarifer has been remarkably successful. In intensive hatcheries this has consisted of some or all of the following strategies: no recycling of culture water, chemical disinfection of influent water and larval tanks between batches, and reduction of larval density from 15-30 larvae/litre to not more than 15 larvae/litre (preferably less than 10/litre). Extensive culture in 'green ponds' is also associated with a low prevalence of clinical disease and/or histological lesions.

**DIAGNOSTIC PROCEDURES**

Presumptive diagnosis can be made on the basis of the light microscopic appearance of the brain and/or retina. However, individual fish with the presence of only a few vacuoles in the neuropil pose a difficult diagnostic problem.

Electron microscopy is a very useful confirmatory technique. In particular, negative staining yields a rapid result and can be performed on both unfixed (preferable) and formalin-fixed material.

SJNNV can be detected by the fluorescent antibody technique (FAT), enzyme-linked immunosorbent assay (ELISA), or polymerase chain reaction (PCR) amplification of RNA. The FAT is sufficiently broad in specificity to be used to detect at least four other viruses in this group.
To date it has proved impossible to culture these viruses in a range of fish tissue cell cultures.

1. **Histopathology**

Normal histological methods, including haematoxylin and eosin staining, are used. Small larvae are embedded whole and serially sectioned to provide sections of brain and eyeballs. Larger fish (juvenile) usually require removal of eyes and brain for embedding after preliminary fixation.

All the diseases are characterised by vacuolation of the brain. Usually there is also vacuolation of the nuclear layers of the retina, although this lesion is not included in the description of the disease in Japanese parrotfish or turbot. In general, younger fish have more severe lesions whereas older fish have less extensive lesions and these may show a predilection for the retina. Intracytoplasmic inclusions (up to 5 µm in diameter) have been described in sections of European and Australasian sea bass nervous tissues, and neuronal necrosis has been described in most species. Vacuolation of the gut is not caused by these nodaviruses, but is a typical physiological response.

2. **Electron Microscopy**

Virus particles can be visualised in affected brain and retina by both positive and negative staining.

In positively stained material the virus is mainly associated with vacuolated cells and, especially, any inclusions. The particles vary in size from 22-32 nm (European sea bass) to 34 nm (Japanese parrotfish) arranged intracytoplasmically in crystalline arrays, or as aggregates and single particles both intra- and extracellularly. The virus is non-enveloped and icosahedral in shape.

(i) **Negative staining**

Fresh or frozen brain or eye is macerated with 1 or 2% phosphotungstic acid (adjusted to pH 6.8 with KOH) and mounted on copper grids for transmission electron microscopy.

Non-enveloped, round to icosahedral particles about 25-30 nm will be present. It may be possible to detect capsomeres.

(ii) **Whole larvae or eyes and brains of juveniles are fixed in cold 1-2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.1) for 24 hours. The samples are then rinsed three times with 0.1 M cacodylate buffer and postfixed in 1% osmium tetroxide for one hour. The tissues are next dehydrated in a series of acetone and placed in a 1:1 mixture of 100% acetone:Spurr's resin and allowed to infiltrate overnight. Infiltration is completed by transfer to Spurr's resin for 12 hours and then the tissues**
are embedded. Ultrathin sections are cut and stained with uranyl acetate and lead citrate and examined under a transmission electron microscope. Virus particles are especially associated with inclusions and the cytoplasm of vacuolated cells.

3. **FLUORESCENT ANTIBODY TECHNIQUE**

Fish samples fixed in 10% buffered formalin are immersed in 0.1 M PBS containing 5% sucrose (pH 7.2) overnight. After successive immersions in 10%, 30% and 40% sucrose-PBS for 1-2 h, samples are embedded in OCT compound (Miles Inc.), cut at 7 μm by a cryostat apparatus, and washed with cold PBS. Samples are incubated with anti-SJNNV rabbit serum (dilution 1:100) at 37°C for 30 min, washed with PBS, and then reacted with FITC labelled anti-rabbit Ig goat antibody at 37°C for 30 min, washed with PBS, and examined by fluorescence microscopy. Specific fluorescence is observed in the cytoplasm of the affected cells in brain and retina. This FAT is also applicable to paraffin embedded sections.

4. **ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)**

At present, ELISA using anti-SJNNV rabbit serum can be used only to detect VNN from diseased larvae of striped jack.

The fish sample (usually 10 larvae) is homogenised with nine volumes of 0.05 M carbonate-bicarbonate buffer (pH 9.6) and centrifuged at 10,000 g for 30 min. Serially two-fold diluted supernatant is added in a 96-well microplate (0.2 ml/well) and incubated at 25°C for 2 h or at 4°C overnight. After washings with PBS containing 0.05% Tween 20 and blocking with 2% bovine serum albumin, wells are filled with 0.2 ml of anti-SJNNV rabbit serum (dilution 1:1,000) and incubated at 37°C for 2 h. Following washes, 0.2 ml of alkaline phosphatase conjugated anti-rabbit Ig goat antibody is added to each well and incubated at 37°C for 2 h. Following the final washing, 0.2 ml of p-nitrophenyl phosphate disodium salt in diethanolamine solution (pH 9.8) are added and the plates are incubated for 60 min. The absorbance of each well is read at 405 nm using a microplate reader. The absorbance for normal larvae is usually lower than 0.1 in every dilution.

5. **POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION**

SJNNV contains two single-stranded, positive-sense, non polyadenylated RNAs (RNA1 and RNA2). RNA2 encodes a 42 kDa structural protein of the virus. The two primers, a reverse primer (5'-CGAGTCAACACGGGTGAAGA-3') and a forward primer (5'-CGTGTCACTGATGTTGCT-3'), are used for amplification of a target sequence (about 430 bases) of SJNNV RNA2 by PCR. This PCR procedure can be used for detection of the viruses from not only striped jack but also other fish (O. fasciatus, E. akaara, T. rubripes, P. olivaceus, E. moara, and O. punctatus).
The fish sample (0.1 g) is homogenised with 0.5 ml distilled water treated with 0.1% diethyl pyrocarbonate and centrifuged at 10,000 g for 10 min. The resultant supernatant is mixed with 0.04 ml of proteinase K (1 mg/ml) and 0.04 ml of 1% SDS, and incubated at 37°C for 30 min. After centrifugation, total nucleic acids are extracted through the phenol-chloroform method. The total nucleic acids are preheated at 90°C for 5 min and incubated at 42°C for 30 min in 20 μl of PCR buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl) containing 2.5 U of M-MLV reverse transcriptase (USB), 1.0 U of ribonuclease inhibitor, 0.5 μM of reverse primer, 1 mM each of four deoxynucleotide triphosphates (dNTP), and 5 mM of MgCl₂.

The mixture is incubated at 99°C for 10 min to inactivate the reverse transcriptase and then diluted five-fold with PCR buffer containing 0.1 μM of forward primer, 2.5 U of Tth Version 2.0 DNA polymerase (Toyobo) and 2 mM of MgCl₂. The mixture is incubated in an automatic thermal cycler programmed for one cycle at 72°C for 10 min and 95°C for 2 min, then 25 cycles at 95°C for 40 s, 55°C for 40 s, and 72°C for 40 s, and finally held at 72°C for 5 min. Amplified DNA (430 bp) is analysed by agarose gel electrophoresis.

REFERENCES


CHAPTER 9
INFECTIONOUS PANCREATIC NECROSIS
(No OIE number)

SUMMARY

Infectious pancreatic necrosis (IPN) is a highly contagious viral disease of young fish of salmonid species held under intensive rearing conditions. The disease most characteristically occurs in rainbow trout (Onchorhyncus mykiss), brook trout (Salvelinus fontinalis), brown trout (Salmo trutta), Atlantic salmon (Salmo salar), and several Pacific salmon species (Oncorhynchus spp.). IPN virus, or viruses showing serological relatedness to IPN virus, have been reported to cause diseases in some farmed marine fish species, such as cod (Gadus morhua), yellowtail (Seriola quinqueradiata), turbot (Scophthalmus maximus), and halibut (Hippoglossus hippoglossus), and subclinical asymptomatic infections have been detected in a wide range of estuarine and freshwater fish species in the families Anguillidae, Atherinidae, Bothidae, Carangidae, Cotostomidae, Cichlidae, Clupeidae, Cobitidae, Coregonidae, Cyprinidae, Esocidae, Moronidae, Paralichthyidae, Percidae, Poecilidae, Sciaenidae, Soleidae and Thymallidae.

The causative agent, infectious pancreatic necrosis virus (IPNV), is a bi-segmented double-stranded RNA virus belonging to the family Birnaviridae.

Monitoring for IPN is based upon isolation of the virus in tissue culture and its immunological identification. Diagnosis of clinical cases is normally based on characteristic pathological changes, particularly to the pancreas (as detected by standard histological techniques) and/or immunological demonstration of IPNV antigen in infected tissues, confirmed by isolation and immunological identification of IPNV in tissue culture.

Control methods currently rely on the implementation of control policy rules and of hygiene practices in the operating of salmonid husbandry, through the avoidance of fertilised eggs originating from IPNV carrier broodstock, and use of a protected water supply (e.g. spring, borehole pond) into which ingress of fish, particularly possible virus carriers, is prevented. In outbreaks, a reduction of the population density ('thinning out') may help reduce the overall mortality. No treatment or commercial vaccine is available at present.
INTRODUCTION

Infectious pancreatic necrosis (IPN) is a highly contagious viral disease, principally of young fish of salmonid species, held under intensive rearing conditions. Susceptibility generally decreases with age, with resistance to clinical disease in salmonid fish usually being reached at about 1,500 degree-days (value obtained by multiplying the age in days by the average temperature in degrees Centigrade during the lifespan) except for Atlantic salmon smolts which can suffer from disease shortly after transfer from fresh water to seawater. The causative agent is a birnavirus (bi-segmented double-stranded RNA) displaying wide antigenic diversity and marked differences in degrees of virulence.

The disease has a wide geographical distribution, occurring in most, if not all, major salmonid farming countries of North and South America, Europe and Asia.

The first sign of an outbreak in salmonid fry is frequently a sudden and usually progressive increase in daily mortalities, particularly in the faster growing individuals. Clinical signs include darkening pigmentation, a pronounced distended abdomen and a corkscrewing/spiral swimming motion. Cumulative mortalities may vary from less than 10% to more than 90% depending on the combination of several factors such as virus strain, host and environment.

Clinical diagnosis is most often confirmed by histology of the internal organs (particularly of the pancreas) with isolation of the virus in tissue culture, followed by its identification using a serological method such as serum neutralisation, ELISA or fluorescent antibody tests (FAT). Virus isolation in tissue culture is the standard method for detection of asymptomatic carriers.

The disease is transmitted both horizontally through the water route and vertically via the egg. Surface disinfection of eggs is not entirely effective in preventing vertical transmission.

There is no treatment or commercial vaccine yet available. Prevention can be achieved by avoidance of fertilised eggs originating from IPN virus carrier broodstock and use of a protected water supply (e.g. spring, borehole) into which ingress of fish is prevented. In outbreaks, a reduction of the population density ('thinning out') may help reduce the overall mortality.

DIAGNOSTIC PROCEDURES

Monitoring for IPN is based upon isolation of IPN virus (IPNV) in cell culture followed by its immunological identification. Diagnosis of clinical cases is normally based on histology and/or immunological demonstration of IPNV antigen (Ag) in infected tissues (confirmed by isolation and immunological identification of IPNV in tissue culture).
Due to insufficient knowledge on the fish serology of virus infections, the detection of fish antibodies to viruses has not thus far been accepted as a routine diagnostic method for assessing the virus status of fish populations. However, the validation of some serological techniques for diagnosis of certain fish virus infections could arise in the near future, rendering the use of fish serology more widely acceptable for diagnostic purposes.

Infected fish material suitable for virological examination is:

- **during overt infection**: whole alevin (body length ≤ 4 cm), viscera including kidney (4 cm ≤ body length ≤ 6 cm) or, for larger size fish, liver, kidney and spleen.

- **during dormant infection** (asymptomatic virus carrier fish): liver, kidney and spleen (any size fish) and/or ovarian fluid from broodfish at time of spawning.

**Sampling procedures**: see Chapter 1 Part B.

1. **STANDARD MONITORING METHODS FOR IPN**

1.1. **Isolation of IPNV in cell culture**

Cell line(s) to be used: BF-2 and CHSE-214.

a) **Inoculation of cell monolayers**

i) Make a 1:100 dilution of the organ homogenate supernatant (1:100 of tissue). Incubate at 15-20°C for 1 hour or overnight at 4°C. Inoculate an appropriate volume of the primary dilution and a 1:10 dilution thereof onto 24- to 48-hour-old BF-2 monolayers.

ii) Allow to adsorb for 1 hour at 15-20°C, then remove the 1:100 dilution and replace with cell culture medium. Incubate at 15°C. All dilutions are to be made in cell culture medium buffered at pH 7.6 and supplemented with 10% FCS, antibiotics and antimycotics.

b) **Monitoring incubation**

i) Follow the course of infection in positive controls and other inoculated cell cultures, by daily microscopic examination at magnification 40x to 100x, for 7 days. The use of a phase contrast microscope is recommended.

ii) Maintain the pH of the cell culture medium between 7.3 and 7.6 over the whole incubation period. This can be achieved by
addition to the inoculated cell culture medium of sterile bicarbonate buffer (for tightly closed cell culture flasks) or 2 M Tris buffer solution (for cell culture plates) or, even better, by using HEPES-buffered media.

iii) If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of the homogenate, identification procedures must be undertaken immediately (see below).

iv) If no CPE occurs after 7 days of incubation (except in positive control cell cultures), subcultivation of the inoculated cell cultures must be performed.

c) Subcultivation procedures

i) Collect cell culture monolayers and subject them to one freeze-thaw cycle. Pool aliquots of the supernatants from all cell monolayers inoculated with dilutions of organ homogenates.

ii) Dilute 1:20 and 1:100 and inoculate BF-2 cell monolayers as described above (1.1.a.).

iii) Incubate and monitor as in 1.1.b.

iv) If no CPE occurs the test may be declared negative.

1.2. Virus identification

a) Neutralisation test

i) Dilute virus-containing medium 10⁻² and 10⁻⁴.

ii) Mix aliquots (for example 200 μl) of each dilution with equal volumes of an antibody solution for IPNV, and similarly treat aliquots of each virus dilution with cell culture medium.

(The titre of neutralising antibody (NAb) solution must be at least 2,000 for 50% plaque reduction.)

iii) Incubate all the mixtures at 15°C for 1 hour.

iv) Transfer aliquots of each of the above mixtures onto cell monolayers (inoculate 2 cell cultures per dilution).

v) Incubate at 15°C.

vi) Check the cell cultures for the onset of CPE and read the results as soon as it occurs in non-neutralised controls (cell
monolayers being protected in positive neutralisation controls). Results are recorded either after a simple microscopic examination (phase contrast preferable) or after discarding cell culture medium and staining cell monolayers with a solution of 1% crystal violet in 20% ethanol.

vii) The tested virus is identified as IPNV when CPE is prevented or noticeably delayed in the cell cultures which received the virus suspension treated with the IPNV-specific antibody, whereas CPE is evident in all other cell cultures.

b) Enzyme-linked immunosorbent assay (ELISA)

i) Coat the wells of microplates designed for ELISA tests, with appropriate dilutions of purified immunoglobulins (Ig) or serum specific for IPNV, in carbonate buffer 0.02 M, pH 9.5 (200 μl/well). Ig may be polyclonal or monoclonal Ig originating most often from rabbit or mouse, respectively. For the identification of IPNV, monoclonal antibodies specific for certain domains of the nucleocapsid protein (N) are suitable.

ii) Incubate overnight at 4°C.

iii) Rinse 2 times with PBS 0.01 M.

iv) Block with bovine serum albumin (1.5% in carbonate buffer) or other blocking solution for 1 hour at 37°C (200 μl/well).

v) Rinse 4 times with PBST containing 0.05 % Tween 20 (PBST).

vi) Add an equal volume of PBST to the virus suspension to be identified.

vii) Dispense 100 μl/well of 2 or 4 step dilutions of the virus to be identified and of IPNV control virus, and allow to react with the coated antibody to IPNV for 30 min at 37°C.

viii) Rinse once with PBST followed by three washes, allowing the strips to soak for 3 min between washes.

ix) Add to the wells, horse radish peroxidase (HRP)-conjugated polyclonal antibody to IPNV; or MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin.

x) Incubate 30 min at 37°C.

xi) Rinse and wash with PBST as in step viii above.
xii) Add the substrate (H₂O₂) and chromogen (tetrakis-methylbenzidine [TMB] or other approved chromogen). Stop the course of the test when positive controls react, and read the results.

xiii) Alternatively: add H₂O₂ + chromogen to those wells containing the peroxidase conjugated antibody and proceed as above.

