CHAPTER 1.1.1.

QUALITY MANAGEMENT IN VETERINARY TESTING LABORATORIES

SUMMARY

Valid laboratory results are essential for diagnosis, surveillance, and trade. Such results may be achieved by the use of good management practices, valid test and calibration methods, proper technique, quality control, and quality assurance, all working together within a quality system. These subjects comprise one complex area of critical importance in the conduct of testing and in the interpretation of test results. This subject may be called laboratory quality management, and includes managerial, operational, and technical elements. A quality management programme should enable the laboratory to demonstrate that it operates a viable quality system, is technically competent, and is able to generate technically valid results. Additionally, a laboratory should implement a quality management programme that is appropriate for its mandate, clients, needs, and goals, and that can be shown to be effective in meeting quality objectives. The need for the mutual recognition of test results for international trade and the acceptance of international standards such as the ISO/IEC\(^1\) International Standard 17025 (4) for laboratory accreditation also affect the need and requirements for laboratory quality management programmes. The OIE has published detailed standard on this subject (7). This chapter is not intended to reiterate the requirements of these ISO or OIE documents. Rather, it outlines the important issues and considerations a laboratory should address in the design and maintenance of its quality management programme.

KEY CONSIDERATIONS FOR THE DESIGN AND MAINTENANCE OF A LABORATORY QUALITY MANAGEMENT PROGRAMME

In order to ensure that the quality management programme is appropriate and effective, the design must be carefully thought out. The major categories of consideration and the key issues and activities within each of these categories are outlined in the following seven sections of this chapter.

1. THE WORK, RESPONSIBILITIES, AND GOALS OF THE LABORATORY

   Many factors affect the necessary elements and requirements of a quality management programme. These factors include:

   i) The type of testing done;
   ii) The use of the test results;
   iii) The impact of a questionable or erroneous result;
   iv) The tolerance level of risk and liability;
   v) Client needs (e.g. sensitivity and specificity of the test method, costs, turnaround time);
   vi) The role of the laboratory in legal work or in regulatory programmes;

\(^{1}\) International Organization for Standardization/International Electrochemical Commission.
vii) The role of the laboratory in assisting with, confirming, and/or overseeing the work of other laboratories; and

viii) The business goals of the laboratory, including the need for any third party recognition and/or accreditation.

2. STANDARDS, GUIDES, AND REFERENCES

It is recommended that the laboratory choose reputable and accepted standards and guides to assist in designing the quality management programme. The OIE standard on this subject is a useful guideline (7). For laboratories seeking accreditation, the use of ISO/IEC 17025 (4) and/or the OIE standard (7) will be essential. Further information on standards may be obtained from the national standards body of each country, from the International Laboratory Accreditation Cooperation (ILAC), and from accreditation bodies (e.g. the National Association of Testing Authorities [NATA], Australia; the American Association for Laboratory Accreditation [A2LA], United States of America; the United Kingdom Accreditation Service [UKAS], United Kingdom; and the Standards Council of Canada [SCC], Canada). Technical and international organisations such as the AOAC International (formerly the Association of Official Analytical Chemists) and the ISO publish useful references, guides, and/or standards that supplement the general requirements of ISO/IEC 17025. ISO International Standard 9001 (5), a general standard for quality management systems and one of the many standards in the group commonly termed the ‘ISO 9000 series’, is not usable for accreditation, as conformity with its requirements does not necessarily ensure or imply technical competence (see Section 3. below). While a laboratory may implement a quality management system meeting the requirements of ISO 9001, registration or certification is used to indicate conformity with this standard.

3. ACCREDITATION

If the laboratory has determined that it needs formal recognition of its quality management programme, then third party verification of its conformity with the selected standard(s) will be necessary. ILAC has published specific requirements and guides for laboratories and accreditation bodies. Under the ILAC system, ISO/IEC 17025 is to be used for accreditation. Definitions regarding laboratory accreditation may be found in ISO/IEC International Standard 17000 (2). Accreditation is tied to competence and this is significant as it means much more than having and following documented procedures. Having competence also means that the laboratory:

i) Has technically valid and validated test methods, procedures, and specifications that are documented in accordance with the requirements of the selected standard(s) and/or guidelines;

ii) Has adequate qualified personnel who understand the science behind the procedures;

iii) Has correct and adequate equipment;

iv) Has adequate facilities and environmental control;

v) Has procedures and specifications that ensure accurate and reliable results;

vi) Can foresee technical needs and problems;

vii) Can cope with and prevent technical problems that may arise;

viii) Can accurately estimate and control the uncertainty in testing; and

ix) Can demonstrate proficiency to conduct the test methods used.

4. SELECTION OF AN ACCREDITATION BODY

In order for accreditation to facilitate the acceptance of the laboratory’s test results for trade, the accreditation must be recognised by the international community. Therefore, the accreditation body should be recognised as competent to accredit laboratories. Programmes for the recognition of accreditation bodies are, in the ILAC scheme, based on the requirements of ISO/IEC International
Standard 17011 (3). One may obtain information on recognised accreditation bodies from the organisations that recognise them, such as the National Cooperation for Laboratory Accreditation (NACLA), the Asia-Pacific Laboratory Accreditation Cooperation (APLAC), the Interamerican Accreditation Cooperation (IAAC), and the European Co-operation for Accreditation (EA).

5. **DETERMINATION OF THE SCOPE OF THE QUALITY MANAGEMENT PROGRAMME AND/OR OF THE LABORATORY’S ACCREDITATION**

The quality management programme should ideally cover all areas of activity affecting all testing that is done at the laboratory. However, for the purpose of accreditation, the laboratory should determine the scope of testing to be included in the accreditation. Factors that might affect the laboratory’s choice of scope of accreditation include:

i) The availability and cost of necessary personnel, facilities and equipment;

ii) The cost of environmental monitoring against the possibility of cross contamination;

iii) The deadline for accreditation;

iv) The impact of the test results;

v) The number of tests done;

vi) Whether the testing done is routine or non-routine;

vii) Whether any part of testing is subcontracted out;

viii) The quality assurance necessary for materials, reagents and media;

ix) The availability of reference standards (e.g. standardised reagents, internal quality control samples, reference cultures);

x) The availability of proficiency testing;

xi) The availability, from reputable sources, of standard and/or fully validated test methods;

xii) The evaluation and validation of test methods to be done; and

xiii) The technical complexity of the method(s).

Accreditation bodies also accredit the providers and operators of proficiency testing programmes, and may require the use of an accredited provider in order to issue the laboratory a certificate of accreditation.

6. **TEST METHODS**

ISO/IEC 17025 requires the use of appropriate test methods and has requirements for selection, development, and validation. The OIE document (7) also provides requirements for selection and validation.

In the veterinary profession, other standard (methods published in international, regional, or national standards) or fully validated methods (methods having undergone a full collaborative study and that are published or issued by an authoritative technical body such as the AOAC International), while preferable to use, may not be available. Many veterinary laboratories develop or modify methods, and most of these laboratories have test programmes that use non-standard methods, or a combination of standard and non-standard methods. In veterinary laboratories, even with the use of standard methods, some in-house evaluation, optimisation, and/or validation generally must be done to ensure valid results.

Clients and laboratory staff must have a clear understanding of what performance can be expected from a test. Many veterinary testing laboratories will therefore need to demonstrate competence in the development, adaptation, and validation of test methods.
This *Aquatic Manual* provides more detailed and specific guidance on test selection, optimisation, standardisation, and validation in Chapter 1.1.2. The following items discuss test method issues that are of most interest to those involved in the quality management of the laboratory.

### a) Selection of the test method

Valid results begin with the selection of an appropriate test method for the diagnostic issues at hand. Considerations for the selection of a test method include:

1. International acceptance;
2. Scientific acceptance;
3. Method not outdated;
4. Performance characteristics (e.g. analytical and diagnostic sensitivity and specificity, repeatability, reproducibility, isolation rate, lower limit of detection, precision, trueness, and uncertainty);
5. Behaviour in species and population of interest;
6. Resources and time available for development, adaptation, and/or evaluation;
7. Performance time and turnaround time;
8. Type of sample (e.g. serum, tissue) and its expected quality or state on arrival at the laboratory;
9. Analyte (e.g. antibody, antigen);
10. Resources and technology of the laboratory;
11. Nature of the intended use (e.g. export, import, surveillance, screening, confirmatory, individual animal use, herd use);
12. Client expectations;
13. Safety factors;
14. Number of tests to be done;
15. Cost of test, per sample;
16. Existence of reference standards, including reference materials; and
17. Availability of proficiency testing schemes.

### b) Optimisation and standardisation of the test method

Once the method has been selected, it must be set up at the laboratory. Whether the method was developed in-house or imported from an outside source, generally some additional optimisation is necessary. Optimisation is a series of experiments and subsequent data analysis. Optimisation establishes critical specifications and performance standards for the test process and for use in monitoring the correct performance of the test. Optimisation should ensure that a method is brought under statistical control. Optimisation should also determine:

1. Critical specifications for equipment and instruments;
2. Critical specifications for reagents (e.g. chemicals, biologicals);
3. Critical specifications for reference standards, reference materials, and internal controls;
4. Robustness (if applicable);
5. Critical control points and acceptable ranges, attributes or behaviour at critical control points, using statistically acceptable procedures;
6. The quality control activities necessary to monitor critical control points;
vii) The type, number, range, frequency, and/or arrangement of test run controls needed;
viii) The requirements for control behaviour for the non-subjective acceptance or rejection of test results;
ix) The elements of a fixed, documented test method for use by laboratory staff; and
x) The level of technical competence required of those who carry out and/or interpret the test.

c) Validation of the test method

Validation further evaluates the test for its fitness for a given use. Validation establishes performance characteristics for the test method, such as sensitivity, specificity, and isolation rate; and diagnostic parameters such as positive/negative cut-off, and titre of interest or significance. Validation should be done using an optimised, documented, and fixed procedure. Depending on logistical and risk factors, validation may involve any number of activities and amount of data, with subsequent data analysis using appropriate statistics. Test validation is covered in Chapter 1.1.2 Principles of validation of diagnostic assays for infectious diseases, and Chapter 1.1.3 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases.

Validation activities might include:

i) Field and/or epidemiological studies;
ii) Comparison with other methods, preferably standard methods;
iii) Comparison with reference standards (if available);
iv) Collaborative studies with other laboratories using the same documented method, and including the exchange of samples, preferably of undisclosed composition or titre. It is preferable that these be issued by a qualified conducting laboratory that organises the study and evaluates the results provided by the participants;
v) Reproduction of data from an accepted standard method, or from a reputable publication;
vi) Experimental infection studies; and
vii) Analysis of internal quality control data.

Validation is always a balance between costs, risks, and technical possibilities. Experienced accreditation bodies know that there are many cases in which quantities such as accuracy and precision can only be given in a simplified way.

d) Uncertainty

Laboratories should be able to estimate the uncertainty of the test methods as performed in the laboratory. This includes methods used by the laboratory to calibrate equipment (4).

The determination of measurement uncertainty (MU) is not new to some areas of measurement sciences. However, the application of the principles of MU to laboratories for the life sciences is new. Most of the work to date regarding MU is for areas of testing other than the life sciences, and much of the work has been theoretical. However, as accreditation becomes more important, applications are being developed for the other areas. It is important to note that MU does not imply doubt about the validity of a test result or other measurement, nor is it equivalent to error, as it may be applied to all test results derived from a particular procedure. It may be viewed as a quantitative expression of reliability, and is commonly expressed as a number after a +/− sign (i.e. the true value lies within the stated range, as MU is expressed as a range). Standard deviation and confidence interval are examples of the expression of MU. An example of the use of standard deviation to express uncertainty is the allowed limits on the test run controls for an enzyme-linked immunosorbent assay, commonly expressed as +/− n SD.
Although the determination and expression of MU has not been standardised for veterinary testing laboratories (or medical, food, or environmental), some sound guidance exists.

MU must be estimated in the laboratory for each method included in the scope of accreditation. This can be estimated by a series of tests on control samples. MU can also be estimated using published characteristics (6), but the laboratory must first demonstrate acceptable performance with the method. Government agencies may also set goals for MU values for official methods (e.g. Health Canada). Reputable technical organisations, including accreditation bodies (e.g. AOAC International, ISO, NATA, A2LA, SCC, UKAS, Eurachem, and the Co-Operation on International Traceability in Analytical Chemistry [CITAC]) teach courses and/or provide guidance on MU for laboratories seeking accreditation. Codex Alimentarius, which specifies standards for food testing, has taken the approach that it is not necessary for a laboratory to take a further estimate of MU if the laboratory complies with Codex principles regarding quality: i.e. that the laboratory is accredited to ISO/IEC 17025, and therefore uses validated methods (e.g. knows applicable parameters such as sensitivity and specificity, as well as the confidence interval around such parameters), participates in proficiency testing programmes and collaborative studies, and uses appropriate internal quality control procedures.

The requirement for “use of appropriate internal quality control procedures” implies that the laboratory must use quality control procedures that cover all major sources of uncertainty. There is no requirement to cover each component separately. Components can be estimated with experiments in the laboratory (Type A estimates), or from other sources (reference materials, calibration certificates, etc.) (Type B estimates). A traditional control sample procedure, run many times by all analysts and over all shifts, usually covers all the major sources of uncertainty except perhaps sample preparation. The variation of the control samples can be used as an estimate of those combined sources of uncertainty.

ISO/IEC 17025 requires the laboratory to identify all major sources of uncertainty, and to obtain reliable estimates of MU. Laboratories may wish to establish acceptable specifications, criteria, and/or ranges at critical control points for each component. Where appropriate, laboratories can implement appropriate quality control at the critical points associated with each source, or seek to reduce the size of a component. Sources of uncertainty include sampling, storage conditions, sample effects, extraction and recovery, reagent quality, reference material purity, volumetric manipulations, environmental conditions, contamination, equipment effects, analyst or operator bias, and other unknown or random effects. The laboratory would also be expected to account for any known systematic error. (See also Section 6.b. steps i–vii.) Systematic errors (bias) must either be corrected by changes in the method, adjusted mathematically, or have the bias noted in the report statement. If an adjustment is made to the procedure, there may or may not be a need to reassess uncertainty. If there is an adjustment made to correct for bias, then a new source of uncertainty is introduced (the uncertainty of the correction). This must be added to the MU estimate.

There are three principal ways to estimate MU:

1. The components approach (or ‘bottom-up’ approach), where all the sources of uncertainty are identified, reasonable estimates are made for each component, a mathematical model is developed that links the components, and the variations are combined using rules for the propagation of error (1).

2. The control sample approach (or ‘top-down’ approach), where measurements on a stable control material are used to estimate the combined variation of many components. Variation from additional sources needs to be added.

3. The method characteristics approach, where performance data from a valid collaborative study are used as combined uncertainties (other sources may need to be added). Laboratories must meet defined criteria for bias and repeatability for the MU estimates to be valid. These
should be larger than would be obtained by competent laboratories using their own control samples or components model.

e) **Implementation and use of the test method**

Analysts should be able to demonstrate proficiency in using the test method prior to producing reported results, and on an ongoing basis.

The laboratory should ensure, using appropriate and documented project management, records keeping, data management, and archiving procedures, that it can recreate at need all events relating to test selection, development, optimisation, standardisation, validation, implementation, and use. This includes quality control and quality assurance activities.

7. **STRATEGIC PLANNING**

Continuous improvement is essential. It is recommended that the laboratory be knowledgeable of and stay current with the standards and methods used to demonstrate laboratory competence and to establish and maintain technical validity. The methods by which this may be accomplished include:

i) Attendance at conferences;

ii) Participation in local and international organisations;

iii) Participation in writing national and international standards (e.g. participation on ILAC and ISO committees);

iv) Consulting publications;

v) Visits to other laboratories;

vi) Conducting research;

vii) Participation in cooperative programmes (e.g. Inter-American Institute for Cooperation in Agriculture);

vii) Exchange of procedures, methods, reagents, samples, personnel, and ideas; and

ix) Wherever possible, accreditation by a third party that is itself recognised as competent to issue the accreditation.

**REFERENCES**


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Chapter 1.1.2.

PRINCIPLES OF VALIDATION OF DIAGNOSTIC ASSAYS FOR INFECTIOUS DISEASES

Summary

Validation is the evaluation of a process to determine its fitness for a particular use and includes assay optimization and demonstration of performance characteristics. A validated assay yields test results that identify the presence of a particular analyte (e.g., an antibody) and allows predictions to be made about the status of the test subjects. Assays applied to individuals or populations have many purposes, such as aiding in: documenting freedom from disease in a country or region, preventing spread of disease through trade, eradicating an infection from a region or country, confirming diagnosis of clinical cases, estimating infection prevalence to facilitate risk analysis, identifying infected animals toward implementation of control measures, and classifying animals for herd health or immune status post-vaccination. A single assay may be validated for one or several intended purposes by optimizing its performance characteristics for each purpose (e.g., setting diagnostic sensitivity [DSe] high [such as 99.99%] with associated lower diagnostic specificity [DSp] for a screening assay, or conversely, setting DSp high with associated lower DSe for a confirmatory assay).

The principles of validation discussed in this chapter will focus primarily on methods to detect antibody in sera. However, these same principles could be applied to validation of tests for other analytes in sera or tissues. Chapter 1.1.3 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases extends the principles outlined here to a direct method of infectious agent detection, the molecular diagnostic assays.

By considering the variables that affect an assay’s performance, the criteria that must be addressed in assay validation become clearer. The variables can be grouped into three categories: (a) the sample – host/organism interactions affecting the analyte composition and concentration in the serum sample; (b) the assay system – physical, chemical, biological and technician-related factors affecting the capacity of the assay to detect a specific analyte in the sample; and (c) the test result – the capacity of a test result, derived from the assay system, to predict accurately the status of the individual or population relative to the analyte in question.