2. **Diagnostic procedures for confirmation of IPN in suspected outbreaks**

Confirmation of IPNV can be achieved by any of the following methods:

2.1. **Conventional virus isolation with subsequent serological identification**

As Section 1.

2.2. **Virus isolation with simultaneous serological identification**

a) **Sampling procedures**

As B.1.1. in Chapter 1 (General Information) for the selection of fish specimens.

As B.2.1. & 2.2. in Chapter 1 (General Information) for the selection of materials sampled.

b) **Processing of organ samples**

See the following sections in Chapter 1 (General Information):

B.3.1. for transportation
B.3.2. for virus extraction and obtaining of organ homogenates
B.3.3. for treatment to neutralise birnaviruses (if required).

c) **Virus identification by neutralisation test**

i) Dilute organ homogenates 1:100, 1:1,000 and 1:10,000 in cell culture medium.

ii) Mix with equal volume of a solution of antibody to IPNV as in 1.2.a, inoculate the cell monolayers, incubate at 15°C and monitor the fate of cell infection as in 1.2.a.

iii) Subcultivation: if no CPE appears after one week subcultivate the cell culture fluids of non antibody-treated controls as in 1.1.c.
2.3. ELISA

a) Microplate processing

As 1.2.b. of this chapter up to point iv (inclusive).

b) Sampling procedures

See the following sections in Chapter 1 (General Information):
B.1.1. for the selection of fish specimens
B.2.1. & 2.2. for the selection of materials sampled.

c) Processing of organ samples

See the following sections in Chapter 1 (General Information):
B.3.1. for transportation
B.3.2. for virus extraction and obtaining of organ homogenates.

d) Carrying out the ELISA

i) Set aside an aliquot of 1/4 of each homogenate in case further virus isolation in cell culture is required.

ii) Treat the remaining part of homogenate with 0.5% PBST (vol./vol.) as 1.2.c point v and 2 mM of phenyl methyl sulfonide fluoride (PMSF); mix gently.

iii) Complete the other steps of procedure 1.2.b.

REFERENCES


SUMMARY

Infectious salmon anaemia (ISA) is a viral infection of Atlantic salmon (Salmo salar). It is so far only reported to occur in Norway. For more detailed information on the condition, see reference 12. Atlantic salmon is the only susceptible fish species known to develop the disease, but experimentally, sea trout and rainbow trout have been shown to act as asymptomatic carriers of the disease agent.

Clinically, the initial infection appears as a systemic and lethal condition which is characterised by anaemia, ascites, congestions and enlargement of the liver (dark in colour) and spleen, as well as peritoneal petechiae. Haemorrhages in the eyes may also be seen. Hepatocellular degeneration and necrosis is a consistent histopathological finding. The infection is only seen in fish held in sea water or in fish exposed to sea water. A virus, probably the ISA agent, has recently been isolated and is currently under investigation.

The reservoirs of ISA are not known, but spread of the disease has occurred as a result of the purchasing of subclinically infected Atlantic salmon smolts; from farm to farm; and from fish slaughterhouses or industries from which organic material (especially blood and processing water) from ISA infected fish has been discharged directly into sea water without further treatment.

Few environmental factors have been identified that can be directly linked to outbreaks of the disease. In a latent carrier population, various stress factors such as treatment against salmon lice, cestodes or infectious diseases may be followed by disease outbreaks some 2-3 weeks later.

Due to lack of sufficient information on the ISA virus, the diagnostic procedures for ISA are still based upon clinical, pathological, histopathological and haematological changes.

The incidence of ISA has been greatly reduced by implementation of general legisatory measures regarding movement of fish, mandatory health control, and slaughterhouse and transport regulations, as well as specific measures including restrictions on affected, suspected and neighbouring farms, epizootiological studies, enforced sanitary slaughtering, generation segregation ('all in/all out'), and disinfection of offals and wastewater, etc. from fish slaughterhouses.
INTRODUCTION

Infectious salmon anaemia (ISA) is an infectious disease in Atlantic salmon (Salmo salar).

The infective agent is possibly a virus but its real nature has not yet been identified. ISA has so far only been described in Norway. Atlantic salmon is the only fish species known to be susceptible to ISA, but the ISA agent may survive for some time in sea trout (Salmo truttae) and rainbow trout (Onchorhynchus mykiss). The ISA agent has not been shown to survive in turbot (Psetta maxima), ballan wrasse (Labrus bergylta), sea bass (Dicentrarchus labrax), or cod (Gadus morhua).

DIAGNOSTIC PROCEDURES

There are no established methods for identifying the agent, and diagnosis has to rely upon identification of typical pathological changes. None of the described lesions are pathognomonic to ISA. The following requirements must be fulfilled when making the diagnosis:

- typical macroscopic findings
- typical histological findings
- typical haematological findings.

1. TYPICAL MACROSCOPIC FINDINGS

Necessary finding

- Dark livers
  
  Not necessarily present in all individuals, but there have to be some fish with dark livers. Livers can alternatively become yellow with haemorrhagic spots or be pale.

Supportive findings

- Pale gills and heart
  
  Result of anaemia

- Ascites
  
  Always present, early sign

- Enlarged spleen
  
  Always present, early sign

- Visceral fat petecchiae
  
  Always present

- Dark foregut
  
  Sometimes present

All these findings are typical of ISA, with some variation with regard to severity. The more of them that are observed, the more confident one can be regarding the diagnosis. Dark livers is an absolute criterion, as this finding is the one most specific to ISA. Dark livers are also seen with cardiomyopathic syndrome (CMS), which is distinguished from ISA by typical gross and histological heart lesions.
2. **TYPICAL HISTOLOGICAL FINDINGS**

**Necessary finding**

Multifocal haemorrhagic hepatic necroses, that may become confluent to give the changes a 'zonal' appearance, leaving areas around large veins intact (late stage of disease development).

**Supportive findings**

Focal congestion and dilatation of hepatic sinusoids, sometimes with distribution as described for the necroses (early stage). Rupture of sinusoidal endothelium with presence of erythrocytes within the space of Disse (early sign).

Findings described as supportive are present in early stages of disease development at haematocrit values of 15-25. The ISA-typical liver changes are observed at heamatocrits below 10. Comparable although not identical liver changes (usually without rupture of endothelium) may be seen in advanced stages of CMS.

ISA is most frequently diagnosed in springtime. Mortality may be low for months after introduction of ISA, until an 'outbreak' occurs. Necropsy and examination of a large number of diseased or dead fish increase the probability of detecting ISA.

Always suspect ISA when extremely dark livers are observed.

3. **TYPICAL HAEMATOLOGICAL FINDINGS**

**Necessary finding**

Haematocrit <10.

**Supportive finding**

Blood smears with degenerate and vacuolised erythrocytes, and presence of erythroblasts with irregular nuclear shape. Reduced proportion of leucocytes relative to erythrocytes, the highest reduction among lymphocytes and thrombocytes. Reduction of several plasma parameters, except for some enzymes indicating liver damage, and for electrolytes (fish in seawater).

A haematocrit below 10 is not a unique finding for ISA. Fish with ulcerations and fish suffering from erythrocytic inclusion body syndrome (EIBS), may regularly demonstrate haematocrit values this low.

4. **VIROLOGICAL EXAMINATION**

These have not yet been established for routine diagnostic purposes.
5. Zoosanitary Precautions

- Sanitary slaughtering
- Cleaning and disinfection of farm premises and equipment
- Restricted movement of live fish
- Disease surveillance
- Disinfection of offal/waste water from fish slaughterhouses.

REFERENCES


Chapter 11

Epizootic ulcerative syndrome (No OIE number)

Summary

Epizootic ulcerative syndrome is an epizootic infectious disease of wild and farmed fresh and brackish water fish. The disease is currently extending through South East to South Asia and is believed to be the same disease as red spot disease in Eastern Australia, and probably the same as mycotic granuloma in Japan.

The disease occurs in almost all freshwater fish species and to a lesser extent in brackish water fish. EUS is more prevalent in snakeheads (Channidae) and barbs (Puntius spp.). Tilapias seem to be completely resistant. Chinese and European carp are rarely affected.

The primary causative agent of the disease appears to be a fungus of the genus Aphanomyces, recently termed Aphanomyces invadens. However, the opportunistic bacteria Aeromonas hydrophila and Aeromonas sobria and a number of viruses, including two distinct groups of the rhabdoviridae and a retrovirus, may also be involved in the pathogenesis.

Diagnosis is based on clinical signs and histological evidence of the typical aggressive invasiveness of the non-sporing hyphae of the fungi, in the context of heavy losses. Isolation of the fungus allows its characteristic growth profile to be used as an aid to its speciation.

Control of EUS is almost impossible in the context of fish movements since it seems to move between watersheds via wild species. Regulation of tropical fish importation should control its movement to islands but on land masses, spread of the disease appears inevitable.

During outbreaks, liming of water and improvement of water quality, together with removal of infected fish, is often very effective.

Introduction

Epizootic ulcerative syndrome (EUS) is an epizootic infectious disease of great importance in wild and farmed species of fresh and brackish water fish. The disease extended its range from Australia, where it was previously called red spot disease (RSD), through Papua New Guinea to South East Asia and into South Asia, where it has reached as far south as Sri Lanka. It is now
approaching Pakistan.

The most probable primary causative agent is the fungus *Aphanomyces invaderis*, which causes severe liquefactive necrosis of muscle tissue as it invades the body, extending down into the visceral organs. Affected fish usually also suffer from severe bacterial septicemia, involving a variety of opportunistic pathogens. The most frequently found bacterial pathogens are *Aeromonas hydrophila* or *Aeromonas sobria*. A variety of parasites have also been found among diseased fish, but their presence is inconsistent. An associated virus infection is also frequent, involving rhabdoviridae or myxoviridae, but retroviruses have also been isolated from affected populations. By itself the fungus cannot normally invade fish and some co-factor such as severe environmental stress or a virus infection is postulated as the initiating factor to this complex and exceedingly important disease.

Almost all freshwater fish species native to a particular area and a number of brackish water species are susceptible to EUS. Snakeheads (*Channidae*) and barbs (*Puntius* spp.) are particularly susceptible. Chinese and European carp are rarely affected and tilapias seem to be completely resistant.

**CLINICAL SIGNS AND PATHOLOGY**

The initial feature is usually mass mortality, with distinct dermal lesions and ulcers, of fish in a water body. The surviving fish will show lesions and ulcers of varying degrees of severity. They may appear as red spots, blackish burn-like marks, or deeper ulcers with red centres and white rims. Some fish, especially snakeheads, survive a long time with such ulcers which may erode down to the vertebrae or so deep as to expose the brain or abdominal viscera.

Histologically, in less severely affected fish, typical non-sporulating, centrally invasive very delicate fungi can be observed encompassed within a granulomatous coating of epitheloid cells, relentlessly extending deep into the visceral organs such as the kidney and liver, after it has spanned the musculature.

**DIAGNOSIS**

Diagnosis is based on clinical signs and histopathology. So far, there are no specific diagnostic tests available.

The fungus can, with some difficulty, be isolated and cultured. Apart from its typical vulnerability to temperatures above 30°C, it is very similar to non-pathogenic opportunistic *Aphanomyces* spp., which readily contaminate the surface of affected fish and often interfere with isolation attempts. Experimentally, the fungus has also been shown to be pathogenic to salmonids, after intramuscular injection of spores, with similar pathology and mortality.
CONTROL

The control of EUS in wild populations is an impossible task. However, in small closed water bodies, the disease can be effectively controlled by liming of water and improvement of water quality together with removal of infected fish.

REFERENCES


CHAPTER 12
BACTERIAL KIDNEY DISEASE
(B408)

SUMMARY

Bacterial kidney disease (BKD) is a chronic infection with a protracted course and an insidious nature. Fish of the Salmonidae family are clinically susceptible, in particular those of the Oncorhynchus (Pacific salmon) genus. The diagnosis depends on increased low mortality, clinical classical and pathological changes associated with the disease as well as histopathological granulomatous changes, and isolation of Renibacterium salmoninarum in cysteine enriched media (KDM2 - SKDM) from the lesions or from asymptomatic (latent) carriers of the pathogen.

Identification of the agent. Isolation of R. salmoninarum may be achieved by streaking material from diseased fish onto KDM2 or its selective analogue SKDM plates with subsequent incubation period at 15-18°C. Maximum incubation time reported for visible growth is 8 and 12 weeks for KDM2 and SKDM respectively, but growth is usually obtained after 3 weeks. Identification is made according to morphological criteria, staining reactions, catalase and oxidase production, enzyme profiles (API-Zym) as well as immunological techniques (agglutination, FAT, IFAT, ELISA).

Requirements for biological products. Several papers in the literature report vaccination trials carried out against BKD. But no commercial vaccine is presently available. Although some success could be claimed experimentally, all the trials conducted in the field resulted in rather questionable protection.

A. DIAGNOSTIC TECHNIQUES

Bacterial kidney disease (BKD) is caused by Renibacterium salmoninarum, a coryneform Gram positive bacterium which is the sole species belonging to the genus Renibacterium and has been reported to occur in North America, Japan, Western Europe and Chile. Its economic importance for salmonid husbandry, and especially for Pacific salmons (Oncorhynchus spp.), results from this wide distribution both in fresh and saline environments, from its chronicity which does not allow the disease to be suspected before late clinical or debilitating manifestations, from its vertical transmission through sexual products, and from the inefficacy of the main therapeutical compounds used in treating fish. The special nutritive requirements and fastidious growth of the bacterium make its diagnosis difficult. Although health control presently appears to be the best way to control the disease, and although different methods have been suggested for
improving the detection of the agent in infected fish populations, there is not yet general agreement on the respective value of these methods.

The overt disease only appears in advanced cases of infection, when the fish have completed their first year of life. Exophthalmia and abdominal distension resulting from the impairment of the excretory function can be associated with superficial lesions and haemorrhagia. Internal pathology is more typical. Systemic granulomatous lesions can be found in all the organs, but develop especially in the kidney. Greyish necrotic abscesses tend to multiply and merge until the diffuse granulomatosis results in enlargement and necrosis of the whole kidney, which appears swollen and bloated with irregular greyish areas. The condition must be distinguished from proliferative kidney disease (PKD), in which the kidney hypertrophy is not associated with any discolouration, and from nephrocalcinosis, in which only the urinary conducts are impaired and exhibit a white porcelain colour. In doubtful cases, microscopic observation of smears or prints of kidney samples, stained with Gram or metachromatic dyes (toluid blue, thionin), allows visualisation of large numbers of small bacteria that must not be confused with the melanin granules also commonly present in kidney tissues. In any case, clinical diagnosis provides only a suspicion of BKD, as other Gram positive bacteria, namely lactic bacteria, have been demonstrated to produce similar infections in salmonids. Diagnosis ought to be confirmed by laboratory procedures.

1. **Identification of the agent**

Specimens can be taken from dead and preferably moribund fish showing lesions that give reasons for suspicion of BKD. The diagnostic procedure should follow the sequence: direct microscopy of stained smears (Gram, PAS, Ziehl-Neelsen) from lesions in kidney or other internal organs, possibly direct detection of antigens in tissues samples using specific antisera against *R. salmoninarum*, inoculation of material onto suitable medium for bacterial growth and identification.

Although the fastidious growth of *R. salmoninarum* and its special requirements for serum and cysteine make its culture difficult, whenever possible culturing should be used for confirmation. Special media (KDM2, SKDM) and procedures have been developed to improve the isolation of the bacterium, which is usually obtained after two to three weeks. Morphological criteria, staining reactions, catalase and oxidase production, and enzyme profiles in API-Zym panels are useful.

1.1. **Isolation and bacteriological identification**

   a) **Sampling**

   Tissue samples for diagnosis and identification of *R. salmoninarum* should be taken aseptically from the lesions in kidneys or other organs. When no lesions are present the kidney should be preferred
for sampling, but in mature females the coelomic fluid may also represent a convenient material.