Factors that affect the concentration and composition of analyte in the serum sample are mainly attributable to the host and are either inherent (e.g., age, sex, breed, nutritional status, pregnancy, immunological responsiveness) or acquired (e.g., passively acquired antibody, active immunity elicited by vaccination or infection). Nonhost factors, such as contamination or deterioration of the sample, may also affect the analyte in the sample.

Factors that interfere with the analytical accuracy of the assay system include instrumentation, technician error, reagent choice (both chemical and biological) and calibration, accuracy and acceptance limits of controls, reaction vessels, water quality, pH and ionicity of buffers and diluents, incubation temperatures and durations, and error introduced by detection of closely related analytes, such as antibody to cross-reactive organisms, rheumatoid factor, or heterophile antibody.

Measures that influence the capacity of the test result to predict accurately the infection or analyte status of the host are DSe, DSp, and prevalence of the disease in the population targeted by the assay. DSe and DSp are derived from test results on samples obtained from selected reference animals of known status for

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1 In this chapter, the terms ‘positive’ and ‘negative’ have been reserved for test results and never refer to infection or antibody/antigen status of the host. Whenever reference is made to ‘infection’ or ‘analyte’, any method of exposure to an infectious agent that could be detected directly (e.g., antigen) or indirectly (e.g., antibody) by an assay, should be inferred.
the target analyte. The methods used to select the reference animals are critical to the accuracy of the estimates (5). The degree to which the reference animals represent all of the host and environmental variables in the population targeted by the assay has a major impact on the accuracy of test-result interpretation. For example, experienced diagnosticians are aware that an assay, validated by using samples from northern European cattle, may not give valid results for the distinctive populations of cattle in Africa.

The capacity of a positive or negative test result to predict accurately the infection and/or exposure status of the animal or population of animals is the most important consideration of assay validation. This capacity is not only dependent on a highly precise and accurate assay using well characterised and standardised reagents and carefully derived estimates of DSe and DSp, but is heavily influenced by prevalence of the infection in the targeted population or the likelihood that an animal is infected based on clinical criteria. Without a current estimate of the disease prevalence in that population or likelihood of infection in an individual animal, the interpretation of a positive or negative test result may be compromised.

Many factors obviously must be addressed before an assay can be considered to be "validated" (5, 16). However, there is no consensus whether the concept of assay validation is a time-limited process during which only those factors intrinsic to the assay are optimised and standardised, or whether the concept includes an ongoing validation of assay performance for as long as the assay is used. Accordingly, the term 'validated assay' elicits various interpretations among laboratory diagnosticians and veterinary clinicians. Therefore, a working definition of assay validation is offered as a context for the guidelines outlined below. Ideally, all diagnostic assays would be fully validated for one or more purposes, but in practice there are sometimes limitations to the completeness of validation.

A. DEFINITIONS OF ASSAY VALIDATION

Definition 1. From the perspective of laboratory results obtained from an assay over time, a validated assay consistently provides test results that identify animals as positive or negative for an analyte or process (e.g. antibody, antigen, or induration at skin test site) and, by inference, accurately predicts the infection and/or exposure status of animals with a predetermined degree of statistical certainty. This is referred to as analytical validity.

Definition 2. From the perspective of an assay developer or user, assay validation is the development and verification of test method performance characteristics at a defined level of statistical confidence for a particular target population. This is also known as diagnostic validity.

For either definition, the assay is valid only insofar as its performance characteristics are consistent with the purpose for which the assay is intended.

This chapter will focus on the principles underlying development and maintenance of a validated assay. Previous iterations of this chapter (12) were condensed renditions of a review article (9). At that time, the goal was to fulfil Definition 1 of assay validation. In this expanded update, the content is reorganised into the parts of assay validation consistent with the format of the OIE Validation Template, and embraces both Definitions 1 and 2 of assay validation. In addition to the validation process per se, guidance is offered on scientifically sound methods for development, maintenance, and extension of validation criteria for a given assay.

It must be emphasised that an assay, when applied to target populations, will minimise misclassifications of animals as false positive or false negative only to the extent that validity is assured for all elements of the assay validation process (see Section C). This assumes that the assay is fit for the purpose for which it

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2 In this definition, the DSe and DSp are performance characteristics of the assay for a given target population. They determine - together with the disease prevalence in the population - the probability that a given test result reflects the true status of the animal. An assay can be recognised as validated if reliable estimates of DSe and DSp for a given target population are available. This does not imply any minimum threshold values for these parameters. In practical applications, low values of DSe and DSp or diagnostic problems due to low disease prevalence are compensated by the sampling design or by combining multiple diagnostic assays into parallel or serial testing regimens. The selection of assays, the sampling process, the combination of multiple assays into a testing regimen and the interpretation rule for the results define the diagnostic process.
is intended (e.g. a confirmatory assay will likely yield many false-negative results if used as a screening assay). It also assumes that a well designed and documented test method and proper standardised reagents, in combination with well-trained technicians, will give a stable assay within the laboratory. Furthermore, it assumes a thorough use of the most rigorous experimental design and epidemiological and statistical tools. These are required to reduce bias, random error, and false assumptions about the reference population of animals upon which the assay performance estimates are made (5). Finally, it assumes that when placed in practice, the assay is conducted within the context of a rigorous quality assurance programme.

B. ASSAY VALIDATION – INTRODUCTION

1. SELECTION OF AN ASSAY FIT FOR ITS INTENDED PURPOSE

The OIE Standard for Management and Technical Requirements for Laboratories Conducting Tests for Infectious Animal Diseases (14) is a specific interpretation of the more generally stated requirements of the ISO/IEC 17025:2005 international quality standard for testing laboratories (8). The OIE Quality Standard clearly states that test methods and related procedures must be appropriate for specific diagnostic applications in order for the test results to be of any relevance. In other words, the assay must be ‘fit for purpose’. The Quality Standard further states that in order for a test method to be considered appropriate, it must be properly validated and that this validation must respect the principles outlined in the validation chapters of this Aquatic Manual.

While this chapter deals with validation and fitness for purpose from a scientific perspective, it should also be noted that other factors may impact the relevance of an assay with respect to fitness for purpose. These factors include not only the diagnostic suitability of the assay, but also its acceptability by scientific and regulatory communities, acceptability to the client, and feasibility given available laboratory resources. Operational requirements are often overlooked and may include running costs, equipment requirements, level of technical sophistication and interpretation skills, kit/reagent availability, shelf life, transport requirements, safety, biosecurity, sample throughput, test turn-around times, aspects of quality control and quality assurance.

As outlined in the background information in Certification of diagnostic assays on the OIE website (www.oie.int), the first step is selection of an assay type that likely can be validated for a particular use. The intended purpose(s) of an assay have been broadly defined as:

a) to demonstrate population ‘freedom’ from infection (prevalence apparently zero)
   i) ‘free’ with and/or without vaccination
   ii) historical ‘freedom’
   iii) re-establishment of ‘freedom’ following outbreaks;

b) to demonstrate freedom from infection in individual animals or products for trade purposes;

c) to demonstrate efficacy of eradication policies;

d) to confirm diagnosis of clinical cases;

e) to estimate prevalence of exposure or infection to facilitate risk analysis (surveys, classification of herd health status, implementation of disease control measures);

f) to determine immune status of individual animals or populations (post-vaccination).

As previously stated, when considering an assay for a specific purpose, other ‘fitness’ factors should be considered in the initial decision making process. Operational requirements are often overlooked and may include; running costs, equipment requirements, kit/reagent availability, shelf life, transport requirements, safety, biosecurity, sample throughput, test turn-around-times, etc.

2. INITIAL ASSAY DEVELOPMENT CONSIDERATIONS
An indirect enzyme-linked immunosorbent assay (ELISA) for detection of antibody will be used in this chapter to illustrate the principles of assay validation. It is a type of assay that can be difficult to validate because of signal amplification of both specific and nonspecific components (2). This methodology serves to highlight the problems that need to be addressed in any assay validation process. The same basic principles are used in validation of other complex or simple assay formats. Chapter 1.1.3 Validation and quality control of polymerase chain reaction methods used for the diagnosis of infectious diseases describes the principles for validating gene-amplification techniques.

Selection of appropriate samples, calibrated instrumentation, and a relevant methodology to achieve the intended purpose are critical elements in assay validation. Continuity in experiments is assured when reagents and samples are chosen, properly prepared, aliquoted, and stored for use in each experiment. This reduces the variability and provides data needed to establish appropriate controls for ensuring each run of the assay is valid.

a) Control samples

It is useful to select four or five samples (serum in our example) that range from high to low levels of antibodies against the infectious agent in question. In addition, a sample containing no antibody is required. These samples will be used to optimise the assay reagents and protocol during feasibility studies, and later as control samples. The samples ideally should represent known infected and uninfected animals from the population that eventually will become the target of the validated assay. The samples should have given expected results in one or more serological assay(s) other than the one being validated. The samples are preferably derived from individual animals, but they may represent pools of samples from several animals. A good practice is to prepare a large volume (e.g. 10 ml or more if possible) of each sample and divide it into 0.1 ml aliquots for storage at or below −20°C. One aliquot of each sample is thawed, used for experiments, and ideally then discarded. If it is impractical to discard the aliquot, it may be held at +4°C between experiments for up to about 2 weeks; however, there is a possibility of sample deterioration under these circumstances. Then, another aliquot is thawed for further experimentation. This method provides the same source of serum with the same number of freeze–thaw cycles for all experiments (repeated freezing and thawing of serum can denature antibodies so should be avoided). Also, variation is reduced when the experimenter uses the same source of serum for all experiments rather than switching among various sera between experiments. This approach has the added advantage of generating a data trail for the repeatedly run samples. After the initial stages of assay validation are completed, one or more of the samples can become the serum control(s) that are the basis for data expression and repeatability assessments both within and between runs of the assay. They may also serve as standards if their activity has been predetermined; such standards provide assurance that runs of the assay are producing accurate data (16).