For routine controls for detecting infected individuals in a population a sufficient number of fish must be sampled.

b) Isolation

*R. salmoninarum* is a fastidious growing organism which requires prolonged incubation (2 to 12 weeks at 15°C) to produce colonies. Cysteine and serum are requisite factors, and different media or ingredients have been proposed to improve its growth or reduce the development of associated microorganisms. Two of these special media are currently used:

**Kidney disease medium 2 (KDM2)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-cysteine (chlorhydrate)</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Tryptone</td>
<td>1 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.05 g</td>
</tr>
<tr>
<td>Agar</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Adjust the pH to 6.5-6.8 with NaOH, distribute in flasks or tubes and autoclave for 20 min at 120°C. Can be stored for 1 month at 4°C.

Regenerate prior to use and add 5-10% fetal calf serum.

**Selective kidney disease medium (SKDM)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-cysteine (chlorhydrate)</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>0.005 g</td>
</tr>
<tr>
<td>Tryptone</td>
<td>1 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.05 g</td>
</tr>
<tr>
<td>Agar</td>
<td>1 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Adjust to pH 6.8 with NaOH and autoclave for 15 min at 121°C. Cool to approximately 48°C, and add 10% fetal calf serum, and the following components, previously filter sterilised (0.22 μm):

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-cycloserine</td>
<td>0.00125 g</td>
</tr>
<tr>
<td>Oxolinic acid</td>
<td>0.00025 g</td>
</tr>
<tr>
<td>Polymyxin B sulphate</td>
<td>0.0025 g (final concentrations)</td>
</tr>
</tbody>
</table>

Dishes with isolation medium are dried at room temperature for 24-48 h, inoculated with 0.1 to 0.2 ml drops of infectious material, and incubated at 15°C in plastic bags or moist chambers when the absorption is complete.
Antibiotic supplementation of KDM2 medium may reduce the problems with fast-growing organisms (bacteria and fungi), but also inhibit _R. salmoninarum_ itself. This supplementation must thus be done carefully. Another possibility is to inspect the dishes regularly at intervals of 2-3 days, and aseptically remove the colonies produced by fast-growing organisms. In order to respect the viability of the KD bacterium, Evelyn recommends the preparation of tissue suspensions in physiological saline 9 p.1000 enriched with peptone 1 p.1000.

When a stock culture of _R. salmoninarum_ is already available, it is possible to take advantage of a "satellitism" phenomenon described by Evelyn for accelerating the growth of the isolates. A heavy suspension of the laboratory feeding strain is dropped onto the centre of the plate, and the samples to be tested are inoculated in the periphery. The growth rate and the colony size of the isolates are noticeably increased. The growth enhancement may also be achieved by adding 1.5% v/v sterile spent KDM2 broth to the medium.

c) Characteristics

After sufficient time of incubation on KDM2 and SKDM, _R. salmoninarum_ produces white or creamy shiny smooth, round, raised, entire, pin point to 2 mm colonies. Bacteria from diseased fish will on the average produce visible colonies after 2 to 3 weeks, however up to 8 weeks have been reported for initial growth on KDM2, and 12 weeks on the selective medium SKDM. Old cultures may achieve a granular or crystalline appearance. Transverse sections through such colonies will reveal the presence of Gram positive rods in a crystalline matrix. The crystalline material is thought to be cysteine precipitated from the medium. No growth occurs on blood agar medium without cysteine supplement or trypticase yeast agar. For some strains a uniformly turbid growth occurs in broth, but for others a sediment may develop. _R. salmoninarum_ appears as small (0.3-1.5 x 0.1-1 μm) Gram positive, PAS positive, asporogenous, non-motile, non-acid fast rods, frequently in pairs, short chains or pleomorphic forms as "Chinese letters", especially in fish tissue.

_R. salmoninarum_ is catalase positive and oxidase negative. Its phenotypic characteristics have been established using API-Zym systems and conventional tests (Tables 1 and 2). However the slow growth of the organism does not render such tests very useful in practice, and serological methods are more usually employed to confirm the identity of the isolated strains.
### Table 1

Distinguishing profiles of Gram-positive bacteria morphologically similar to *R. salmoninarum*, obtained with API-Zym

<table>
<thead>
<tr>
<th>Character</th>
<th>Control</th>
<th>Alkaline phosphatase</th>
<th>Esterase (butyrate)</th>
<th>Esterase (caprylate)</th>
<th>Lipase (myristate)</th>
<th>Leucine arylamidase</th>
<th>Valine arylamidase</th>
<th>Cystine arylamidase</th>
<th>Trypsinase</th>
<th>Chemotrypsinase</th>
<th>Acid phosphatase</th>
<th>Phosphoamidase</th>
<th>1-galactosidase</th>
<th>1-glucosidase</th>
<th>1-glucuronidase</th>
<th>N-acetyl-1-talosidase</th>
<th>1-mannosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taxon (and source of strains)</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>11</td>
<td>12</td>
<td>13</td>
<td>14</td>
<td>15</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Actinomyces viscosus (ATCC 15887)</td>
<td></td>
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<tr>
<td>Corynebacterium acnes (NCTC 737)</td>
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<td></td>
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<tr>
<td>C. pyogenes (NCTC 5224)</td>
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<tr>
<td>C. xerosis (NCTC 7929)</td>
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<tr>
<td>Lactobacillus spp. (pseudokidney disease, 3 isolates)</td>
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<tr>
<td>Mycobacterium aqua (Körmenty)</td>
<td></td>
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<td></td>
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<tr>
<td>Myc. fortuitum (Körmenty)</td>
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<tr>
<td>Myc. marinum (Körmenty)</td>
<td></td>
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<tr>
<td>Mycobacterium sp. (Ashburner, SC 744)</td>
<td></td>
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<td></td>
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<tr>
<td>Nocardia asteroides (ATCC 14759)</td>
<td></td>
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<td></td>
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<tr>
<td>Renibacterium salmoninarum (46 isolates)</td>
<td></td>
<td></td>
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<tr>
<td>Rothia dentocariosa (ATCC 17931)</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Presumptive coryneform (laboratory isolate 1988)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
</tbody>
</table>

Modified (extracted) from Table 15.5 of reference 1.
### Table 2

**Characteristics of Renibacterium salmoninarum**

<table>
<thead>
<tr>
<th>Character</th>
<th>Response</th>
<th>Character</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Production of:</strong></td>
<td></td>
<td><strong>Response</strong></td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>+</td>
<td>Acid phosphatase</td>
<td>-</td>
</tr>
<tr>
<td>Starch</td>
<td>-</td>
<td>Alkaline phosphatase</td>
<td>-</td>
</tr>
<tr>
<td>Testosterone</td>
<td>+</td>
<td>Butyrate esterase</td>
<td>-</td>
</tr>
<tr>
<td>Tributyrin</td>
<td>+</td>
<td>Caprylate esterase</td>
<td>+</td>
</tr>
<tr>
<td>Tween 40</td>
<td>+</td>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Tween 60</td>
<td>+</td>
<td>Chymotrypsinase</td>
<td>-</td>
</tr>
<tr>
<td>Testosterone</td>
<td>-</td>
<td>Cystine arylamidase</td>
<td>-</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>-</td>
<td>(\alpha)-fucosidase</td>
<td>-</td>
</tr>
<tr>
<td>Xanthine</td>
<td>-</td>
<td>(\alpha)-galactosidase</td>
<td>-</td>
</tr>
<tr>
<td>Acid production from sugars</td>
<td>-</td>
<td>(\beta)-galactosidase</td>
<td>-</td>
</tr>
<tr>
<td>pH 7.8</td>
<td>+</td>
<td>(\beta)-glucosaminidase</td>
<td>-</td>
</tr>
<tr>
<td>Bile salts (0.025% w/v)</td>
<td>-</td>
<td>(\alpha)-glucosidase</td>
<td>+</td>
</tr>
<tr>
<td>Crystal violet (0.0001% w/v)</td>
<td>-</td>
<td>(\beta)-glucosidase</td>
<td>+</td>
</tr>
<tr>
<td>Methylene blue (0.001% w/v)</td>
<td>-</td>
<td>(\beta)-glucoronidase</td>
<td>-</td>
</tr>
<tr>
<td>Nile blue (0.0001% w/v)</td>
<td>+</td>
<td>Leucine arylamidase</td>
<td>-</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>+</td>
<td>(\alpha)-mannosidase</td>
<td>-</td>
</tr>
<tr>
<td>Potassium thiocyanate (1% w/v)</td>
<td>-</td>
<td>Myristate esterase</td>
<td>-</td>
</tr>
<tr>
<td>Sodium chloride (1% w/v)</td>
<td>+</td>
<td>Oxidase</td>
<td>+ (poor)</td>
</tr>
<tr>
<td>Sodium selenite (0.01% w/v)</td>
<td>+</td>
<td>Tryptsinase</td>
<td>-</td>
</tr>
<tr>
<td>Thallous acetate (0.001% w/v)</td>
<td>-</td>
<td>Valine arylamidase</td>
<td>-</td>
</tr>
<tr>
<td><strong>Degradation of:</strong></td>
<td></td>
<td><strong>Utilisation of:</strong></td>
<td></td>
</tr>
<tr>
<td>4-umbelliferyl (4MU)-acetate</td>
<td>+</td>
<td>4MU-butyrate</td>
<td>+</td>
</tr>
<tr>
<td>4MU-butrate</td>
<td>+</td>
<td>4MU-(\beta)-D-cellobiopyranoside monohydrate</td>
<td>-</td>
</tr>
<tr>
<td>4MU-(\beta)-D-cellobiopyranoside monohydrate</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4MU-elaidate</td>
<td>-</td>
<td>4MU-(\alpha)-L-arabinopyranoside</td>
<td>-</td>
</tr>
<tr>
<td>4MU-(\alpha)-L-arabinopyranoside</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4MU-2-acetamido-2-deoxy-(\beta)-D-galactopyranoside</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4MU-(\beta)-L-fucopyranoside</td>
<td>-</td>
<td>4MU-heptanoate</td>
<td>+</td>
</tr>
<tr>
<td>4MU-(\beta)-L-fucopyranoside</td>
<td>-</td>
<td>4PU-heptanoate</td>
<td>+</td>
</tr>
<tr>
<td>4MU-oleate</td>
<td>+</td>
<td>4PU-laurate</td>
<td>+</td>
</tr>
<tr>
<td>4MU-oleate</td>
<td>+</td>
<td>4PU-nonanoate</td>
<td>+</td>
</tr>
<tr>
<td>4MU-palmitate</td>
<td>+</td>
<td>4PU-palmitate</td>
<td>+</td>
</tr>
<tr>
<td>4MU-propionate</td>
<td>+</td>
<td>4PU-propionate</td>
<td>+</td>
</tr>
<tr>
<td>Lecithin</td>
<td>-</td>
<td>4MU-propionate</td>
<td>+</td>
</tr>
</tbody>
</table>
1.2. Antigen detection and identification by serological methods

Although some risk of cross-reactions has been reported to occur with other bacteria the antigenic homogeneity of *R. salmoninarum* is a characteristic which favours the use of specific antisera in identification procedures. Such techniques have been applied to direct detection of the KD bacterium in infected fish and are favoured by the existence of a soluble, heat-stable, major antigen (antigen F) abundantly released in infected tissues. The amino acid and DNA sequences of the main soluble antigen have been determined. Rapid immunological tests have found broad application in monitoring disease outbreaks and screening of brood fish to sort out the least infected fish for propagation purposes. Slide agglutination and coagglutination are now supplemented by direct or indirect fluorescent antibody tests (FAT and IFAT) and by enzyme-linked immunosorbent assays (ELISA), using either specific sera or monoclonal antibodies. A diagnostic ELISA kit approved by the United States Department of Agriculture has recently been developed and commercialised.

*a) Serum*

It is of course necessary to obtain good antisera. When polyclonal antisera are required this can be achieved by immunising rabbits. One of the most effective methods to obtain sera with fair antibody titers is the multispot intradermal schedule described in Chapter 1 (C.3.2.).

Monoclonal antibodies have also been produced against a major surface protein which is a part of the antigen, antigen F, mentioned above. However, the epitopes the Mabs react with are not heat stable. Mabs are currently used in a diagnostic ELISA kit recently commercialised (1.2.d.).

In certain cases, purified immunoglobulins, or at least enriched fractions, may prove useful and should be prepared by classical methods.

*b) Agglutination test*

Isolated organisms can be identified quickly using slide agglutination tests. Some colonies are gently mixed into a drop of sterile saline, on a clean glass slide, and a drop of antiserum is added. The agglutination of the bacteria can be observed by comparing a similar suspension in normal rabbit serum as control. The appropriate dilution of reacting serum will have been previously determined by testing a control *R. salmoninarum* strain in twofold dilutions of this serum.

In order to improve and facilitate the agglutination, coagglutination
using *Staphylococcus aureus* (Cowan I strain) sensitised with specific immunoglobulins has been described. *Staphylococcus*, cultivated 24 h at 37°C in liquid medium, is washed and successively treated by formalin 0.5% and heating 30 min at 100°C. The suspension is adjusted to 10% (v/v) in phosphate buffer and added to 0.1 volume of anti-*Renibacterium* Ig. Incubation is 3 h at 25°C, with periodic stirring. The sensitised bacteria are then centrifuged and adjusted to the initial volume after washing 3 times. Agglutination tests are performed on glass slides maintained in moist chambers and read after 30, 60 and 120 min.

According to the authors this test provides better and quicker results when the antigen to be tested is heated 30 min at 100°C. It appears, therefore, that the specific reaction is supported mainly by the heat stable antigen known as antigen F. As antigen F is soluble and widely released in infected tissues coagglutination has been applied to direct detection of *R. salmoninarum* in kidney tissue preparations. Despite its low requirements in materials it does not seem to ensure as good a sensitivity as immunofluorescence or ELISA tests.

c) Immunofluorescence

Direct and indirect immunofluorescent antibody tests (FAT, IFAT) have been commonly used in demonstrating the presence of the bacterium in tissues. In the first case, rabbit or goat anti-BK bacterium immunoglobulins conjugated to fluorescein are used. In the second case, heterospecific Igs prepared against the anti-BKD serum are labelled. Tissue smears prepared from infected kidney are air-dried and heated 2 min at 60°C before being incubated with the reagents and observed microscopically through UV light. This technique has proved sensitive enough to permit detection of carrier fish, as well as to control eggs and ovarian fluids of mature fish selected as breeders. However, an interlaboratory comparison of the IFAT revealed that reproducibility was poor for the lowest levels of infection and moreover several infections were missed as shown by parallel culture. To obviate risks of cross-reaction with other coryneform bacteria the alternative use of monoclonal antibodies against specific determinants has been recommended.

d) Enzyme-linked immunosorbent assay (ELISA)

ELISA can be used for the detection of soluble antigen extracted from infected tissue and is probably the most sensitive method. It allows testing of large numbers of samples and is specially well adapted to the requirements of health certification. The most common technique is the double antibody sandwich one: specific antibodies are coated onto the polystyrene or PVC surface of
microplate wells, and samples containing the antigen, conjugate serum, and substrate are successively added.

Two different ELISA procedures for soluble antigens have been used. One uses polyclonal antibodies reacting with heat stable antigenic determinants, while the other uses monoclonal antibodies towards heat labile antigenic determinants. The heat treatment limits cross reactions, but not completely, as shown by cross reactions with feather meal used in fish feed. The monoclonal antibodies appear very specific, but the samples must be kept cold or frozen after sampling. Diseased and subclinically infected fish give ELISA reactions that are clearly distinct from uninfected fish. However, the exact positive threshold is difficult to set as representative non-infected fish, for determining normal background variation, are not always easy to find.

Problems linked to cross-reactions are the same as in immunofluorescence and could be solved definitively using monoclonal antibodies. A diagnostic ELISA kit approved by the US Department of Agriculture has been developed by DiagXotics Inc. (126 Old Ridgefield Rd, Wilton, CT 06897, USA). All the materials and reagents are supplied to perform quantitative tests which run exactly as previously described, except for two modifications: microplates are sold ready for use, coated with a first monoclonal antibody. After sample distribution a second Mab of different specificity and conjugated to biotin is added. This allows the sensitivity to be improved by using the avidin-biotin system, streptavidin being conjugated to the horse radish peroxidase added in the subsequent step. Control antigen solutions are also supplied to allow a standard curve to be established.

2. Serological tests

Serological tests are not used routinely for the diagnosis of BKD. In fact, the development of the antibody response does not correlate clearly with the course of the infection, and several authors disagree with the adoption of serology as a health inspection method.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

No commercial vaccines against BKD are available, but they are greatly needed as *R. salmoninarum* responds poorly to medical treatment. There is evidence that *Renibacterium* under some conditions elicits an immune response in fish, and there are some reports of vaccination in the literature. The protective ability of a vaccine is however questionable and basically one of the problems is the intracellular nature and vertical transmission of the agent. This will require a much better understanding of the pathogenesis of *R. salmoninarum*, and how it
interacts with the immune system of the fish. It may only be possible to
vaccinate non-exposed fish, as exposed and subclinically infected fish may not
respond to vaccination. When developed, suitable vaccines should give
protection to vaccinated fish throughout the whole lifecycle in fish farms under
normal conditions.

For the moment, health control based on specific detection strategies and
elimination of carrier or diseased fish still represents the best way to combat
BKD.

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   Horwood Ltd., 384 pp.

   reaction in the indirect fluorescent antibody test for Renibacterium
   5, 8-9.


   salmoninarum, the causative agent of bacterial kidney disease, in rainbow
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   Fish Biol., 30, 327-334.

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CHAPTER 13
ENTERIC SEPTICAEMIA OF CATFISH
(Edwardsiellosis)
(B414)

SUMMARY

Enteric septicaemia of catfish or edwardsiellosis is a bacterial disease first described in channel catfish in the USA and caused by Edwardsiella ictaluri, a Gram negative motile bacillus belonging to the family Enterobacteriaceae. There are indications that E. ictaluri consists of more than one serotype, recent isolates from Thailand being different from most of the US isolates.

All strains of E. ictaluri are reported virulent and cause high mortality in warm water fish species, in particular fishes belonging to the family Ictaluridae. This disease condition is also known as enteric septicaemia.

Identification of the agent: The identification of E. ictaluri is based on the isolation of the causative agent and characterisation by biochemical tests. E. ictaluri can easily be differentiated from E. tarda by its inability to produce indole and H₂S. E. tarda produces both. Additionally the two species do not cross react serologically.

Serological tests. For rapid results, and due to its antigenic homogeneity, E. ictaluri may be identified by means of slide agglutination, the indirect fluorescent antibody technique (IFAT), or the enzyme-linked immunosorbent assay (ELISA). Serologically, the antibody response against the agent may be assessed by direct quantitative microagglutination, passive haem-agglutination or ELISA using polyclonal or monoclonal reagents. However the ELISA and certain other techniques are still limited to specialised research laboratories.

Requirements for biological products: Since E. ictaluri is immunogenically active, production of vaccines soon appeared feasible, and indeed, a commercial bacterin has appeared on the US market. The vaccine consists of inactivated bacteria and may be administered by immersing young fish in properly diluted preparations. Boosters can be given orally prior to the disease peak of autumn. However, international standards are not yet available.
A. DIAGNOSTIC TECHNIQUES

Enteric septicaemia of catfish (ESC) is caused by the bacterium *Edwardsiella ictaluri* which belongs to the Enterobacteriaceae family. Since its first recognition in 1976 in channel catfish (*Ictalurus punctatus*) from Alabama and Georgia, USA, ESC has become the foremost bacterial disease problem in the commercial catfish industry in the South Eastern United States. However, *E. ictaluri* has also been reported from *Clarias batrachus* in Thailand and from several ornamental species, and the susceptibility of other species including salmonids has been shown experimentally. There are indications that *E. ictaluri* consists of more than one serotype, recent isolates from Thailand being different from most of the US isolates.

The natural reservoir of *E. ictaluri* seems to be the intestine of the fish, from which the faeces disseminate the organism into the environment. *E. ictaluri* can survive in pond bottoms for an extended period of time and these may thus be a source of infection. The disease occurs only within a limited temperature range, from 18 to 28°C. This results in seasonal fluctuations, spring and autumn being the critical periods. Other environmental factors (water quality, organic compounds, stocking density and stress factors) are known to modulate the virulence of the agent. In spite of these characteristics *E. ictaluri* is generally considered to be a true obligate parasite.

The clinical signs may differ according to the fish species. Two clinical forms of ESC have been described. Most frequently the infection seems to begin in the olfactory sacs through the nasal route, and to progress slowly upwards to generate granulomatous inflammation in brain tissues. This chronic meningoencephalitis can account for behavioural manifestations, with alternating listlessness and chaotic swimming. In typical cases, "hole in the head", a deep erosion of the dorso-cranial part of the head which may extend to the frontal bone is observed. Although general infection can result from such chronic forms, it is more frequently associated with acute septicemia following enteritic infection. Then, as in many other bacterioses, skin petechia and haemorrhages are observed around the mouth, on the throat and at the base of the fins. Anaemia and exophthalmia are frequent. Internally, haemorrhages and necrotic foci are scattered in the liver and other internal organs. Enteritis, systemic oedema, accumulation of ascitic fluid in the general cavity and enlargement of the spleen are not really specific. Histological examination reveals a systemic infection of all organs and skeletal muscles, with diffuse granulomatosis.

The epidemiology and pathogenesis of the disease are poorly documented. However, there are indications that *E. ictaluri* is able to survive in sediments for some months. The natural reservoir of the organism seems to be the intestine of fish, from which it disseminates with faeces into the environment.

Disease outbreaks have been controlled by management procedures to reduce stress, or by feeding the catfish with oxytetracycline (Terramycin®) at the rate
of 25-30 mg/kg fish/day for 10 days. Potentiated sulphonamides have also been used to control *E. ictaluri* outbreaks, but resistances to these products appeared recently. It seems that vaccination might now be tried as a preventive treatment.

1. IDENTIFICATION OF THE AGENT

1.1. Sampling

Bacteriological sampling from freshly dead or moribund fish, taken aseptically from either spleen, liver or kidney tissue, can be streaked onto blood agar plates, brain heart infusion agar or nutrient agar plates. The bacterium grows rather slowly but does not require special nutrients. Optimal temperature for incubation is 25°C.

For routine sampling of fish populations, the rules are the same as those defined for other fish infectious agents (cf. Chapter 1 Part B.1.2. and ref. 13).

1.2. Characteristics

The bacterium grows rather slowly but does not require special nutrients, and its biochemical characteristics appear homogeneous in all isolates.

Following incubation at 26°C for 48 hours, *E. ictaluri* appears as smooth, circular (1-2 mm diameter), slightly convex with entire edges, non-pigmented colonies. It is a Gram negative rod, measuring 0.75-2.5 μm, weakly motile by means of a peritrichous flagellation. This motility generally fails to be observed at 37°C. Growth at a higher than optimal temperatures may result in development of avirulent strains.

After having obtained the bacterium it should be identified by biochemical and serological characteristics. Table 1 shows some of the characteristics of the species and biogroups of the genus *Edwardsiella* as given in Bergey's Manual of Systematic Bacteriology. The bacterium prefers to grow in low temperatures (25-30°C), and although biochemical characteristics can be studied at 37°C, variations can be found, namely in the motility and the production of gas from formate and glucose. *E. ictaluri* is biochemically less active than the other *Edwardsiella* species, but it appears homogeneous. No clearcut biotype variation is detected. *E. ictaluri* and *E. tarda* may be differentiated from each other biochemically by the production of indole and hydrogen sulphide (H₂S). *E. tarda* produces both, while *E. ictaluri* does not. The capacity of all isolates of *E. ictaluri* of degrading chondroitin sulfate might be an important virulence factor in the formation of the "hole in the head" lesions in affected fish.
Table 1
Differentiation of the species and biogroups of the genus *Edwardsiella*

<table>
<thead>
<tr>
<th>Characteristic acid production from:</th>
<th><em>E. tarda</em></th>
<th><em>E. hoshinae</em></th>
<th><em>E. ictaluri</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Biogroup 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Trehalose</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>-</td>
<td>+</td>
<td>(-)</td>
</tr>
<tr>
<td>Tetrathionate reduction</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Malonate utilisation</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Indole production</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>H₂S production in TSI</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Citrate (Christensen's)</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
</tr>
</tbody>
</table>

* Bergey's Manual of Systematic Bacteriology
** Weakly motile according to Hawkes, 1979.

1.3. Detection of the bacterial antigens by serological methods

In addition to well defined biochemical characteristics, there is a strong homogeneity in the antigenic structure of the species, which does not share any serological relationship with *E. tarda*. This confers a real value to serological methods for achieving rapid diagnosis. Slide agglutination with specific antisera against *E. ictaluri* as well as fluorescent antibody techniques and enzyme immunoassay methods have been used to provide confirmatory diagnosis. Specific sera are obtained using formalin killed bacterins to immunise rabbits according to classical standards, but in certain cases monoclonal antibodies may also be used.

a) Agglutination test

Isolated colonies are gently mixed into a drop of sterile saline on a clean glass slide, and a drop of antiserum is added. The agglutination of the bacteria can be evaluated comparing a similar suspension in normal rabbit serum as control. The appropriate dilution of reacting serum will have been previously determined by testing a control *E. ictaluri* strain in twofold dilutions of this serum.
b) **Specific immunofluorescence**

The indirect fluorescent antibody technique (IFAT) may be employed on bacterial smears, or smears from infected organs, for rapid confirmation of a clinical diagnosis. Smears are air-dried and heated 2 min at 60°C before being flooded and incubated for 5 min with specific rabbit antibody. They are washed in phosphate buffered saline pH 7.2, flooded for 5 min with heterospecific immunoglobulins prepared against the anti-*E. ictaluri* rabbit serum, and conjugated with fluorescein isothiocyanate. After rinsing, the slides are observed microscopically through UV light. Improvement of the technique by the use of monoclonal antibody has also been reported.

c) **Enzyme immunoassay**

An enzyme immunoassay adapted to the direct identification of the bacteria in tissues smears from infected fish has been described. Smears are prepared as for IFAT, and the first steps are similar. But the second incubation step (5 min) uses heterospecific immuno-globulin against rabbit antiserum, conjugated to horseradish peroxidase. A third incubation step with a substrate (DMOB Sigma) is performed for 10 min, and after washing and drying the smears are mounted in buffered glycerin and microscopically observed. It may happen that smears are too thick and produce non-specific retention of the staining. This can be solved by rinsing the smear again for 1 or 2 min in IN HCl.

2. **SEROLOGICAL TESTS**

Although antibody detection tests are rarely used for routine diagnostic purposes and are not yet approved as official procedures, they could be of great value for the mass control of large numbers of fish required with the development of health control policies. This is supported by the specificity of the bacterium and the demonstration of circulating antibodies against *E. ictaluri* in the serum of fish recovering from or surviving the disease.

2.1. **Microagglutination test**

Direct microagglutination, performed in 96-well round-bottom microplates as described for other bacterial pathogens, can provide quick quantitative data at minimal cost when high sensitivity is not specially required. It just needs a formalin-killed bacterial suspension prepared according to the usual techniques (i.e. formalin 3.5 p.1000 overnight) and adjusted at 5 x 10^8 ufc/ml. Twofold dilutions of the sera are made in physiological saline, so that the final volume is 0.25 µl/well. Antigen is added at 75 µl and the plates are incubated 2 h at 37°C and overnight at 4°C before reading. Controls include a rabbit
reference serum of previously established titre and antigen incubated in saline.

2.2. Passive haemagglutination

This technique has been described using *E. ictaluri* LPS (1 mg/ml in PBS, pH 7.2) passively coated on human O red blood cells at 4%. Tested sera must be absorbed with group O human red blood cells to remove non-specific agglutinins, and heated 30 min at 45°C before dilutions are done in veronal buffered saline. Coated blood cells are used at 1%. Incubation is 6 h at room temperature and overnight at 4°C. Controls include coated and uncoated red cells in buffer and coated cells in serum.

2.3. Indirect enzyme-linked immunosorbent assay (ELISA)

The ELISA method has also been proposed, but should be standardised to be adopted on a large scale. The best method seems to be to use soluble major antigen obtained by sonication to coat the microplate wells. The subsequent steps consist of diluting sera to be tested, adding conjugated anti-fish immunoglobulin serum (monoclonal antibody can be used), and the substrate. This technique could prove useful to follow the antibody response to *E. ictaluri* in fish subjected to vaccination programmes. But it needs to be further evaluated for accuracy and reproducibility of the results.

B. REQUIREMENTS FOR BIOLOGICAL PRODUCTS

*E. ictaluri* is known to induce an antibody response after natural disease or active immunisation. The possibility of protecting channel catfish populations by vaccination has been comprehensively studied, and reviewed by Plumb in 1988. However, no commercial vaccine against ESC was available until 1991, when an inactivated bacterin was licensed in the USA. The recommended procedure for use is in two steps: 1) immersion vaccination of young fish (1 litre of bacterin for 100 kg fish); 2) oral booster just prior to the critical period of autumn, feeding a medicated diet at 3% body weight for 5 days. The vaccine was marketed after three years of successful field trials, but it will be of course necessary to wait for large scale results before assessing its value in an anti-ESC strategy. Although it is generally produced and harvested according to norms given by the US Food and Drug Administration for the use of vaccines for fish, no international directives or standards have as yet been published.

REFERENCES


CHAPTER 14
PISCIRICKETTSIOSIS
(No OIE number)

SUMMARY

Piscirickettsiosis is a disease in salmonids caused by Piscirickettsia salmonis and first described in farmed coho salmon (Oncorhynchus kisutch, Walbaum). The disease was first described from Chile in 1989. P. salmonis is placed in the family Rickettsiaceae.

**Identification of the agent:** The identification of P. salmonis is based on isolation on CHSE-214 cell line of the causative agent with subsequent testing regarding characteristics for rickettsiae. The organism may easily be distinguished from Chlamydia, not demonstrating the characteristic chlamydia developmental cycle. The identity may also be confirmed by means of serological tests.

**Serological tests:** For a rapid result, the identity of P. salmonis isolated in cell cultures or observed in smears from pathological material may be confirmed by means of the fluorescent antibody test (FAT) or immunohistochemical methods with type-specific antiserum.

**Control measures:** The implementation of hygienic measures and control policy rules are the only control methods currently available.

INTRODUCTION

Piscirickettsiosis is a septicaemic condition in salmonids. The causative agent of the disease is a Rickettsia named Piscirickettsia salmonis. The disease has so far been described from Canada, Chile, Ireland, and Norway.

P. salmonis has been detected in coho salmon (Oncorhynchus kisutch), chinook salmon (O. tschawytscha), Sakura salmon (O. masou), rainbow trout (O. mykiss), pink salmon (O. gorbuscha), and Atlantic salmon (Salmo salar).

The first evidence of disease may be the appearance of small white lesions in the skin or shallow haemorrhagic ulcers. Affected fish appear dark and lethargic, hanging at the net sides. The major gross pathological changes are gill pallor, peritonitis, ascites, slightly enlarged spleen, swollen grey kidneys and liver with large pale necrotic lesions. Mortality is reported to be 30-90% in Pacific salmon.

The means by which the disease is transmitted is not understood. It may be transmitted horizontally in sea water, or vector-borne. The disease has primarily...
been reported as a problem in marine farming, but has also been described from fresh water farms.

Treatment with anti-bacterials may have some benefit, but is not optimal for controlling the disease. Eggs may be disinfected. Stamping out procedures may be used.

**DIAGNOSTIC PROCEDURES**

The monitoring for and diagnosis of piscirickettsiosis is based upon two methods: isolation of the rickettsial agent in cell culture, or detection in acridine orange (AO) or Giemsa stained tissue smears, followed by the FAT for further identification.

Infected fish material suitable for virological examinations is:

- **during overt infection**: kidney and liver.

**Sampling procedures**: See Chapter 1 Part B.

1. **STANDARD MONITORING METHODS FOR PISCIRICKETTSIOSIS**

1.1. **Isolation of *P. salmonis* in cell culture**

Cell line(s) to be used: CHSE-214 without antibiotics added.

*a) Preparation of tissue*

i) The kidney must be aseptically removed and transferred to a sterile container. No antibiotics can be used at any step in the isolation procedure. Tissues must be kept at 4°C or on ice until processed, and must not be frozen.

ii) Kidney tissue should be homogenised at 1:20 in antibiotic-free balanced salt solution (BSS) then, without centrifugation, further diluted 1:5 and 1:50 in antibiotic-free BSS for inoculation onto cell cultures. Final dilutions for use are 10⁻² and 10⁻³.