It is highly desirable to include OIE International Standard Sera or other international standard sera if they are available. This may lead to harmonisation between the assay under development and a standard test method in which international standard sera are normally used (15).

b) Selection of method to achieve normalised results

Normalisation adjusts raw test results of all samples relative to values of controls included in each run of the assay (not to be confused with transformation of data to achieve a ‘normal’ [Gaussian] distribution). The method of normalisation and expression of data should be determined, preferably no later than at the end of the feasibility studies. Comparisons of results from day to day and between laboratories are most accurate when normalised data are used. For example, in ELISA systems, raw optical density (absorbance) values are absolute measurements that are influenced by ambient temperatures, test parameters, and photometric instrumentation. To account for this variability, results are expressed as a function of the reactivity of one or more serum control samples that are included in each run of the assay. Data normalisation is accomplished in the indirect ELISA by expressing absorbance values in one of several ways (16).
A simple and useful method is to express all absorbance values as a percentage of a single high-positive serum control that is included on each plate (Sample/Positive or S/P ratio). (This control must yield a result that is in the linear range of measurement.) This method is adequate for most applications. More rigour can be brought to the normalisation procedure by calculating results from a standard curve generated by several serum controls. It requires a more sophisticated algorithm, such as linear regression or log-logit analysis. This approach is more precise because it does not rely on only one high-positive control sample for data normalisation, but rather uses several serum controls, adjusted to expected values, to plot a standard curve from which the sample value is extrapolated. This method also allows for exclusion of a control value that may fall outside expected confidence limits.

For assays that are end-pointed by sample titration, such as serum (viral) neutralisation, each run of the assay is accepted or rejected based on whether control values fall within predetermined limits. Because sample values usually are not adjusted to a control value, the data are not normalised by the strict definition of the term.

Whatever method is used for normalisation of the data, it is essential to include additional controls for any reagent that may introduce variability and thus undermine attempts to achieve a validated assay. The normalised values for those controls need to fall within predetermined limits (e.g. within an appropriate multiple of the standard deviation of the mean of many runs of each control). The chosen limits should reflect a reasonable and tolerable assay run rejection rate and an acceptable risk that some test samples may be misclassified.

C. ASSAY VALIDATION – PART 1

1. OPTIMISATION AND STANDARDISATION OF REAGENTS

Using control sera as outlined in section B.2.a of this chapter, the optimal concentrations/dilutions of the antigen adsorbed to the plate, serum, enzyme–antibody conjugate, and substrate solution are determined through 'checkerboard' titrations of each reagent against all other reagents, following confirmation of the best choice of reaction vessels (usually evaluation of two or three types of microtitre plates, each with its different binding characteristics, to minimise background activity while achieving the maximum spread in activity between negative and high-positive samples). Additional experiments determine the optimal temporal, chemical, and physical variables in the protocol, including incubation temperatures and durations; the type, pH, and molarity of diluent, washing and blocking buffers; and equipment used in each step of the assay (for instance pipettes and washers that give the best reproducibility).

a) Linear operating range of the assay

The range of values that constitute the linear operating range of an assay is best determined by a dilution series in which a high positive serum is serially diluted in a negative serum. Each dilution is then run at the optimal working dilution in buffer, and the results plotted in the form of a 'response-curve'. This curve, sometimes referred to as a 'dose–response curve' as in pharmacological applications, establishes the linear range of assay values that are valid for use in the assay.

b) Calibration against reference reagents

i) International standards

Serum standards and other reagents, available from OIE, WHO, FAO, or other international organisations can be used to harmonise the assay with expected results gained from reference reagents of known activity.

ii) In-house standards
The in-house serum controls (used for normalisation of data) and additional secondary serum standards, such as low positive, high positive, and negative sera (used for repeatability estimates in subsequent routine runs of the assay) can be fitted to the response curve to achieve expected values for such sera.

2. REPEATABILITY

Evidence of repeatability (agreement between replicates within and between runs of the assay) is necessary to warrant further development of the assay. This is accomplished by evaluating results from a minimum of three in-house samples representing activity within the linear range of the assay. Quadruplicates of these samples are tested in at least four runs of the assay to determine within-run (intraplate) variation. Between-run variation is determined by using the same samples in a minimum of 20 runs (total), by two or more operators, preferably on separate days. All runs must be independent of each other.

For reporting purposes, ELISA, raw absorbance values are usually used to calculate repeatability during this part of validation because it is uncertain whether the results of the high-positive control serum, which could be used for calculating normalised values, are reproducible in early runs of the assay format. Also, expected values for the controls have not yet been established. Coefficients of variation (CV: standard deviation of replicates ÷ mean of replicates), generally less than 20% for raw absorbance values for most samples (low-titred samples may have larger CVs), indicates adequate repeatability at this stage of assay development. However, if evidence of excessive variation (>30%) is apparent for most samples within and/or between runs of the assay, more preliminary studies should be done to determine whether stabilisation of the assay is possible, or whether the test format should be abandoned. This is important because an assay that is inherently variable has a high probability of not withstanding the rigours of day-to-day testing on samples from the targeted population of animals.

When a serum control sample is nearing depletion, it is essential to prepare and repeatedly test a replacement before the serum control is depleted. The prospective control sample is included in 10–20 runs of the assay before depletion of the original control to establish its proportional relationship to the nearly depleted control. If the depleted sample was a positive control in ELISAs where the normalised value is expressed as a per cent of that positive control, the proportional difference in ELISA activity between the original and replacement sera must be factored into the normalisation algorithm to retain the same cut-off, and thus the same DSe and DSp in the assay. When other reagents, such as antigen for capture of antibody, must be replaced, they should be produced using the same criteria as for the original reagents, and tested in at least five runs of the assay using a panel of sera that has been designed for this purpose. Reagent lots (serials) need to be evaluated for consistency so variability is minimised in the assay as new lots are required. Whenever possible, it is important to change only one reagent at a time to avoid the compound problem of evaluating more than one variable at a time. Variability is minimised when reagents are well characterised using methods other than that of the target assay.

3. DETERMINATION OF ANALYTICAL SPECIFICITY AND SENSITIVITY

Analytical specificity of the assay is the degree to which the assay does not cross-react with other analytes and analytical sensitivity is the smallest detectable amount of the analyte in question.

Analytical specificity is assessed by use of a panel of samples derived from animals that have been exposed to genetically related organisms that may stimulate cross-reactive antibodies, or sera from animals with similar clinical presentations. This 'near neighbour analysis' is useful in determining the probability of false-positive reactions in the assay. It is also appropriate to document a group specificity criterion that includes detection of the analyte of interest in sera from animals that have experienced infections/exposure to an entire group or serotype of organisms of interest.

Analytical sensitivity is assessed by end-point dilution analysis, which indicates the dilution of serum in which the analyte is no longer detectable, or at least, is indistinguishable from the activity of negative
sera. The earliest time after exposure to an infectious agent that antibody can be detected affects analytical sensitivity. This effect can be deduced by testing serially-drawn blood samples from animals post-exposure to the agent in question. The duration of antibody presence also affects analytical sensitivity, which can be determined by long-term serial testing of experimentally infected/exposed animals.

If the intended purpose of the assay is for screening of animals for antibody activity, analytical sensitivity needs to be high to achieve the greatest probability possible for detecting infected animals. If very high analytical sensitivity is not achievable, the assay may not be fit as a screening assay. Alternatively, if confirmation of another independent diagnostic procedure is the purpose for which the assay is intended, analytical specificity is required that minimises the amount of cross-reactivity. If neither of these objectives is obtainable, the reagents need to be recalibrated, replaced, or the assay should be abandoned.