*b) Inoculation of cell monolayers*

i) A 10⁻² and 10⁻³ dilution of the organ homogenates should be inoculated on to cultured cells. The CHSE-214 cell line should be used for isolation. Cultures must be maintained in antibiotic-free medium.

ii) The diluted homogenate can be inoculated directly (0.1
ml/culture) into the antibiotic-free culture medium overlaying the cells.

iii) The cell cultures must be incubated at 15-18°C for 28 days and observed for the appearance of cytopathic effect (CPE). The rickettsial CPE consists of plaque-like clusters or rounded cells. With time, the CPE progresses until the entire cell sheet is destroyed.

iv) If no CPE occurs (except in the positive control) cultures should be incubated at 15-18°C for an additional 14 days.

1.2. Identification of *P. salmonis* in cell culture

a) Fluorescent antibody test (FAT)

i) The identity of rickettsiae isolated in cell culture or observed in AO stained smears must be verified by serological methods, e.g. FAT.

ii) Fluid from cell cultures showing extensive CPE can be spotted directly onto microscope slides and tested in the FAT.

iii) Tissue smears to be examined by FAT must be freshly prepared or stored at ≤-20°C. Preliminary results indicate that storage at temperatures ≥-4°C is not advisable.

1.3. Identification of *P. salmonis* in acridine orange tissue smears

a) Preparation of acridine orange (AO) stain (Lauer et. al., 1981)

The following stain formulation should be used: Add 20 mg AO powder to 190 ml of sodium acetate buffer (100 ml 1 M CH₃CO₂Na.3H₂O and 90 ml 1 N HCl). Adjust the pH to 3.5 with 1 N HCl. Store in a brown bottle at ambient temperature.

b) Preparation and observation of slides

i) Smears or impressions of the kidney, liver and spleen should be prepared, air dried, and fixed for five minutes in absolute methanol. The slides should then be flooded with AO stain for two minutes, rinsed with tap water, and air dried.

ii) Slides should be observed under oil with a fluorescence microscope equipped with the appropriate filter blocks.

iii) In AO stained preparations, the rickettsiae can appear bright red-orange or green in colour. Individual organisms are 0.5-
1.5 μm in diameter. They are pleomorphic, predominantly coccoid but also appear as rings, and pairs of curved rods are frequently observed.

2. **DIAGNOSTIC PROCEDURES FOR CONFIRMATION OF PISCIRICKETTSIOSIS IN SUSPECTED OUTBREAKS**

Confirmation of piscirickettsiosis can be achieved by any of the following methods:

2.1. Conventional isolation of *P. salmonis* in cell culture with subsequent serological identification as described in Section 1.

2.2. Detection in tissue smears using AO or Giemsa stain as described in Section 1.

**REFERENCES**


DISEASES OF BIVALVE MOLLUSCS

DISEASES NOTIFIABLE TO THE OIE

CHAPTER 15

DIAGNOSTIC TECHNIQUES: GENERAL INFORMATION

1. PRELIMINARY REMARKS

Diagnostic procedures for molluscan pathogens are limited, and histological examination is the conventional method applicable to these hosts. Consequently we can present in a single document all the techniques currently available for the diagnosis of diseases believed to be contagious. This presentation takes into account the performance of these techniques in terms of sensitivity, ease of use, and samples to be examined.

2. SAMPLING

2.1 Sampling points

For each zone referred to, a number of sampling points must be selected so as to maximise the chances of detecting pathogens. Account must be taken of parameters having an effect on the development of the pathogenic agents, such as stocking density, water flows, and the development cycle of the molluscs.

For a given zone at least three sampling points must be selected. The number of points must be increased for large zones containing several discrete areas of cultivation of the susceptible species.

Whenever possible, at least one sample must be taken from natural beds. Any molluscs showing abnormalities (abnormal growth, gaping shells) must be selected.

2.2 Sampling period and frequency

The frequency of inspection is based on the infection period, and inspections have to take place thereafter. Inspection periods must also take account of the transfer of molluscs, which generally takes place in spring and autumn. Sampling should therefore be carried out:

- once a year after the summer period (i.e. in September-October in the northern hemisphere) for the genera Marteilia, Haplosporidia and Perkinsus;
- twice a year (spring/autumn) for the genera Bonamia and Mikrocytos and for iridovirus.
2.3 Sampling size

During the initial two-year period which precedes the granting of approved status, the sample size for each sampling point is 150 or a sufficient number to ensure detection at a 95% confidence level of pathogen carriers at a prevalence of 2%.

During the subsequent years (maintenance of approved status), the sample size must be maintained at 150 to ensure detection at a 95% confidence level of pathogen carriers at a prevalence of 2%.

3. Shipment of Samples

All molluscs sampled must be delivered to the approved laboratory within 24 h after sampling. They must be packed in accordance with current standards in order to keep them in good condition. A label clearly stating the place of sampling and the health history (if any) must be attached to the sample.

4. Macroscopic Examination

The molluscs must be opened carefully so as not to damage the tissues, in particular the mantle, gills, heart and digestive gland. Anomalies and lesions of the tissues, as well as any shell deformities, shell-boring organisms and conspicuous mantle inhabitants, must be noted.

5. Examination of Stocks Where Abnormal Mortalities Occur

Whenever abnormal mortalities occur in stocks of bivalve molluscs an urgent investigation must be carried out to determine the aetiology. (An abnormal mortality is a sudden sizeable mortality [more than 15% of the stock] which occurs in a short time between two observations [15 days]. In the hatchery an abnormal mortality is a failure of successive productions of larvae coming from different broodstock.)

The sample taken must consist of 150 individual oysters and must be handled in accordance with the procedure defined for histological analysis. This technique must be used initially, before any other type of examination. The samples are fixed, preferably in Carson's fixative, which allows re-use of the sample for electron microscopy.
CHAPTER 16
BONAMIOSIS
(B431)

SUMMARY

Bonamiosis is a lethal infection of the blood cells of common oysters, accompanied by nonspecific branchial lesions. It is caused by two protozoa of the phylum Ascetospora, Bonamia ostreae and Bonamia sp.

The disease may occur throughout the year, and can be transmitted experimentally by cohabitation or inoculation.

Bonamiosis affects the following oysters: Ostrea edulis, O. angasi, O. conchaphila and Tiostrea chilensis. Its geographical distribution is: Spain, USA, France, Greece, Irish Republic, Italy, Netherlands, UK, New Zealand and Australia.

In the natural environment, diagnosis is possible after an oyster has spent 3-4 months in an infected area, and it is accomplished by histological techniques in sections or smears of infected organs.

For diagnosis, the rules for sampling are those stated in the general section (Chapter 15).

DIAGNOSTIC PROCEDURES

1. PREPARATION AND EXAMINATION OF SAMPLES FOR BONAMIA

1.1 Cytological examination: blood smears

For larvae and oysters, after the samples have been air-dried, make a squash for larvae or put the cardiac tissues on a histological slide. The slides are air-dried, then fixed in methanol. The prepared larvae and oysters are stained using a commercially available blood staining kit, in accordance with the manufacturer's instructions. After staining they are rinsed using tap water and allowed to dry completely with cold or warm air, and mounted in a synthetic resin.

The parasite (2 μm in size) shows up as blue (cytoplasm) with a red nucleus. It may be observed inside or outside the haematocytes. An observation time of 5 min per slide is sufficient.

1.2 Histology

Cut the bivalve through the digestive gland, gills and heart and place the sample in a fixative liquid such as Davidson's, Bouin's or Carson's
fluid. Carson's fixative enables the samples to be used for electron microscopy when necessary. The sample volume:fixative volume ratio of 1:10 must be respected.

Several non-specific stains, e.g. haematoxylin-eosin or Masson's trichrome process, enable Bonamia to be visualised. These examples are not exhaustive. It is recommended that two sections per oyster be examined.

The parasite (2 μm in size) occurs freely in the connective tissue and haematocytes. Detection of microcells in haematocytes, connective tissue or extracellularly should be confirmed by transmission electron microscopy.

REFERENCES


CHAPTER 17

HAPLOSPORIDIOSIS
(B432)

SUMMARY

Haplosporidiosis is an infection, sometimes lethal, of blood cells, connective tissue and digestive tissues, often accompanied by brownish-red discolouration of gills and mantle. It is caused by protozoa of the phylum Ascetospora: Haplosporidium nelsoni, H. costale, H. armoricanum and Haplosporidium spp. The following species are affected: Crassostrea virginica, Ostrea edulis, Ruditapes decussatus, and Ostrea angasi. Only H. nelsoni, a parasite of C. virginica, is responsible for epizootics. This species is found in the bays of Delaware and Chesapeake (eastern coast of USA). It is also reported from Florida to Massachusetts and in Maine.

Infection takes place between mid-May and the end of October. It has not been possible to transmit the disease experimentally in the laboratory.

Haplosporidia may be detected in smears or stained sections of infected organs. For diagnosis, the rules for sampling are those defined in the general section (Chapter 15).

DIAGNOSTIC PROCEDURES

1. PREPARATION AND EXAMINATION OF SAMPLES FOR HAPLOSPORIDIA

1.1 Cytological examination

Cut the digestive gland and the gills along a sagittal plane, soak up excess water by applying absorbent paper to the sample, then press that part of the cut surface with the digestive gland, gills and mantle against a glass slide. The slides are dried in air and then fixed with methanol (for 2-3 min).

The slides are stained using a commercially available staining kit in accordance with the manufacturer's instructions. Dip the slides in the first bath for 4-5 seconds, then immerse immediately in the second bath (3 sec.). Rinse with tap water, dry completely in cold or warm air, and mount in synthetic resin (Eukitt). Plasmodium stages (4 to 30 μm in size) show up as blue cytoplasm with red nuclei. They affect mainly the gills, palps, connective tissue and epithelium of the digestive gland.
1.2. Histological examination

Cut the visceral mass along a sagittal plane with small scissors, and place the sample in a fixative (Davidson's, Bouin's or Carson's fluid); the last-named is suitable for samples which may be examined later by electron microscopy if necessary. There should be at least 10 parts of fluid to every part of sample by volume.

The sections are subsequently treated by conventional histological procedures. Many nonspecific stains show up Ascospora: haemalum-eosin, Masson's trichrome and others. Two sections from each oyster should be examined. The different stages of the parasite can be observed in the gills, palps, connective tissue and epithelium of the digestive gland.

REFERENCES


CHAPTER 18
MARTEILIOSIS
(B434)

SUMMARY

Marteiliosis is caused by protozoan parasites of the phylum Ascetospora, genus Marteilia, which develop mainly in the epithelial cells of the digestive gland and are associated with emaciation of the oyster and exhaustion of its reserves of energy (glycogen).

The type species, Marteilia refringens, is a lethal parasite of the common European oyster, Ostrea edulis. Marteiliosis affects mainly the following species of shellfish: Ostrea edulis, Saccostrea commercialis, Mytilus edulis, Mytilus galloprovincialis, Saccostrea cucullata, Crassostrea virginica and Cardium edule. Diseases of economic significance are caused by Marteilia refringens in Ostrea edulis, and Marteilia sydneyi in S. commercialis.

The geographical distribution of these diseases is: Australia, Spain, France, Italy, Morocco. The period of infection for M. refringens is confined to the summer, when water temperature is greater than 17°C. M. sydneyi infections occur all year round. The mode of infection and the life cycle outside the host are unknown.

Diagnosis is by examination of smears of infected organs, or stained sections. For diagnosis, the rules for sampling are those stated in the general section (Chapter 15).

DIAGNOSTIC PROCEDURES

1. PREPARATION AND EXAMINATION OF SAMPLES FOR Marteilia

1.1 Cytological examination

In order to prepare the smears, cut a section through the digestive gland and the gills, remove the excess water by placing the sample on blotting paper, then place on a slide the sample corresponding to the section which passes through the digestive tract. The slides are dried in air and then fixed in methanol (2-3 min).

The samples are stained using a commercially available staining kit. Stain in accordance with the manufacturer's instructions. After staining, rinse using tap water and allow to dry completely with cold or warm air and place in a synthetic resin.
The parasite is 5-8 µm in size in the early stages and may reach up to 40 µm during sporulation. The cytoplasm of the cells stains a blue colour of a greater or lesser intensity, the nucleus being bright red. The secondary cells or sporoblasts are surrounded by a bright halo. An observation time of 5 min per slide is sufficient.

1.2 Histological examination

For histological sections, cut a section through the digestive gland using small scissors, and place the sample in a fixative liquid such as Davidson's, Bouin's or Carson's. The last-named enables the samples to be reused for electron microscopy if necessary. The ratio of volume of tissue to volume of fixative must be no more than 1:10.

The samples are subsequently handled in accordance with the classical histological methods. Several non-specific stains allow Marteilia refringens to be observed: haemalum-eosin, Masson's trichrome. These examples are not exhaustive. It is recommended that two sections per oyster be examined.

The young stages of Marteilia are present in the epithelium of the stomach; later developed stages can be found in the epithelium of the digestive diverticulata. Free sporangiae can also be observed in the lumen of the intestine.

REFERENCES


Mikrocytosis is a lethal disease of two genera of oysters, Crassostrea gigas and Saccostrea commercialis, caused by two protozoa (Protista incerta sedis), Mikrocytos mackini and M. roughleyi respectively.

The disease induces pustules, abscesses and ulcers, mainly on the mantle, with corresponding brown scars on the shell. Abscesses are composed of granular haemocytes and hyalinocytes which contain small cells of 1-3 μm. The mortality rate varies around 40%. The disease occurs more often during the spring for C. gigas and during the austral winter for S. commercialis.

The geographical distribution of M. mackini is the west coast of Canada, probably ubiquitous throughout the strait of Georgia including Henry Bay, Denman Island and confined to other specific localities around Vancouver Island. M. roughleyi occurs in New South Wales, Australia.

Diagnosis is by examination of smears of infected organs or stained sections. For diagnosis, the rules for sampling are those stated in the general section (Chapter 15).

DIAGNOSTIC PROCEDURES

1. PREPARATION AND EXAMINATION OF SAMPLES FOR MIKROCYTOSIS

1.1 Cytological examination

Cut a section through the abscesses or ulcers, remove the excess water by placing the sample on blotting paper, then blot on a slide the sample corresponding to the section which passes through the infected tissue. The slides are dried in air and then fixed with methanol (2-3 min).

The slides are stained using any equivalent Wright-Giemsa stain (e.g. Merck's Hemacolor Kit or Baxter's Diff-Quick) in accordance with the manufacturer's instructions. Dip the slides in the first bath for 4-5 seconds, then immerse immediately in the second bath (3 sec). Rinse with tap water, dry completely in cold or warm air, and mount in synthetic resin (Eukitt).
The parasite, 1-3 μm in diameter, appears included in haemocytes or free of the host cells and has blue cytoplasm and a small red nucleus. An observation time of 5 min per slide is sufficient.

1.2 Histological examination

For histological sections, cut a section through the body of the oyster including pustules, abscesses and ulcers if any are present. Then place the sample in a fixative liquid such as Davidson's, Bouin's or Carson's. The last-named enables the samples to be reused for electron microscopy if necessary. The ratio of volume of tissue to volume of fixative must be no more than 1:10.

The samples are subsequently handled in accordance with the classical histological methods. Several non-specific stains allow *Mikrocytos* to be observed: haemalum-eosin, Masson's trichrome. These examples are not exhaustive. It is recommended that two sections per oyster be examined.

The stages of *Mikrocytos* are present in the haemocytes concentrated in and around the abscesses or the ulcers for *M. roughleyi* and in the vesicular connective tissue cells on the periphery of the lesions for *M. mackini*.

REFERENCES


Perkinsosis is a lethal infection of connective tissue, caused by protozoan parasites of the phylum Apicomplexa, genus Perkinsus (formerly Dermocystidium). The type species, Perkinsus marinus, is a lethal parasite of the American oyster, Crassostrea virginica.

The geographical distribution of the disease caused by various Perkinsus species is: Australia, Spain, Portugal, and the USA (East Coast).

Principally the following shellfish are affected: Crassostrea virginica, Ruditapes decussatus, R. philippinarum, Haliotis rubra, H. laevigata, Tridacna gigas and recently Argopecten irradians. Diseases of economic importance are caused by Perkinsus marinus in C. virginica (USA), Perkinsus atlanticus in R. decussatus (Portugal), and Perkinsus olseni in Haliotis rubra (Australia) and H. laevigata. Some 50 species of bivalves may harbour Perkinsus species without harmful effect.

The main period of infection is summer, when the water temperature exceeds 20°C. The infective stage is a biflagellate zoospore, which develops into a trophozoite. Trophozoites multiply within the host by successive binary fissions.

Perkinsus is detectable in stained sections and by culture in thioglycollate medium. For diagnosis, the rules for sampling are those stated in the general section (Chapter 15).

**DIAGNOSTIC PROCEDURES**

1. **PREPARATION AND EXAMINATION OF SAMPLES FOR PERKINSUS**

   1.1 **Histological examination**

   Cut the visceral mass with small scissors along a sagittal plane, and place the sample in a fixative (Davidson's, Bouin's or Carson's fluid); the last-named is suitable for samples which may be examined later by electron microscopy if necessary. There should be at least ten parts of fluid to every part of sample by volume.

   Samples are then treated by conventional histological procedures.
Many nonspecific stains show up Apicomplexa: haemalum-eosin, Masson's trichrome and others. Two sections from each oyster should be examined.

According to species, the size of trophozoites varies from 5-7 μm for *P. marinus*, 13-16 μm for *P. olseni* and 10-15 μm for *P. atlánticas*. Trophozoites are usually characterized by the presence of a vacuole which displaces the nucleus towards the periphery. With haemalum-eosin stain, the cytoplasm of trophozoites stains pink and the nucleus violet.

In the case of *Ruditapes decussatus*, the presence of trophozoites elicits a cystic reaction, and the cysts may be large enough to be seen with the naked eye.

1.2. Diagnosis by culture in thioglycollate medium

Tissue samples measuring 5 x 10 mm are cut out (giving preference to rectal samples from oysters) and placed in liquid thioglycollate medium (Difco) plus antibiotics. The antibiotics (benzylpenicillin 0.5 mg/ml and streptomycin sulphate 0.5 mg/ml) are added under sterile conditions after having been sterilised by filtration (0.22 μm). Incubation is done at 22-25°C for 48-72 hours.

The size of cultured parasites increases from 3-10 to 70-125 μm. After incubation, the fragments of tissues are collected and placed in Lugol's 1:5 solution for 10 min, then mounted between a slide and a cover slip for microscopic examination in the fresh state. *Perkinsus* parasites are present as round cells, stained blue or bluish-black by Lugol's solution, and they may reach 120 μm.

REFERENCES


CHAPTER 21
IRIDOVIROSES
(B435)

SUMMARY

Three iridovirus infections have been reported in bivalve molluscs. Although the viruses have not yet been purified and studied in detail, they have been classified as iridovirus because of the presence of DNA and the shape and size of their virions, 380 nm in diameter. The first cases of infection described were in the Portuguese oyster, Crassostrea angulata, in which the virus elicited necrosis of branchial tissues and infections of haemocytes. Virions develop from inclusion bodies, present in infected cells.

Iridoviruses can affect both C. angulata and C. gigas (larvae). Lethal iridoviral infections in C. angulata produce lesions of the gills, or lesions of haemocytes and connective tissues. In C. gigas the infection is particularly responsible for the death of larvae, and it is known as oyster velar virus disease (OVVD).

The geographical distribution of these diseases, which are no longer being reported, was Spain, France and Portugal. Larval mortality has affected hatcheries on the western coast of USA.

In the absence of established cell lines from bivalve molluscs, the only diagnostic procedure is to examine stained smears or sections of infected organs, supported in presumptive cases by electron microscopy. More sensitive and easier methods are needed for routine diagnosis.

For diagnosis, the rules for sampling are those stated in the general section (Chapter 15).

DIAGNOSTIC PROCEDURES

1. PREPARATION AND EXAMINATION OF SAMPLES FOR IRIDOVIRUS

1.1 Cytological examination

Cut the digestive gland and gills along a sagittal plane, soak up excess water by applying absorbent paper, then press that part of the cut sample which passes through affected organs against a glass slide. The slides are dried in air and then fixed with methanol (2-3 min).
The slides are stained using Merck's Hemacolor Kit (with reagent solution 2 [ref. 11956] for red staining and reagent solution 3 [ref. 11957] for blue staining). Dip the slides in the first bath for 4-5 seconds, then immerse immediately in the second bath (3 sec). Rinse with tap water, dry completely in cold or warm air, and mount in synthetic resin (Eukitt).

It is sufficient to examine each slide for 5 min.

The cytoplasm of infected cells stains blue, and it contains a weakly-staining red nucleus and an inclusion body, variable in size, stained bright red.

1.2. Histological examination

Cut the visceral mass and the gills with small scissors along a sagittal plane, and place the sample in a fixative (Davidson's, Bouin's or Carson's fluid); the last-named is suitable for samples which may be examined later by electron microscopy if necessary. There should be at least ten parts of fluid to every part of sample by volume.

The samples are then treated by conventional histological procedures.

Many nonspecific stains show up iridoviral inclusion bodies: haemalum-eosin, Masson's trichrome and others. Two sections from each oyster should be examined.

Infected cells contain a highly chromophilic inclusion body and are larger than normal.

1.3. Electron microscopy

With EM, the viruses described during the outbreaks of gill disease and the massive mortalities of 1970 were present in large polymorphic cells of 30-40 μm and in conjunctive tissue cells respectively. These cells contained inclusion bodies from which extruding viral particles could be observed. The virions were icosahedral and they were 380 nm and 350 nm in diameter respectively (according to the disease), with electron-dense nucleoids of 250 nm and 190 nm.

The iridovirus of oyster velar velum disease infects only the epithelial cells of the velum. It is also icosahedral and is 230 nm in diameter.
REFERENCES


DISEASES OF CRUSTACEANS

OTHER SIGNIFICANT DISEASES

CHAPTER 22

BACULOVIRAL MIDGUT GLAND NECROSIS VIRUS (B443)

SUMMARY

Baculoviral midgut gland necrosis virus (BMN) has been found in cultured kuruma shrimp, Penaeus japonicus, in Japan and Korea. In Japan it is considered one of the major problems in hatcheries. As with Baculovirus penaei (BP) and Penaeus monodon-type baculovirus (MBV), the main target organ for BMN is also the hepatopancreas, and the virus may initiate mass mortality for the infected larvae. However, unlike BM and MBV, it does not produce an occlusion body in the infected hepatopancreatocyte.

So far BMN epizootics have been reported in Japan. BMN-like virus (non-occluded, type C baculovirus) has been reported from Penaeus monodon in the Philippines, Australia and Indonesia.

Experiments on artificial infection revealed that BMN caused high mortality to healthy P. japonicus larvae. BMN has been experimentally transmitted to P. monodon, P. chinensis and P. semisulcatus.

Two diagnostic techniques, wet-mount and histopathology of the hepatopancreas, have been used for the demonstration of BMN infection in P. japonicus. Under electron microscopy, enveloped virions measuring approximately 310 nm x 72 nm were observed. No occlusion bodies have been found in the infected nuclei of hepatopancreatocytes.

Since the oral route has been demonstrated to be the main infection pathway for BMN infection, complete or partial eradication of viral infection may be accomplished by thorough washing of fertile eggs or nauplii using clean sea water.

A. INTRODUCTION

Epizootics of baculoviral midgut gland necrosis (BMN) of larval Penaeus japonicus have occurred in the Kyushu and Chugoku area of Japan since 1971. BMN-like virus (non-occluded, type C baculovirus) has been reported from P. monodon in the Philippines and possibly in Australia and Indonesia. BMN virus
was experimentally transmitted to *P. monodon*, *P. chinensis* and *P. semisulcatus*.

During recent years, *P. japonicus* has become one of the most important cultured shrimps in Taiwan; BMN has not been detected in that species or in other cultured species in that country (unpublished finding).

The BMN infected moribund shrimp larvae showed a nuclear hypertrophy and remarkable cellular necrosis or collapse of the midgut gland (hepatopancreas).

Results of infectivity trials and field surveys showed that BMN may initiate mass mortality for the healthy larvae. It was also noted that, as for MBV and BP, viruses released with faeces into the environmental water of intensive culture systems of *P. japonicus* play an important role in disease spread.

**B. DIAGNOSTIC TECHNIQUES**

The morbid or heavily infected larvae reveal the clinical sign of a cloudy midgut gland, which may easily be observed by the naked eye. In addition, wet-mounts and histopathology of hepatopancreas are more reliable techniques for a definite diagnosis of BMN virus infection.

1. **WET-MOUNT TECHNIQUE**

   Infection of BMN virus can be rapidly diagnosed by demonstration of fresh squashes of hepatopancreas under dark field or light microscopy. In hepatopancreas of diseased post larvae with no polyhedral or round occlusion bodies, but with hypertrophied nuclei in midgut gland epithelial cells as viewed under dark field illumination equipped with a wet type condenser, infected nuclei appear white against the dark background due to the increased reflected and diffracted rays produced by numerous virus particles in the nucleus. Feulgen stain makes the difference clearer between normal nuclei (about 10 μm in diameter) and infected hypertrophied nuclei (about 20-30 μm in diameter). The healthy hepatopancreas only presents very few fat droplets. A similar result may also be obtained when 10% formalin is used for fixation.

2. **HISTOPATHOLOGICAL TECHNIQUE**

   Shrimps for observation were fixed in neutral 10% formalin and Bouin's solution, stained with hematoxylin and eosin by Vago-Amargier's method and observed under conventional microscopy. The MBV infected shrimps showed greatly hypertrophied nuclei within hepatopancreatic epithelial cells that are undergoing necrosis. The abnormal nuclei reveal marginated chromatin, a laterally disassociated nucleolus, and the absence of occlusion bodies that characterise infections by type A and type B baculoviruses.
C. ERADICATION PROCEDURES

Studies on the virucidal effect of disinfectants on BMN virus demonstrated that this virus was inactivated by 5 ppm of chlorine, 25 ppm of iodine, 100 ppm of benzalkonium chloride or benzethonium chloride and 0.5% of formalin. However, these solutions at each described concentration level were toxic to shrimp larvae. Therefore, no disinfectants can be used in practice in hatcheries for eradication of BMN virus infection. It is suggested that washing nauplii or fertile eggs with clean sea water to eradicate the digested excrement of shrimp is a better way for the production of BMN virus-free larvae. Suggested procedures for eradication of BMN virus from hatcheries are shown in Table 1.

<table>
<thead>
<tr>
<th>Broodstock</th>
<th>Collection of fertile eggs for 1 minute</th>
<th>Pass through a soft gauze with pore size of 800 μm to remove digested excrement or faeces of shrimp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>=&gt; Wash with running sea water at salinity level of 28-30% for 3-5 minutes to make sure all the faecal debris have been removed.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>=&gt; Pass through a soft gauze with pore size of 100 μm to collect eggs or nauplii.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>=&gt; Wash with running sea water at salinity level of 28-30% for 3-5 minutes to remove the adhesive viral particles.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>=&gt; Hatchery pond.</td>
<td></td>
</tr>
</tbody>
</table>

REFERENCES


CHAPTER 23
NUCLEAR POLYHEDROSIS BACULOVIROSES
(Penaeus monodon-type baculovirus and Baculovirus penaei)
(B441 / B442)

SUMMARY

Penaeus monodon-type baculovirus (MBV) and Baculovirus penaei (BP), two nuclear polyhedrosis viruses, have been considered to be potentially serious pathogens in the larval stages of host shrimp. They possess a wide distribution and diverse host range. Both MBV and BP are characterised by the presence of occlusion bodies (OBs), which may be referred as polyhedral occlusion bodies (POBs), or polyhedral inclusion bodies (PIBs), in hepatopancreatocytes, gut cells and digested excrement.

Studies on the epizootiology of MBV infection in P. monodon showed a very high incidence of MBV in postlarvae, juvenile and broodstock of P. monodon obtained from Asia and Southeastern Asia. P. monodon from Texas, Ecuador and Brazil also revealed positive MBV infection. The other species revealed a low MBV incidence rate in these areas. BP was also demonstrated to be widespread in distribution in cultured and wild penaeid shrimps in the Northern and Southern Americas.

Crowding and chemical or environmental stress may enhance the pathogenicity and increase the prevalence of these two viruses in their hosts.

MBV can be diagnosed by wet-mount and histopathological observation of hepatopancreas. Observation of digested excrement for the presence of MBV occlusion bodies is also feasible.

Prevention of MBV and BP infection in hatcheries may be obtained by first washing nauplii or fertile eggs with formalin, iodophores and clean sea water.

A. INTRODUCTION

Baculoviruses of shrimps including Penaeus monodon-type baculovirus (MBV) and Baculovirus penaei (BP), two nuclear polyhedrosis viruses, have been considered to be potentially serious pathogens in the larval stages of host shrimp. They possess a wide distribution and diverse host range. Both MBV and BP are characterised by the presence of occlusion bodies (OBs), which may be referred to as polyhedral occlusion bodies (POBs) or polyhedral inclusion bodies (PIBs), in hepatopancreatocytes, gut cells and digested excrement. Penaeus monodon-type baculovirus (MBV) is found in shrimps from the
Indopacific and Pacific Coasts of Asia, Australia, Africa, Southern Europe, and Northern and Southern America.

MBV has been found to infect various species of shrimps including *Penaeus esculentus*, *P. kerathurus*, *P. merguiensis*, *P. monodon*, *P. plebejus*, *P. penicillatus*, *P. semisulcatus*, *P. vannamei*, and *Metapenaeus ensis*. The most serious infection is found in the cultured black tiger prawn, *P. monodon*. Epizootiological studies on MBV infection in *P. monodon* in 1989 showed an MBV incidence of more than 50% in postlarval juveniles and broodstocks obtained from Taiwan and Southeastern Asia (unpublished result). BP was found in cultured and wild penaeid shrimps in the Americas, ranging from the Northern Gulf of Mexico south through the Caribbean and reaching at least as far as central Brazil, and from Peru to Mexico along the Pacific Coast. No BP has ever been found in wild or cultured penaeids in Asia. This virus was demonstrated to infect *Penaeus aztecus*, *P. brasiliensis*, *P. duorarum*, *P. marginatus*, *P. paulensis*, *P. penicillatus*, *P. schmitti*, *P. setiferus*, *P. stylirostris*, *P. subtilis*, and *P. vannamei*.

Experiments performed in hatcheries showed that MBV initiated mortality for infected postlarval *P. monodon*. Results obtained from pathogenicity studies showed that environmental stress significantly affects the survival of MBV-infected *P. monodon* and *P. penicillatus*. Mass mortality which occurred in MBV-infected *P. monodon* postlarvae may have been initiated by mixed infection with *Vibrio* spp. However, when the shrimps were kept in a grow-out pond with good environmental conditions, no mortality or growth retardation occurred in the MBV-infected juveniles. Eradication of MBV infection in *P. monodon* or *P. penicillatus* in hatcheries, which will be described in the following section, is therefore important.

It was also demonstrated that BP is pathogenic to *P. vannamei*, *P. aztecus*, *P. duorarum*, and *P. marginatus*. As for MBV, physical or chemical stress on BP-infected shrimps may enhance the severity of virus infection and increase the prevalence rate.

**B. DIAGNOSTIC TECHNIQUES**

Several diagnostic procedures are used in screening shrimp stocks for MBV infections:

1) Wet-mount examination of hepatopancreas
2) Histopathological observation of hepatopancreas
3) Examination of excrement
4) Recombinant DNA technique.

The first two techniques are employed for studies on the epizootiology and pathogenicity of MBV and BP. However, the third technique is suitable for screening of MBV and BP-free broodstocks for hatcheries. The fourth technique provides a highly specific and sensitive method for detection of MBV and BP in
shrimp stocks. Furthermore, this technique can be used to identify the target organs of the viral infection and to determine which life stages are most susceptible.

1. **Wet-Mount Technique**

MBV infection may readily be diagnosed by the demonstration of spherical, round, pyramidal or tetrahedral shaped occlusion bodies in wet-mounts of hepatopancreatocytes or midgut of shrimp under bright field or phase contrast microscopy. The occlusion bodies may also be pyramidal or tetrahedral. BP infected hepatopancreatocytes reveal tetrahedral occlusion bodies. However, the sensitivity of this technique is rather limited since spherical or tetrahedral occlusions are difficult to distinguish from lipid droplets and secretory granules in unstained wet-mounts of tissue squashes. Aqueous malachite green (0.05%) may be used in preparing for MBV and BP diagnosis to aid in the observation of occlusion bodies. Also, occlusion bodies (in squashes or histology) fluoresce under ultraviolet light following staining with aqueous 0.18% phloxine, thereby providing a rapid and specific diagnosis.