D. ASSAY VALIDATION – PART 2

1. DETERMINING ASSAY PERFORMANCE CHARACTERISTICS AFTER ESTABLISHMENT OF A STANDARD ASSAY METHOD AND REAGENT CRITERIA

Estimates of DSe and DSp are the primary performance indicators established during validation of an assay. These must be established after the assay and reagents are standardised; alteration of protocols or reagents may require reestablishment of performance characteristics. They are the basis for calculation of other parameters from which inferences are made about test results. Therefore, it is imperative that estimates of DSe and DSp are as accurate as possible. Ideally, they are derived from testing a series of samples from reference animals of known history and infection status relative to the disease/infection in question and relevant to the country or region in which the test is to be used, but that is not always possible. A sampling design must be chosen that will allow estimation of diagnostic performance characteristics. However this is a difficult process complicated by logistical and financial limitations. It is also limited by the fact that reference populations and gold standards may be lacking. The following are examples of reference populations and methodologies that may aid in determining performance characteristics of the test being validated.

a) Reference animal populations

i) Infected or exposed and uninfected or non-exposed reference animals

Selection of reference animals to evaluate performance characteristics requires that the variables attributable to the target population are represented in the infected/exposed and uninfected/unexposed reference animal populations. The variables include but are not limited to species, age, sex, breed, nutritional status, pregnancy, stage of infection, immunological status including vaccination history, and historical, epidemiological, and/or clinical data including herd disease history should be noted and considered.

ii) Reference animal status determined by other assays

In serology, the ‘standard of comparison’ is the results of a method or combination of methods with which the new assay is compared. Although the term ‘gold standard’ is commonly used to describe any standard of comparison, it should be limited to methods that unequivocally classify animals as infected or uninfected. Some isolation methods themselves have problems of repeatability and sensitivity. Gold standard methods include unequivocal isolation of the agent or pathognomonic histopathological criteria.

Because a true gold standard may be lacking or is impossible to achieve, relative standards of comparison are often necessary; the most common of these include results from other serological assays. Calculations of DSe and DSp are most reliable when the gold standard of comparison is available. When only relative standards of comparison are available, estimates of DSe and DSp for the new assay may be compromised because the error in the estimates of DSe and DSp for the relative standard is carried over into those estimates for the new
ash. Indeed, when using imperfect reference tests without efforts to control for any biases, the DSe and DSp performance estimates of the new test will be flawed and thus unacceptable.

iii) Experimentally infected or vaccinated reference animals

Sera obtained sequentially from experimentally infected or vaccinated animals have been used to ‘validate’ a new assay. Such repeated observations from the same animals are not acceptable for establishing estimates of DSe and DSp because the statistical requirement of independent observations is violated. Thus, time-point sampling of individual experimental animals is necessary. Also, exposure to organisms under experimental conditions, or vaccination may elicit antibody responses that are not quantitatively and qualitatively typical of natural infection in the target population (9). The strain of organism, dose, and route of administration to experimental animals are examples of variables that may introduce error when extrapolating DSe and DSp estimates to the target population. For these reasons, validation of an assay should not solely be based on experimental animals.

iv) Reference animals – Status unknown

When it is not possible to assemble sera from animals of known infection status, it is possible to estimate DSe and DSp by non-gold standard methods or latent class models (3, 7). As these statistical models are complex, an expert should be consulted to provide assistance on proper ways to conduct and describe the sampling from the target population(s), the characteristics of other tests included in the analysis, the appropriate choice of model and the estimation methods based on peer-reviewed literature.

2. THRESHOLD DETERMINATION

To achieve performance estimates of DSe and DSp of the new assay, the test results first must be reduced to categorical (positive or negative) status. This is accomplished by insertion of a cut-off point (threshold or decision limit) on the continuous scale of test results. Although many methods have been described for this purpose, three examples will illustrate different approaches, together with their advantages and disadvantages. The first is a cut-off based on the frequency distributions (9) of test results from uninfected and infected reference animals. This cut-off can be established empirically by visual inspection of the frequency distributions, by receiver-operator characteristics (ROC) analysis (6, 17), or by selection that favours either DSe or DSp, depending on the intended use for a given assay (11). A second approach is establishing a cut-off based only on uninfected reference animals, for instance the 99th percentile in a frequency distribution of assay values for uninfected reference animals; this provides an estimate of DSp but not DSe. The third method provides an ‘intrinsic cut-off’ based on test results from sera drawn randomly from within the target population with no prior knowledge of the animals’ infection status (4).

If considerable overlap occurs in the distributions of test values from known infected and uninfected animals, it is difficult to select a cut-off that will accurately classify these animals according to their infection status. Rather than a single cut-off, two cut-offs can be selected that define a high DSe (e.g. inclusion of 99% of the values from infected animals), and a high DSp (e.g. 99% of the values from uninfected animals). The values that fall between these percentiles would then be classified as suspicious or equivocal, and would require testing by a confirmatory assay or retesting for detection of seroconversion.

The selection of the cut-off will typically reflect the intended purpose of the assay. For example, a screening assay designed for high DSe versus a confirmatory assay designed for high DSp will require different cut-offs in the same assay system. Although the intended purpose will dictate the cut-off, a ROC analysis is still desirable, as it will show the potential performance of the assay in other epidemiological settings.

3. ASSAY PERFORMANCE ESTIMATES
a) Number of reference animals required

The number and source of reference samples coupled with the methodologies used to derive DSe and DSp estimates are of paramount importance if the assay is ever to be properly validated for use in the population of animals targeted by the assay. It is possible to calculate the number of reference samples, from animals of known infection/exposure status, required for determinations of DSe and DSp that will have statistically defined limits. Formulae and tables for determining the number of samples required are provided elsewhere (5, 9).

b) DSe and DSp estimates based on reference animals with defined infection status

The selection of a cut-off allows classification of test results into positive or negative categories. Calculations of DSe and DSp are aided by associating the positive/negative categorical data with the known infection status for each animal using a two-way (2 × 2) table (Table 1). After the cut-off is established, results of tests on standard sera can be classified as true positive (TP) or true negative (TN) if they are in agreement with those of the gold standard (or other standard of comparison). Alternatively, they are classified as false positive (FP) or false negative (FN) if they disagree with the standard. Diagnostic sensitivity is calculated as TP/(TP + FN) whereas diagnostic specificity is TN/(TN + FP); the results of both calculations are usually expressed as percentages (Table 1).

Table 1. Calculations of DSe and DSp aided by a 2 × 2 table that associates infection status with test results from 2000 reference animals

<table>
<thead>
<tr>
<th>Test Result</th>
<th>Infected (n = 600)</th>
<th>Uninfected (n = 1400)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>570</td>
<td>46</td>
</tr>
<tr>
<td>Negative</td>
<td>30</td>
<td>1354</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>TP</th>
<th>FN</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP</td>
<td>570</td>
<td>600</td>
</tr>
<tr>
<td>FN</td>
<td>46</td>
<td>1400</td>
</tr>
</tbody>
</table>

Diagnostic sensitivity = TP/(TP + FN) = 570/(570 + 46) = 95.0%
Diagnostic specificity = TN/(TN + FP) = 1354/(1354 + 1354) = 96.7%

c) DSe and DSp estimates based on animals with infection status not defined

As mentioned above, these statistical models are complex, expert advice should sought not only in the design of the evaluation study but the interpretation of the estimates of DSe and DSp as well. It has been recommended to the OIE that an expert group be formed to address the application of latent class models and to draft guidelines for models as they apply to the validation and certification assays by the OIE.

4. COMPARISON AND HARMONISATION OF ASSAYS

For the most part, new assays are developed to improve on existing techniques. In order to demonstrate that a new assay is an improvement over an existing technique, there must be some form of comparison that demonstrates the improvement. The comparison may be related to analytical and/or diagnostic performance characteristics. It may also be related to operational characteristics such as cost, ruggedness, turn-around-times, throughput, etc. If the new assay is to be incorporated into a diagnostic regimen involving other test methods, the rationale for its use, interpretation of data and decision making should be stated.
When an international standard method (15) is available for detection of an analyte, it is possible to harmonise the performance of that method with the one under development. This process requires use of the same serum controls and/or standards in both assays. If OIE Standard Sera or other international standard sera are available, preferably at least three (negative, low positive, and high positive), they should be included in the assay-comparison study. This could lead to a new assay that is indexed to an international standard method and international standard sera (15). Harmonisation of the two assays may then be realised.

It is critical that all samples, test reagents, and the protocol or instructions for running the assay be controlled. If the reagents will not be supplied from a common source, the laboratories should produce and characterise the reagents independently. This will allow determination of the adequacy of the protocol for reagent production and characterisation. This provides data needed to determine whether it is necessary to establish a single shared source of well characterised reagents. Part of the evaluation is the determination that the protocol or instructions are complete, clear and precise. If verbal instructions are required, the developer should consider revision of the protocol to ensure they are comprehensive. If it is determined that the protocol or instructions were interpreted in a different manner, then they should be rewritten and the reproducibility may need to be re-established using the revised protocol or instructions.

E. ASSAY VALIDATION – PART 3

1. ESTABLISHING REPRODUCIBILITY OF THE ASSAY

An assay intended for distribution to many laboratories (such as a commercial kit) must be evaluated for reproducibility, which is defined as the ability of a test method to provide consistent results when applied to aliquots of the same samples tested at different laboratories. This is accomplished by testing a panel of sera in a minimum of three laboratories using the identical test method and serum panels.