2. **Histopathological Technique**

This technique is a reliable technique for definite diagnosis of MBV and BP infection. Prior to preparation for observations, the specimen should be preserved in Davidson's fixative containing 33% ethyl alcohol (95%), 20% formalin (approximately 37% formaldehyde), 11.5% glacial acetic acid and 33.5% distilled or tap water. In addition, Bouin's fixative, Carnoy's solution and 10% formalin solution may also be used for this purpose. To obtain a better result, no dead shrimps should be used. Live, moribund or compromised shrimps are killed by injection of fixative, then the cuticle over the cephalothorax and abdomen just lateral to the dorsal midline are opened and immersed in fixative for 24-72 hrs, then transferred to 70% ethyl alcohol for storage. These fixed specimens may be shipped by wrapping in cloth or paper towel saturated with 70% ethyl alcohol and packed in plastic bags. Tissues such as the hepatopancreas or gills may be cut into small pieces and fixed directly in the fixative.

After dehydration, the specimens are embedded in paraffin and sectioned into 5-7 μm thickness. Harris hematoxylin and eosin, Giemsa's and Gram's staining solutions may be used for the demonstration of spherical (MBV) or tetrahedral (BP) occlusion bodies in hepatopancreatocytes, gut epithelial cells or gut lumen.

Necrosis and loss of hepatopancreatic and midgut epithelial cells are observed in the sections of diseased shrimps. Single or more often multiple occlusion bodies in hypertrophied nuclei, and chromatin diminution and margination in infected hepatopancreatic epithelial cells, have been observed in advanced infection. Occlusion bodies may be stained bright red with
haematoxylin and eosin, and stained intensely with Gram's stain and toluidine blue. Brown and Benn histological Gram stain, although not specific for baculovirus occlusion bodies, tends to stain occlusions more intensely (either red or purple, depending on section thickness, time of decolourisation, etc.) than the surrounding tissue, aiding in demonstrating their presence in low-grade infections.

3. **DIGESTED EXCREMENT EXAMINING TECHNIQUE**

The shrimps used for examination may be placed in an aquarium or plastic tank for a few hours until digested excrement is present. The faeces are collected and placed in a glass centrifuge tube. Subsequently, digesting solution consisting of 4 parts n-butanol, 1 part n-hexane and 10 parts distilled water is added to each tube and the mixture is then homogenised for 3-5 minutes and centrifuged at a speed of 1,500-2,000 rpm. Similar procedures may be repeated twice for better digestion. The sediments are observed for the presence of MBV or BP occlusion bodies with the aid of staining using eosin, Giemsa, acridine orange or malachite green solutions.

4. **RECOMBINANT DNA TECHNIQUE**

The recombinant DNA technique involves probe cloning, probe labelling, filter hybridisation analysis, and *in situ* hybridisation analysis. Standard procedures are involved in these components of the recombinant DNA technique, and they can be found in most molecular biology manuals. Many of the reagents needed for the procedures can even be purchased as kits. The DNA of both MBV and BP has been successfully detected through the application of gene probes. Details of the methods can be found in the literature. General descriptions of the procedures are provided below.

4.1 **Probe cloning and labelling**

BP or MBV virions can be isolated from hepatopancreatic tissue of infected shrimp; they are then purified by centrifugation. The DNA can also be purified with phenol extraction and ethanol precipitation. The BP or MBV DNA is digested with restriction endonucleases, ligated into the bacterial vector, and used to transform bacterial cells. Clones containing viral DNA inserts are selected. Gene probes can be labelled either radioactively with P or S or with dioxigenin, a non-radioactive label. Convenient kits containing reagents for labelling are available from Amersham, DuPont-NFN, Promega, BRL, and other companies.

4.2 **Filter hybridisation analysis**

Shrimp to be tested are processed for DNA extraction and purification. The DNA is blotted onto a nylon filter and subsequently the filter is prehybridised, then hybridised with a labelled BP or MBV probe. Hybridisation mixtures will usually include formamide (30-50%), sheared fragments of DNA, dextran sulphate, Denhardt's solution, and NaCl in sterile water. After hybridisation, the filter is washed several
times in salt solution to wash off non-specifically bound probe. The hybridisation signal can be detected using suitably sensitive autoradiography film if a radiolabelled probe is used. Detection of the non-radiolabelled probe is usually through an enzyme reporter, such as alkaline phosphatase or peroxidase; a colour precipitate can then be observed at the hybridisation site.

4.3 *In situ* hybridisation

This procedure is now well established for the identification and localisation of BP and MBV DNA in tissue sections of infected shrimp. Before attempting *in situ* hybridisation, it is usual to fix the viral DNA in place in the cell with a fixative such as formalin. The tissue is then washed in PBS, embedded in paraffin, and sectioned at approximately 4-6 μm thickness. Sections can be collected onto precoated slides and then deparaffinised. The sections can then be rehydrated. This is followed by digestion with proteinase K (10 μg/ml) for 30 min at 37°C. Subsequently the sections are prehybridised for 1 hour at 37°C, and then hybridised by adding labelled MBV or BP DNA probes. After hybridisation, the sections can be washed and the presence of viral DNA can be detected within the cells.

Table 1

Suggested procedures for the production of BP or MBV non-infected postlarvae

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Description</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Nauplii*</td>
<td>Collection of nauplii using plankton net</td>
<td>Running sea water for 1-2 minutes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Formalin 400 ppm for 30 seconds to 1 minute</td>
</tr>
<tr>
<td></td>
<td>=&gt;</td>
<td>Running sea water for 3-5 minutes</td>
</tr>
<tr>
<td></td>
<td>=&gt;</td>
<td>Hatchery ponds</td>
</tr>
<tr>
<td></td>
<td>=&gt;</td>
<td>Iodophore 0.1 ppm for 1 minute</td>
</tr>
<tr>
<td>b) Fertilised eggs**</td>
<td>Collection of fertilised eggs</td>
<td>Running sea water for 1-2 minutes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Formalin 100 ppm for 1 minute</td>
</tr>
<tr>
<td></td>
<td>=&gt;</td>
<td>Running sea water for 3-5 minutes</td>
</tr>
<tr>
<td></td>
<td>=&gt;</td>
<td>Hatchery ponds</td>
</tr>
<tr>
<td></td>
<td>=&gt;</td>
<td>Iodophore 0.1 ppm for 1 minute</td>
</tr>
</tbody>
</table>

* Nauplii are much easier to collect than are fertilised eggs in hatcheries
** Fertilised eggs are more sensitive than nauplii to formalin.
5. **ERADICATION PROCEDURES**

Experiments performed on the infection mechanism showed that MBV infection was initiated by the oral route. Eradication of MBV infection in hatcheries may therefore be obtained by several washes of nauplii or fertilised eggs using clean sea water, formalin and iodophore solutions (Table 1). Nauplii are much easier to collect than are fertilised egg in hatcheries, and fertilised eggs are more sensitive to formalin. The procedures used for nauplii are therefore highly recommended for commercial hatcheries.

**REFERENCES**


Infectious hypodermal and haematopoietic necrosis (IHHN) virus, a parvo- or a picorna-like virus, is considered to be a potential pathogen for a serious disease in several species of shrimps. This virus invades all the cells in tissues of ectodermal and mesodermal origin. However, in light cases, the endoderm-derived tissues such as midgut, midgut caeca and hepatopancreas are not infected by this virus.

IHHN is characterised by its worldwide distribution among cultured shrimp. However, no data are available on the distribution of this virus in wild shrimp. The geographic distribution of IHHN virus infection in shrimp has increased very significantly in recent years.

Infection by IHHN virus has been demonstrated to cause disease in Penaeus stylirostris, P. monodon and P. vannamei.

Wet-mount or histopathological observations are the main techniques for demonstration of IHHN virus infection in shrimps. For quarantine purposes, bioassay of a suspect shrimp population with a sensitive indicator species followed by wet-mount or histopathological observation of randomly selected indicator shrimps is recommended. To enhance the infection, shrimps may be reared under relatively crowded and stressful conditions.

Monoclonal antibodies against IHHNV may be applied to detect IHHNV infected shrimps with a very high specificity by using indirect enzyme-linked immunosorbent assay (ELISA), and immunoblot and Western blot assays. Also, the in situ hybridisation technique can be applied on paraffin sections of IHHNV infected shrimps using a DNA probe.

A. INTRODUCTION

Infectious hypodermal and haematopoietic necrosis (IHHN) virus, a parvo- or a picorna-like virus, has been found in shrimps cultured on the Atlantic and Pacific coasts, Pacific Islands, Asia and Middle East. The virus invades all the cells in tissues of ectodermal and mesodermal origin.

This virus is also characterised by its wide host range. Penaeus vannamei, P.
Stylirostris, P. monodon and P. semisulcatus are susceptible to IHHN virus in nature. However, experimental infection of IHHN virus was obtained in P. schmitti, P. japonicus, P. aztecs and P. duorarum. Infection by IHHN virus was demonstrated to cause acute epizootics and mass mortality for P. stylirostris, P. monodon and P. vannamei. In contrast no significant mortality was found in other penaeid shrimps. IHHN virus initiated higher mortality rates (up to 80 or 90%) in populations of small size. Although infection of tissue cells were observed in older or larger shrimps, very little mortality was obtained.

It is concluded that like BP or MBV, IHHN virus affects the larvae or young juvenile stages of shrimp more seriously than it affects larger shrimps.

No eradication technique has been developed. However, IHHNV free larvae may be produced by using virus free broodstock.

B. DIAGNOSTIC TECHNIQUES

The following three diagnostic procedures are employed to diagnose IHHN virus infection in shrimps: a) direct samplings for histopathological observations; b) enhancement of infection followed by direct sampling techniques; c) bioassay of a suspect population with a sensitivity 'indicator' species.

1. DIRECT EXAMINATION

Samples for examining are fixed in Davidson's fixative and stained by haematoyxlin and eosin staining procedures for histopathological observations as described in the chapter on MBV and BP (Appendix 2) under bright field microscopy with 200 or 400x magnification. Cowdry type A eosinophilic inclusion bodies are observed in hypertrophic nuclei of the tissues or organs including gills, periepods, maxillipeds, stomach mucosa, gnathorax, ventral nerve cord and ganglia, and the haematopoietic tissues. Necrosis and inflammatory response of infected tissue cells is the other sign for IHHN virus infection which may result in shrimp mortality.

2. ENHANCEMENT OF INFECTION

The enhancement procedure may be achieved with a quarantined population by rearing shrimps in relatively crowded or stressful conditions. The stressors may include O₂ deficiency in water, unsuitable temperature, insufficient food supply or presence of NH₃ or NO₂ in the water. All these stressful conditions may initiate a heavy infection of IHHN virus in the host shrimps. The moving of larvae from a hatchery pond to an aquarium or tank would also act as a stressor to the infected shrimps.
3. **Bioassay Technique**

Carriers of IHHN virus may be detected with highly sensitive 'clean' (uninfected) shrimps such as juvenile *P. stylirostris* at body weight of 0.05-4 g. This technique is considered to be the best method for examination or screening of IHHNV infected shrimps or carriers.

The following two methods can be applied to expose the suspect carrier shrimps to indicator species:

1) Injection or immersion of 'indicator' shrimps with homogenates derived from suspected samples. Using this technique 'indicator' shrimps will show signs of IHHN virus disease 5-20 days after treatment.

2) Rearing suspect carrier shrimps with indicator shrimps. Using this method 'indicator' shrimps will show signs of IHHN disease within 1 or 2 months after rearing.

In addition, chopped carcases of suspect 'carrier' shrimp may also be fed to 'indicator' shrimps. Fifteen to 60 days following feeding, the indicator shrimps will reveal signs of IHHN disease.

4. **Monoclonal Antibodies to IHHNV**

IHHNV virus may be purified from infected shrimps. Crude homogenate from infected shrimps may be clarified by a series of low-speed centrifugations and the virus pelleted at 145,000 g. The virus suspension is treated with activated charcoal, filtered on a Celite-235 bed and extracted with freon. The virus is repelleted at 145,000 g and further purified on a 15-40% sucrose gradient followed by a 25-40% cesium chloride gradient. Monoclonal antibodies (MAbs) may be obtained by routine techniques using myeloma cells and spleen cells of BALB/C strain mice. MAbs may react specifically with IHHNV in immunoblots and Western blots.

5. **Gene Probes**

IHHNV gene probes may be obtained by purification of virus and extraction of DNA followed by transformation of DNA in competent *E. coli* DH5 cells. Positive reactions may be obtained when the probe is tested on paraffin sections of IHHNV infected shrimps. Tested shrimps may be fixed in Davidson's fixative, embedded and sectioned at 5 μm using standard histological procedures. IHHNV probes react only with IHHNV infected cells.

**REFERENCES**


CHAPTER 25
YELLOWHEAD DISEASE
(No OIE number)

SUMMARY

Yellowhead virus (YHV), a non-occluded baculo-like virus, has been demonstrated to be a potentially serious pathogen for the cultured juveniles of Penaeus monodon. YHV is characterised by initiating massive necrosis and vacuolated cells and hypertrophied nuclei of infected tissues with densely stained basophilic inclusions adjacent to nuclei.

To date YHV epizootics have occurred only in Thailand, causing very serious mass mortality. Experiments on artificial infection using the filtrate from the diseased shrimps showed a very high mortality rate.

Two presumptive tests, copper content in haemolymph and histopathological observations using light microscopy, may be used for the diagnosis of YHV infection in shrimp. However, definite diagnosis should rely on transmission electron microscopy (TEM). Under EM, enveloped virions with bacilliform morphology, ranging from 150-200 nm in length and 40-50 nm in diameter, have been found in the cytoplasm of infected cells.

No eradication procedures have been developed for YHV infection. Good pond management is so far the only prevention technology for yellowhead disease (YHD). Some chemicals such as chlorine and iodine have proved to be effective disinfectants. However, the use of these chemicals during the culture period of shrimp is not recommended.

A. INTRODUCTION

Yellowhead disease (YHD) is a viral infectious disease of the giant tiger prawn, Penaeus monodon. The causative agent has been demonstrated to be a non-occluded baculo-like virus, yellowhead virus (YHV). It may initiate mass mortality for cultured shrimps at the grow-out stage. To date, YHD has been found in central and southern Thailand only.

Epizootics of yellowhead disease (YHD) of Penaeus monodon have occurred in many areas of central and southern Thailand since 1990. The yellowhead virus (YHV) infected moribund shrimps showed histopathological changes in gill lamellae with extensive necrosis of pillar and epidermal cells and haemocytes. Cellular necrosis, with hypertrophied nuclei and densely stained basophilic
globose cytoplasmic inclusions adjacent to nuclei, was also observed in connective tissue underlying the midgut, in cardiac tissue, in haematopoietic tissue, and in gill tissue. Results of the infectivity trials and field surveys demonstrate that YHV may initiate a mass mortality for cultured *P. monodon*. This virus seems to be the most virulent destructive virus so far detected in cultured *P. monodon*.

**B. DIAGNOSTIC TECHNIQUES**

The moribund or heavily infected shrimps exhibit the clinical sign of a light yellow colour in the cephalothorax and gills, that may easily be observed by the naked eye. In addition, total haemocyte count, wet mount of gills, copper content of haemolymph, Giemsa or haematoxylin staining of gills, and electron microscopic observations are used for the diagnosis of YHD (3,4).

1. **WET MOUNT TECHNIQUE**

   Infection of YHV infection can be rapidly diagnosed by the presence of globular materials with a yellowish and fat-like structure in the secondary lamellae of the gills.

2. **HISTOPATHOLOGICAL OBSERVATION**

   Haemolymph collected from diseased shrimps is fixed in cold 10% formalin in sea water. A thin film is then prepared on a glass slide and air dried. Subsequently, Wright-Giemsa staining is performed on the blood film. Under microscopy pyknotic and karyorrhectic nuclei of the haemocytes of diseased shrimps are observed.

   With light microscopy, sections of yellowhead specimens stained with haematoxylin and eosin showed necrotic cells with hypertrophied nuclei and large vacuoles. The disease was characterised by the presence of densely basophilic, globose bodies adjacent to nuclei in the infected tissues in lymphoid tissue, in the interstitial tissues of the hepatopancreas, in the connective tissue underlying the midgut, in cardiac tissue, in haematopoietic tissue and in gill tissue.