A test panel consisting of a minimum of 20 samples is assembled for this purpose. Ideally, these will be individual samples from animals within the target population, representing the range of assay activity anticipated in that population. If such samples are not available, dilution of a high positive with a negative serum to achieve the range of activity is acceptable but not optimal. Replicates of about 20% of the samples are desirable as a check on repeatability within each participating laboratory. Each sample is aliquoted, rendering a series of identical panels for distribution to other laboratories. The sample identity is encoded for blind testing, and each panel is handled, transported to participating laboratories, and stored identically.

The descriptive statistics for test panel data accumulated from the laboratories includes mean, standard deviation, and range of results for each sample as well as controls. Evaluation of precision and accuracy at each laboratory is facilitated by Youden plots. The data will help to inform the legitimacy of the upper and lower control limits of the assay as established by the developer.

F. ASSAY VALIDATION – PART 4

1. PROGRAMME IMPLEMENTATION

Ultimate proof of the usefulness of an assay is its successful application(s). These would include international, regional or national programs. As new and improved assays are developed and come on-line, they will ultimately replace existing assays if they prove a better fitness for purpose. However, this will only happen if they are actually put into routine use and their usefulness documented over time. In the natural progression of diagnostic and/or technological improvement, some new assays will become the new standard of comparison. As such, they may progressively achieve national, regional and international recognition. As a recognised standard, these assays will also be used to develop reference reagents for quality control, proficiency and harmonisation purposes. These reference reagents may also become international standards, as well. The last level of validation in the OIE Registry involves
documentation related to actual application and levels of recognition for the assay in question. This is intended to provide potential users with an informed and unbiased source of information.

2. Monitoring validity of assay performance

a) Interpretation of test results – factors affecting assay validity

An assay’s test results are useful only if the inferences made from them are accurate. A common error is to assume that an assay with 99% DSe and 99% DSp will generate one false-positive and one false-negative result for approximately every 100 tests on animals from the target population. Such an assay may be precise and accurate, but produce test results that do not accurately predict infection status. For example, if the prevalence of disease in a population targeted by the assay is only 1 per 1000 animals, and the false-positive test rate is 1 per 100 animals (99% DSp), for every 1000 tests on that population, ten will be false positive and one will be true positive. Hence, only approximately 9% of positive test results will accurately predict the infection status of the animal; the positive test results will misclassify the animal 91% of the time. This illustrates that the capacity of a positive or negative test result to predict infection status is dependent on the prevalence of the infection in the target population (10). Of course, the prevalence will probably have been determined by use of a serological test with its own inherent misclassification of results.

An estimate of prevalence in the target population is necessary for calculation of the predictive values of positive (PV+) or negative (PV−) test results. When test values are reported without providing estimates of the assay’s DSp and DSe, it is not possible to make informed predictions of infection status from test results (9). It is, therefore, highly desirable to provide an interpretation statement with test results accompanied by a small table indicating PV+ and PV− for a range of expected prevalences of infection in the target population. Without provision of such information, test results from the assay may have failed to accurately classify the infection status of animals, and thus do not reflect a fully validated assay.

b) Maintenance of validation criteria

A validated assay needs constant monitoring and maintenance to retain that designation. Once the assay is put into routine use, internal quality control is accomplished by consistently monitoring the assay for assessment of precision and accuracy (1).

Reproducibility between laboratories should be assessed at least twice each year. It is highly desirable to become part of a consortium of laboratories that are interested in evaluating their output. In the near future, good laboratory practice, including implementation of a total quality assurance programme, will become essential for laboratories seeking to meet national and international certification requirements (see Chapter 1.1.1).

Proficiency testing is a form of external quality control for an assay. It is usually administered by a reference laboratory that distributes panels of samples, receives the results from the laboratories, analyses the data, and reports the results back to the laboratories. If results from an assay at a given laboratory remain within acceptable limits and show evidence of accuracy and reproducibility, the laboratory may be certified by government agencies or reference laboratories as an official laboratory for that assay (13). Panels of sera for proficiency testing should contain a full representation of an analyte’s concentration in animals of the target population. If the panels only have high-positive and low-positive sera (with none near the assay’s cut-off), the exercise will only give evidence of reproducibility at the extremes of analyte concentration, and will not clarify whether routine test results on the target population properly classify infection status of animals.

c) Enhancement and extension of validation criteria

Because of the extraordinary set of variables that impact on the performance of serodiagnostic assays, it is highly desirable to expand the number of standard sera from animals of known
infection status because of the principle that error in the estimates of DSe and DSp is reduced with increasing sample size. Furthermore, when the assay is to be applied in a completely different geographical region, it is essential to re-validate the assay for its new intended use by subjecting it to sera from populations of animals that reside under local conditions. The same is true for establishing DSe and DSp for subpopulations (e.g. age groups, vaccinated/non-vaccinated, etc.).

REFERENCES


* * *
CHAPTER 1.1.3.
VALIDATION AND QUALITY CONTROL OF POLYMERASE CHAIN REACTION METHODS USED FOR THE DIAGNOSIS OF INFECTIOUS DISEASES

SUMMARY

The diagnosis of infectious diseases is performed by direct and/or indirect detection of infectious agents. By direct methods, the particles of the agents and/or their components, such as nucleic acids, structural or nonstructural proteins, enzymes, etc., are detected. The indirect methods demonstrate the antibodies induced by the infections.

The most common direct detection methods are virus isolation, bacteria cultivation (the gold standards), electron microscopy, immunofluorescence, immunohistochimistry, antigen enzyme-linked immunosorbert assay (ELISA), nucleic-acid hybridisation (NAH) and nucleic acid amplification, such as the polymerase chain reaction (PCR). As NAH and PCR assays have nucleic acid molecules as targets, they are also termed methods of molecular diagnosis.

The most common indirect methods of infectious agent detection are virus neutralisation, antibody detection by ELISA and haemagglutination inhibition tests. In general, diagnostic laboratories simultaneously apply both the direct and the indirect methods, in order to assure the certainty of a diagnosis.

OIE principles of validation have been developed for indirect detection methods, i.e. for antibody ELISA (see Chapter 1.1.2.). The purpose of this chapter is to extend the rules to a direct method of infectious agent detection, i.e. to adapt the principles of validation to the PCR assays.

The experiences of the last decade indicate that the PCR techniques will eventually supersede many of the classical direct methods of infectious agent detection. It is clear that in many laboratories, the PCR is replacing virus isolation or bacteria cultivation for the detection of agents that are difficult or impossible to culture. There are several reasons for this trend, including that virus isolation requires: i) the presence of replicating viruses; ii) expensive cell culture and maintenance facilities; iii) as long as several weeks to complete the diagnosis; and iv) special expertise, which is missing or diminishing today in many laboratories. Although PCR assays were initially expensive and cumbersome to use, they have now become relatively inexpensive, safe and user-friendly tools in diagnostic laboratories (2, 7).

A. PCR METHODS USED IN ROUTINE MOLECULAR DIAGNOSTICS

1. THE PRINCIPLES OF THE PCR

Polymerase chain reaction (PCR) implies that there is an amplification reaction in the assay. The term ‘chain reaction’ refers to several cycles of copying a specified stretch of DNA from a target nucleic acid, in this case from the genome of an infectious agent. The amplified region is defined by two (or more) short oligonucleotides, and two primers that are complementary to DNA regions flanking the target sequence. Using a heat-stable DNA polymerase, which is not denatured during heat cycling, it is possible to copy the DNA sequence between the primers. By repeating 20–40 times a heat-cycling regime, the amount of copied target DNA gained is enough for further operations, such as detection, cloning or sequencing. The diagnostic sensitivity of the PCR is very high because several million copies of the selected target are produced. The specificity of the reaction may also be very high, as determined
by the specific nucleotide sequences of the oligonucleotides (primers). The primers are designed to detect specific nucleotide sequences in the genomes of the selected target infectious agents.

a) **DNA amplification**

If the genome of the infectious agent is DNA, the amplification is performed directly, with or without previous purification of the target DNA.

b) **RNA amplification (reverse-transcription PCR)**

The genomes of many infectious agents contain ribonucleic acid (RNA) that cannot be amplified directly by the PCR. For PCR amplification, a double-stranded DNA target is necessary, and this is not available in the case of RNA viruses. This problem can be solved by the addition of a step before the PCR is begun. Using reverse transcriptase it is possible to transcribe the RNA into complimentary DNA (cDNA), which is double-stranded DNA and hence can be used in a PCR assay (the procedure is termed reverse transcriptase PCR: RT-PCR). Traditionally, the reverse transcription reaction is performed in a separate reaction vessel and the cDNA produced is then transferred to a new tube for the PCR. However, heat-stable DNA polymerases with reverse transcriptase activity are now readily available. When used in specific buffers, these enzymes enable the RT-PCR reaction to take place in the same tube and in direct sequence without any further handling.

Some examples of PCR methods currently used are given below.

2. **SINGLE PCR**

‘Single PCR’ (or simply PCR) uses one pair of oligonucleotide primers to amplify a small part of the genome of the infectious agent. Analytical sensitivity is relatively high with a minimum number of 100 to 1000 copies of the genome detectable. Analytical specificity is also rather high. Both analytical sensitivity and specificity can be further improved by applying nested PCR (see point 3 below). Using Southern blotting and checking the PCR products with a labelled probe can further improve specificity, but this is time-consuming and is not a common practice in diagnostic laboratories today.