3. **COPPER CONTENT OF HAEMOLYMPH AND TOTAL CELL COUNT OF HAEMOCYTES**

   Haemolymph may be collected using a 1 ml plastic syringe and 20 G needle. L-cysteine may be used as anticoagulant. The mixture of anticoagulant and haemolymph (1:1) is immediately counted using a haemocytometer. The results show that the haemocyte count of the YHV infected shrimps is significantly lower than that of the normal ones. Haemolymph without addition of anticoagulant is allowed to clot at room temperature and serum is obtained following centrifugation at 6,500 rpm for 10 minutes. The copper content of the serum may be analysed using atomic absorption
spectroscopy. The results reveal that the copper content in haemolymph in normal shrimps is significantly higher than that of the YHD infected shrimps.

REFERENCES


Crayfish plague is a highly infectious disease of all crayfish (Decapoda: Astacidae, Cambaridae) of non-North American origin. The aetiological agent is an Oomycete fungus, Aphanomyces astaci, which is now widespread in Europe as well as in North America. The European crayfish species, the Noble crayfish Astacus astacus of northwest Europe, the stone crayfish Austropotamobius pallipes of southwest and west Europe, the related Austropotamobius torrentium (mountain streams of southwest Europe) and the slender clawed or Turkish crayfish Astacus leptodactylus of eastern Europe and Asia Minor are all highly susceptible. The only other crustacean known to be capable of infection by A. astaci is the Chinese mitten crab (Eriocheir sinensis) and this only under laboratory conditions.

The disease first occurred in Europe in the mid 19th century in North Italy and then on the Franco-German border region. From the latter region a steady spread of infection occurred, principally in two directions - down the Danube into the Balkans and towards the Black Sea, and across the North German plain into Russia and from there south to the Black Sea and northwest to Finland and finally in 1907 to Sweden. In the 1960s the first outbreaks in Spain were reported and in the 1980s further spread of infection to the British Isles, Turkey, Greece and Norway have been reported.

The reservoir for the original infections in the mid 19th century was never established, but the post-1960s extensions are largely linked to movements of North American crayfish introduced more recently for purposes of crayfish farming. These species (Pacifastacus leniusculus [the Signal crayfish] and Procambarus clarki [the Louisiana swamp crayfish]) can act as largely or completely asymptomatic carriers, but can be killed by A. astaci under adverse conditions. Transmission has also resulted from contaminated crayfish traps and other contaminated equipment.

Clinically, infected crayfish may present a wide range of gross signs of infection or none at all. Focal whitening of local areas of musculature beneath transparent areas of thin cuticle, especially of the ventral abdomen and in the periopod (limb) joints, often accompanied by even more localised brown melanisation, is the most consistent sign. In the terminal stages of infection animals show a limited range of behavioural signs, principally a loss of the normal aversion to bright light (they are seen in open water in daylight) later accompanied by a loss of limb co-ordination which
produces an effect which has been described as "walking on stilts". Eventually, moribund animals lose their balance and fall onto their backs before dying.

Diagnosis requires isolation and identification of the pathogen by microscopic morphology; no biochemical or serological methods exist.

Control of spread of infection once a watershed is infected is in practical terms impossible. Prevention of all introductions of crayfish to natural waters and into enclosed waters from which they may escape to natural waters can be effective, although movement of fish can result in the movement of infected water between watersheds and can transmit infection as can contaminated equipment such as boots and fishing gear. Malachite green has been demonstrated to be effective in 'disinfecting' contaminated water and fish, and sodium hypochlorite and iodophores are effective for disinfection of contaminated equipment. Thorough drying of equipment (>24 h) is also effective since the oomycetes are not resistant to desiccation.

INTRODUCTION

Crayfish plague is a highly infectious fungal disease of fresh water crayfish. The disease is of North American origin. North American crayfish species are generally resistant to the disease, whilst all crayfish species from other continents are highly susceptible. The causative agent is an Oomycete fungus, Aphanomyces astaci. Other members of this genus are now implicated in a number of fish diseases, particularly the epizootic ulcerative syndrome of South East Asia.

The disease first appeared in Europe in about 1860. By 1935 only the British Isles, the Iberian Peninsula, Greece, Turkey and Norway remained free of infection. In the period 1960-1980 those areas were also infected. In most cases evidence exists to link these more recent extensions to the transfer of infected carrier crayfish introduced for farming.

The first sign of a crayfish plague mortality may be the presence of numbers of crayfish at large during daylight (crayfish are normally nocturnal), some of which show evident loss of coordination in their movements and easily fall over onto their backs and are unable to right themselves. Often, however, unless waters are carefully observed, the first recognition that there is a problem will be the presence of large numbers of dead crayfish in a river or lake.

In susceptible species where sufficient numbers of crayfish are present to allow infection to spread rapidly, and particularly at summer water temperatures, infection will spread quickly and stretches of over 50 km of river may lose all their crayfish in less than 21 days from the first observed mortality. Crayfish
plague has an unparalleled severity of effect; infected susceptible crayfish do not survive – 100% mortality is the norm. Resistant North American species do survive infection in many cases and then act as largely asymptomatic carriers, although under adverse conditions (stress, other infections), a major crayfish plague mortality may occur.

Infection is transmitted horizontally through river water by means of the fungus' motile biflagellate zoospores, which exhibit a positive chemotaxis towards crayfish. Depending on the site of the initial source of infection, the mortality will spread downstream at the speed of flow of the river, and upstream at 2 to 4 km per year. The upstream spread may be attributed to normal movements of crayfish between infection and the development of clinical disease.

Infection may also be transmitted in transport water (e.g. with movements of fish between farms) and on contaminated equipment (boots, fishing gear, etc.). Fish and fish water may be disinfected by standard therapeutic doses of malachite green during transport, and equipment with hypochlorite or iodophores. No therapy of infected crayfish is possible.

Diagnosis of crayfish plague strictly requires the isolation and characterisation of the pathogen, *A. astaci*, using simple mycological media fortified with antibiotics to control bacterial contamination. Isolation is only likely to be successful before or within 12 hours of the death of infected crayfish. However, there is no other disease or pollution effect which can cause such total mortalities of crayfish, while leaving all other animals in the same water unharmed, so that isolation of the pathogen is desirable but not essential, particularly in regions where further spread of infection is known to be a potential hazard. Clinical signs of crayfish plague include behavioural changes and a range of visible external lesions. The range of these is however very large so that, except for the experienced eye, such clinical signs are of limited diagnostic value.

**B. DIAGNOSTIC PROCEDURES**

**ISOLATION AND IDENTIFICATION OF *A. ASTACI***

Isolation methods are as described by Alderman and Polglase. An agar medium (IM) is used, containing yeast extract and glucose in river water with antimicrobial agents (penicillin G and oxolinic acid) which prevent the growth of most bacteria and enable easy and rapid isolation of the pathogen.

**IM medium:**

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<tr>
<td>Agar</td>
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<tr>
<td>Yeast extract</td>
<td>1.0 g</td>
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<tr>
<td>Glucose</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Oxolinic acid</td>
<td>10 mg</td>
</tr>
<tr>
<td>River water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Penicillin G (sterile)</td>
<td>1.0 g</td>
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<td>added after autoclaving and cooling to 40°C</td>
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Simple aseptic excision of infected tissues which are then placed as small pieces (1-2 mm$^2$) on the surface of IM plates will normally result in successful isolation of *A. astaci* from moribund or recently dead (<24 h) animals. On IM agar growth of new isolates of *A. astaci* is almost entirely within the agar except at temperatures below 7°C, when some superficial growth occurs. Colonies are colourless. Dimensions and appearance of hyphae are much the same in crayfish tissue and in agar culture. Vegetative hyphae are aseptate (5)-7-9(10) μm in width (i.e. normal range 7-9 μm, but observations have ranged between 5 and 10 μm). Young, actively growing hyphae are densely packed with coarsely granular cytoplasm with numerous highly refractile globules. Older hyphae are largely vacuolate with the cytoplasm largely restricted to the periphery with only thin strands of protoplasm bridging the large central vacuole. The oldest hyphae are apparently devoid of contents. Hyphae branch profusely, with vegetative branches often tending to be somewhat narrower than the main hyphae for the first 20-30 μm of growth.

When actively growing thalli or portions of thalli from broth or agar culture are transferred to distilled water, sporangia form readily in 20 to 30 h at 16°C and 12 to 15 h at 20°C. Sporangia are myceloid, terminal or intercalary, developing from undifferentiated vegetative hyphae. Sporangial form is variable: terminal sporangia are simple, developing from new extramatrical hyphae, whilst intercalary sporangia can be quite complex in form. Intercalary sporangia develop by the growth of a new lateral extramatrical branch which forms the discharge tube of the sporangium. The cytoplasm of such developing discharge tubes is noticeably dense and these branches are slightly wider (10-12 μm) than ordinary vegetative hyphae. Sporangia are delimited by a single basal septum in the case of terminal sporangia and by septa at either end of the sporangial segment in intercalary sporangia. Such septa are markedly thicker than the hyphal wall and have a high refractive index. Successive sections of vegetative hypha may develop into sporangia and most of the vegetative thallus is capable of developing into sporangia.

Within developing sporangia the cytoplasm cleaves into a series of elongate units (10-25 x 8 μm) which are initially linked by strands of protoplasm. Although the ends of these cytoplasmic units become rounded, they remain elongate until and during discharge. Spore discharge is aplanoid, that is, the first spore stage is an aplanosporangium which encysts at the sporangial orifice and probably represents the suppressed saprolegniaceous primary zoospore. No evidence has been observed for the existence of a flagellated primary spore, thus, in this description, the terms 'sporangium' not 'zoosporangium' and 'primary spore' not 'primary zoospore' have been used. Discharge is fairly rapid (<5 min) and the individual primary spores (cytoplasmic units) pass through the tip of the sporangium and accumulate around the sporangial orifice. Speed of cytoplasmic cleavage and discharge is temperature dependent. At release, each primary spore retains its elongate irregularly amoeboid shape briefly before encystment occurs.
Encystment is marked by a gradual rounding up followed by the development of a cyst wall which is evidenced by a change in the refractive index of the cell. From release to encystment occupies 2 to 5 min. Some spores may drift away from the spore mass at the sporangial tip and encyst separately. Formation of the primary cyst wall is rapid and once encystment has taken place the group of spores remains together as a coherent group and adheres well to the sporangial tip so that marked physical disturbance is required to break up the spore mass.

Encysted primary spores are spherical (8)9-11(15) µm in diameter and are relatively few, (8)15-30(40) µm per sporangium in comparison to other Aphanomyces spp. Spores remain encysted for 8-12 h. Optimum temperatures for sporangial formation and discharge lie between 16 and 24°C, but the discharge of secondary zoospores from the primary cysts peaks at 20°C and does not occur at 24°C. In new isolates of A. astaci, it is normal for the majority of primary spore cysts to discharge as secondary zoospores, although this varies with staling in long term laboratory culture. Sporangial formation and discharge occurs down to 4°C.

In many cases, some of the primary spores are not discharged from the sporangium and many sporangia do not discharge at all. Instead, the primary spores appear to encyst in situ within the sporangium, often develop a spherical rather than elongate form and certainly undergo the same changes in refractive index that mark the encystment of spores outside the sporangium. Such within sporangial encystment has been observed on crayfish. Spores encysted in this situation appear to be capable of germinating to produce further hyphal growth.

Release of secondary zoospores is papillate, the papilla developing shortly before discharge. The spore cytoplasm emerges slowly in an amoeboid fashion through a narrow pore at the tip of a papilla, rounds up and begins a gentle rocking motion as flagellar extrusion begins and spore shape changes gradually from spherical to reniform. Flagellar attachment is lateral; zoospores are typical saprolegniaceous secondary zoospores measuring 8x12 µm. Active motility takes some 5-20 min to develop (dependent on temperature) and, at first, zoospores are slow and uncoordinated. At temperatures between 16 and 20°C, zoospores may continue to swim for at least 48 h.

REFERENCES


1. LIST OF OIE REFERENCE LABORATORIES FOR FISH, MOLLUSC AND CRUSTACEAN DISEASES

2. LIST OF ORGANISATIONS WITH WHICH THE OIE HAS COOPERATION AGREEMENTS
## 1. LIST OF OIE REFERENCE LABORATORIES FOR FISH, MOLLUSC AND CRUSTACEAN DISEASES IN 1995*

<table>
<thead>
<tr>
<th>Diseases/Viruses</th>
<th>Expert/Laboratory</th>
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</table>
| Viral haemorrhagic septicaemia virus                   | Dr N. Jørgen Olesen  
Statens Veterinaere Serum Laboratorium, 2 Hangovej, DK-8200 Aarhus N, DENMARK  
Tel: (45) 86.16.79.00, Fax: (45) 86.10.74.64 |
| Virus de la septicémie hémorragique virale            |                                                                                   |
| Viral de la septicaemia hemorrágica viral             |                                                                                   |
| Spring viraemia of carp virus                         | Dr B.J. Hill  
Fish Disease Laboratory, MAFF, Barrack Road, The Nothe, Weymouth, Dorset DT4 8UB, UNITED KINGDOM  
Tel: (44) 1305.20.66.00, Fax: (44) 1305.20.66.01 |
| Virus de la virémie printanière de la carpe          |                                                                                   |
| Virus de la viremia primaveral de la carpa            |                                                                                   |
| Infectious haematopoietic necrosis virus (Rhabdoviruses) | Dr Jo-Ann Leong  
Oregon State University, Department of Microbiology, Nash Hall 220, Corvallis, Oregon 93331-3804, USA  
Tel: (1.503) 737.4441, Fax (1.503) 737.0496 |
| Virus de la nécrose hématopoïétique infectieuse (Rhabdoviruses) |                                                                                   |
| Virus de la necrosis hematopoyética infecciosa (Rhabdoviruses) |                                                                                   |
| Onchorhynchus masou virus                             | Dr M. Yoshimizu  
Hokkaido University, Faculty of Fisheries, Hakodate, Hokkaido 014, JAPAN  
Tel: (81.138)41.0131, Fax: (81.138)43.5015 |
| Virus de l'Onchorhynchus masou                        |                                                                                   |
| Virus del Onchorhynchus masou                         |                                                                                   |
| Epizootic haematopoietic necrosis virus                | Dr A. Hyatt  
Australian Fish Health Reference Laboratory, c/o Australian Animal Health Laboratory, P.O. Bag 24, Geelong, Vic. 3220, AUSTRALIA  
Tel: (61)52.27.5000, Fax: (61)52.27.5555 |
| Virus de la nécrose hématopoïétique épizootique       |                                                                                   |
| Virus de la necrosis hematopoyética epizoótica        |                                                                                   |

* This list is updated annually and the revised list will be published in the May issue of the OIE Bulletin.
Reference Laboratories, contd.

| Mollusc pathogens | Dr H. Grizel  
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<tr>
<td>Agents pathogènes des mollusques</td>
<td>IFREMER, Laboratoire de Pathologie des Invertébrés, 17390 La Tremblade, FRANCE</td>
</tr>
<tr>
<td>Agentes patógenos de los moluscos</td>
<td>Tel: (33) 46.36.30.07, Fax: (33) 46.36.37.51</td>
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| Crustacean pathogens | Dr D. Lightner  
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<tr>
<td>Agents pathogènes des crustacés</td>
<td>Department of Veterinary Science, Building 90, Room 202, Tucson AZ 85721, USA</td>
</tr>
<tr>
<td>Agentes patógenos de los crustáceos</td>
<td>Tel: (1.602) 621.6903, Fax: (1.602) 621.6366</td>
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|  
| Prof. S.N. Chen  
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<tbody>
<tr>
<td>Department of Zoology, Director, Institute of Fishery Biology, National Taiwan, University, No. 1, Roosevelt Road, Section 4, Taipei, Taiwan</td>
</tr>
<tr>
<td>TAIPEI CHINA 10764</td>
</tr>
<tr>
<td>Tel: (886.2) 368.71.01, Fax: (886.2) 368.71.22</td>
</tr>
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</table>
2. LIST OF ORGANISATIONS WITH WHICH THE OIE HAS COOPERATION AGREEMENTS

The OIE has signed a number of agreements with international organisations having an interest in animal health or veterinary public health. These agreements aim to ensure that there is a coordination of effort and cooperation in carrying out joint actions where appropriate. The organisations concerned are the following (when two dates are given, these refer to the different dates on which the agreement was officially ratified by the two organisations):

1. Food and Agriculture Organisation of the United Nations (FAO)
   Agreement signed in 1952/1953

2. World Health Organisation (WHO)
   Agreement signed in 1961/1962

3. Inter-American Institute for Cooperation on Agriculture (IICA)
   Agreement signed in 1981

4. Pan American Health Organisation/World Health Organisation
   (PAHO)
   Agreement signed in 1993