3. **NESTED PCR**

Nested PCR assays use two amplification cycles with four primers, termed external and internal primers. In general, nested PCR assays provide higher analytical sensitivity and specificity compared with single PCR. Analytical sensitivity is typically <10 genomic copies of the infectious agent, and analytical specificity is also enhanced because in the nested PCR, four oligonucleotides have to bind specifically to the selected targets in order to yield a positive reaction (2).

4. **REAL-TIME PCR**

Real-time PCR is an amplification where the PCR products are detected directly during the amplification cycles using fluorescence-labelled probes. Various real-time methods, such as TaqMan or Molecular Beacon assays, have become popular tools for detection of infectious agents. Real-time PCR has been used for the detection of bacteria, viruses or parasites from a range of animal species (2, 6, 8). These new assays have several advantages over the ‘classical’ single or nested PCR methods. Only one primer pair is used, providing sensitivity often close or equal to traditional nested PCR but with a much lower risk of contamination. Fluorescence, indicating the presence of the amplified product, is measured through the lid or side of the reaction vessel thus there is no need for post-PCR handling of the products. These procedures are considerably less time-consuming compared with traditional PCR product detection in agarose gels followed by ethidium bromide staining and again, the risk of contamination is reduced. The use of a 96-well microtitre plate format, without the need for nested PCR, allows the procedure to be automated (5). Diagnosis can be further automated by using robots
for DNA/RNA extractions and pipetting. Compared with classical amplification methods, a further advantage of the real-time PCR is that it is possible to perform quantitative assays (6).

5. MULTIPLEX PCR

Probes for real-time PCR can be labelled with a large number of different fluorophores, which function as reporter dyes. The use of fluorescent probes emitting different colours enables ‘multiplexing’ of the assays. In multiplex PCR, various infectious agents can be detected and differentiated in a single reaction vessel at the same time. The ‘classical’ PCR technique was also found to be suitable for the development of multiplex systems. However, the use of ‘classical’ nested PCR methods for the construction of a multiplex assay is complicated because of the large number of primers that may ‘compete’ with each other in the same reaction mix. In contrast, the concept of real-time PCR (single primer pairs) provides excellent possibilities for the construction of highly sensitive multiplex systems (2, 4).

B. VALIDATION OF MOLECULAR DIAGNOSTIC ASSAYS

When performing diagnostic analyses of clinical material it is important to produce data of good quality. For this, some key criteria have to be fulfilled. The establishment of quality assurance (QA) and quality control (QC) systems is required, i.e. a set of quality protocols, including the use of control samples, that ensure that the system is working properly and confirms data quality. QA and QC systems, together with trained and competent personnel, have already been established in many laboratories world-wide. Assay validation is another essential factor for assuring that test results reflect the true status of the samples (3).

To predict the performance of a diagnostic assay, it is necessary to use a validation methodology to validate the assay in question. Validation is the evaluation of a diagnostic assay for the purpose of determining how fit the assay is for a particular use. The general principles of assay validation can be found in Chapter 1.1.2. Principles of Validation of Diagnostic Assays for Infectious Disease. This chapter extends these validation principles to molecular diagnostic assays. For explanations of terms and definitions please consult Chapter 1.1.2.

C. MEASURES OF VALIDITY

Performance characteristics (or assay parameters) give information about how a method functions under specified conditions. Some typical performance characteristics are given in Chapter 1.1.2. and some others, important to PCR methods, are given here.

D. STAGES OF ASSAY VALIDATION

In Chapter 1.1.2., the stages of assay validation are described in detail. In this chapter, these stages are presented briefly with special emphasis on molecular diagnostic assays.

STAGE 1. FEASIBILITY STUDIES

A feasibility study is a preliminary step in validating a new assay. The goal is to determine whether or not a new assay can detect a range of target concentrations without background activity. At least ten samples (for example, infectious agents produced in the laboratory in cell or bacterial culture) are chosen, ranging from low to high levels of the infectious agent. It is also necessary to include at least ten samples containing no target. Usually it is difficult to separate this stage from stage 2, as preliminary optimisation is necessary before further studies can be conducted. Assays that look promising are subjected to further development in stage 2. Note that it is sometimes possible to substantially improve an assay by proper optimisation schemes and thus exclusion of non-optimal assays should be done with caution.
Primer selection is critical, and account should be taken of the nature of the infectious agent, its genome structure and the diversity of genetic sequences among different strains.

The result obtained by PCR may be influenced by the performance of the thermocycler, which should therefore be monitored on a continual basis. Regular temperature calibration is crucial and, for real-time PCR instruments, the optical systems must be controlled regularly. Assays developed and validated using a specific brand of thermocycler should be revalidated or otherwise controlled when new equipment is used.

**STAGE 2. ASSAY DEVELOPMENT AND STANDARDISATION**

1. **SELECTION OF OPTIMAL REAGENT CONCENTRATIONS, PROTOCOL PARAMETERS AND EQUIPMENT**

Sample collection, preparation and transport (see Chapter I.1.) and nucleic acid extraction methods (see Chapter I.1.) are all critical parameters in test performance and should be optimised for disease diagnosis. Suitable methods vary depending on sample and organism type. In general, blood and serum are suitable samples for easy extraction of target nucleic acids, while faeces and semen samples are more difficult to handle. Extraction of RNA targets differs from extraction of DNA targets, and RNA is more prone to degradation. Both commercial (robotic, spin columns, etc.) and in-house methods are used for DNA or RNA extraction. It is crucial to determine the most suitable method before further validation of the assay is performed. If the method of extraction is changed, the entire validation procedure should be repeated.

All equipment used during the process must be properly maintained. Apparatus (heating blocks, refrigerators, freezers, thermocyclers, pipettes, etc.) that require calibration must be calibrated according to the laboratory’s quality protocols.

When developing ‘classical’ or real-time PCR assays, all parameters, protocols and reagents need to be optimised. A standardised assay is a method that consistently gives the same result for a given sample when repeated several times.

2. **REPEATABILITY – PRELIMINARY ESTIMATES**

Agreement between replicates within and between runs of the assay should be accessed at this stage. This gives important information about the assay before further validation is carried out. If excessive variability is encountered, it should be corrected before continuing the validation process.

3. **DETERMINATION OF CRITICAL CONTROL PARAMETERS**

During the optimisation of the PCR assay, it is also possible to estimate the capacity of the method to remain unaffected by small changes in the main parameters. Introduction of intentional variations in the validation process will characterise critical parameters in the assay. Examples of such parameters include: incubation times and temperatures, concentrations of buffers, primers, MgCl₂, etc., pH, amounts of other components added (e.g. dNTP, bovine serum albumin, etc.). The characterisation of critical control parameters is crucial for identifying critical points that must be properly controlled in the assay.

4. **ANALYTICAL SENSITIVITY AND SPECIFICITY**

Analytical sensitivity (or limit of detection) is defined as the smallest number of genome copies of the infectious agent that can be detected and distinguished from a zero result. To determine analytical sensitivity, an end-point dilution is used until the assay can no longer detect the target in question in more than 5% of the replicates (2 standard deviations). Cloned fragments of the PCR products in question can be used as standard samples, either as DNA or for RNA targets, the RNA being transcribed *in vitro* into DNA.
Analytical specificity is defined as the ability of an assay to distinguish the target agent from other infectious agents. This ability is determined by analysing closely related pathogens using the assay in question.

5. RANGE

Analytical techniques can rarely be scaled up or down arbitrarily; the assay should be optimised in the linear phase of the dose–response curve. The range of an assay is defined as the interval between the upper and lower concentration of an infectious agent in a sample in which the agent can be reliably detected.

STAGE 3. DETERMINING ASSAY PERFORMANCE CHARACTERISTICS

1. DIAGNOSTIC SENSITIVITY AND SPECIFICITY

Diagnostic sensitivity (DSe; proportion of known infected reference animals that are tested positive in the assay) and specificity (DSP; proportion of known uninfected reference animals that are tested negative in the assay) are the most important parameters obtained during the validation of an assay. They form the basis for calculating other parameters and hence they are critical to the whole validation process. The number of reference samples required to determine estimates and allowable error of both DSe and DSP can be calculated. To do this, a reasonable prediction of both DSe and DSP must be used. Generally, confidence in the estimate is set at 95%. However, no formula can account for the numerous host/organism factors that can affect the outcome of the test. The number of samples to determine estimates of DSe and DSP is outlined in Chapter 1.1.2. It is recognised that achieving these numbers for molecular assays might be difficult and costly. Testing smaller numbers will result in a reduction in confidence of the estimate. The status of known infected and uninfected animals should be established using comparisons with other assays. The use of spiked samples in PCR is not appropriate as these might not be representative of naturally infected samples and thus the whole validation process could potentially be jeopardised.

2. REPEATABILITY AND REPRODUCIBILITY

Repeatability and reproducibility are both important parameters in assay precision. Repeatability is measured as both the amount of agreement between replicates within the same run or between replicates tested in different runs. Reproducibility is determined in several laboratories using the identical assay (protocol, reagents and controls).

Currently, OIE stage 3 is rarely performed to its full extent in veterinary diagnostic laboratories carrying out PCR assays. Traditionally, many laboratories have used tests developed in-house, probably for practical reasons. Where there are published standardised and validated methods, these should be followed. Inter-laboratory validation processes have to be carried out even if they are costly and labour intensive. This work will lead to standardised assays, allowing harmonised diagnostic activity in various countries.

STAGE 4. MONITORING VALIDITY OF ASSAY PERFORMANCE

The estimation of the prevalence of a virus in the population is necessary for calculating the predictive value of positive (PV+) or negative (PV–) test results. This applies equally to molecular test methods as it does to other methods such as the enzyme-linked immunosorbent assay.

Reference Laboratories are encouraged to determine values for DSe and DSP as accurately as possible, as these are extremely important for judging the real performance of an assay when used in the field. It is also important to estimate the predictive values (PV+ or PV–) in the local situation.
STAGE 5. MAINTENANCE AND ENHANCEMENT OF VALIDATION CRITERIA

When the assay is used as a routine test it is important to maintain the internal QC. The assay needs to be consistently monitored for repeatability and accuracy. Reproducibility between laboratories (ring tests) is recommended by the OIE to be estimated at least twice a year (9).

If the assay is to be applied in another geographical region and/or population, it might be necessary to revalidate it under the new conditions. This is especially true for PCR assays as it is very common for point mutations to occur in many infectious agents (i.e. RNA viruses). Mutations, which may occur within the primer sites, can affect the performance of the assay and by doing so the established validation is no longer valid. It is also advisable to regularly sequence the selected genomic regions in the national isolates of the infectious agents. This is especially true for the primer sites, to ensure that they remain stable so that the validation of the assay cannot be questioned.

1. PRECAUTIONS AND CONTROLS

Considering the uncertainty about the safety and reliability of the PCR in routine diagnosis, special precautions should be applied in any laboratory using PCR for detecting infectious agents, in order to avoid false-positive or false-negative results. These, together with internal controls (mimics) assure the safe evaluation of the results.

a) Precautions to avoid false-positive results

False-positive results (negative samples showing a positive reaction), may arise from either product carryover from positive samples or, more commonly, from cross-contamination by PCR products from earlier experiments. Various practices and tools have been applied to prevent false-positive results. Samples and mixes should be handled in laminar air-flow hoods, which are regularly decontaminated using UV light and bleach. Constructing and using special tube-holders and openers help prevent false-positive PCR. In addition, safe laboratory practices should be applied, i.e. to perform the basic steps of nested PCR (mix and primer preparation, sample preparation, etc.) in separated laboratory areas (1, 2). It is also very important to include negative controls, i.e. samples that are as similar to the test samples as possible but without having the target. At least one negative control per five diagnostic samples should be used.

b) Internal controls (mimics) to avoid false-negative results

False-negative results (infected samples tested as negative) are mostly due to inhibitory effects and/or pipetting errors. Therefore, internal controls (termed ‘mimics’) are used as indicators of amplification efficiency. The mimics have the same primer-binding sequences as the template of the agent, but flank a heterologous DNA fragment of a different size. The identical primer-binding nucleotide sequences allow co-amplification of template and mimic in the same tube with minimal competition. The size differences provide easy discrimination. The internal controls increase the reliability of the diagnostic PCR (1, 2). Caution must be used when designing and validating mimics. Extensive testing is necessary to ensure that the added mimic and its amplification is not competing with the diagnostic PCR and thus lowering the analytical sensitivity. The mimic is used in a concentration slightly higher than the detection limit of the diagnostic PCR to ensure the test’s performance.

In real-time PCR assays, it is also possible to apply internal controls by detecting a selected fragment of the host animal’s genome. By including such an intrinsic control with a specifically coloured reporter fluorophore, it is possible to check the sample quality and to control pipetting errors simultaneously as the target agent is detected (8).

2. PREPARATION OF STANDARDS

Manual of Diagnostic Tests for Aquatic Animals 2006 29
Reference laboratories should provide standard samples representative of a given infectious agent. Such samples can be cultivated infectious agents, clinical specimens, etc., which are distributed in such a manner that the infectious agent is well preserved. Thus, the samples are distributed frozen, in organic solvents (e.g. Trizol) or other suitable ways. The samples can also be sent as nucleic acids (frozen, freeze-dried or in ethanol). For specific details, see the individual disease chapters.

REFERENCES


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NB: There is an OIE Collaborating Centre for the Application of Polymerase Chain Reaction Methods for Diagnosis of Viral Diseases in Veterinary Medicine (consult the OIE Web site for the most up-to-date list of OIE Reference Laboratories and Collaborating Centres: www.oie.int).
CHAPTER 1.1.4.

REQUIREMENTS FOR SURVEILLANCE FOR INTERNATIONAL RECOGNITION OF FREEDOM FROM INFECTION

PART 1

A. INTERNATIONAL RECOGNITION OF FREEDOM FROM INFECTION

1. GENERAL PRINCIPLES

General principles are provided below for declaring a country, zone or aquaculture establishment free from infection in relation to the time of last occurrence, and in particular for the recognition of historical freedom.

An essential prerequisite to provide the guarantees required for the recognition of freedom from infection is that the particular Member Country complies with the requirements of Chapter 1.4.3 of the Aquatic Code for the evaluation of the Competent Authorities.

The general principles are:

• in the absence of infection or vaccination, the animal population would be susceptible to clinical disease, or infection, over a period of time;

• the disease agents to which these provisions apply are likely to produce identifiable clinical or pathological signs in susceptible animals;

• an animal population may be free from some specified pathogens but not from others

• there are competent and effective personnel of the Competent Authority able to investigate, diagnose and report disease or infection, if present;

• the absence of infection over a long period of time in susceptible populations can be substantiated by effective disease investigation and reporting by the Competent Authority of the Member Country.

2. REQUIREMENTS TO DECLARE A COUNTRY, ZONE OR AQUACULTURE ESTABLISHMENT FREE FROM INFECTION WITH A SPECIFIED PATHOGEN

The requirements to declare a country, zone or aquaculture establishment free from infection differ depending on the previous infection status of the country, zone or aquaculture establishment, namely:

• Absence of susceptible species;

• Historically free;
Chapter 1.1.4. - Requirements for surveillance for international recognition of freedom from infection

- Last known occurrence within the previous 25 years;
- Previously unknown infection status.

2.1. Absence of susceptible species

Unless otherwise specified in the relevant disease chapter, a country, zone or aquaculture establishment may be recognised as being free from infection without applying targeted surveillance if there are no susceptible species (as listed in the relevant chapter of the Aquatic Code, or in the scientific literature) present in that country, zone or aquaculture establishment, provided that the prescribed biosecurity conditions have been in place continuously in the country, zone or aquaculture establishment for at least the previous 10 years.

2.2. Historically free

Unless otherwise specified in the relevant disease chapter, a country, zone or aquaculture establishment may be recognised as being free from infection without formally applying targeted surveillance when:

- there has never been any observed occurrence of disease;

or

- eradication has been achieved or the disease has ceased to occur for at least 25 years,

provided that the prescribed biosecurity conditions have been in place continuously in the country, zone or aquaculture establishment for at least the previous 10 years.

2.3. Last known occurrence within the previous 25 years

For countries or zones that have achieved eradication (or in which the disease has ceased to occur) within the previous 25 years, in addition to the prescribed biosecurity conditions, appropriate targeted surveillance must have been applied to demonstrate the absence of the infection, consistent with the provisions of Section B of this chapter.

2.4. Previously unknown infection status

For countries or zones with previously unknown infection status, or which have not previously met the requirements of the Sections A.2.1, A.2.2 or A.2.3 above, the prescribed biosecurity conditions must be introduced in addition to targeted surveillance consistent with the provisions of Section B of this chapter.

3. GUIDELINES FOR THE MAINTENANCE OF CONTINUED RECOGNITION OF FREEDOM FROM INFECTION

A country, zone or aquaculture establishment that has been recognised free from infection following the provisions of Sections A.2.1 or A.2.2, may maintain its official status as infection-free provided that the prescribed biosecurity conditions are continuously maintained.

A country, zone or aquaculture establishment that has been recognised free from infection following the provisions of Sections A.2.3 or A.2.4, may discontinue targeted surveillance and maintain its official status as infection-free provided that the prescribed biosecurity conditions are continuously maintained.

The different paths to recognition of freedom from infection are summarised in the diagram below.
B. TARGETED SURVEILLANCE FOR DEMONSTRATION OF FREEDOM FROM INFECTION

1. INTRODUCTION

This section provides standards to be applied when demonstrating country, zone or aquaculture establishment freedom from infection, in accordance with the principles of Section A. Standards described in this section may be applied to all diseases, their agents and susceptible species as listed in the Aquatic Code, and are designed to assist with the development of surveillance methodologies. More detailed information in each disease chapter (where it exists) of this Aquatic Manual may be used to further refine the general approaches described in this chapter. Where detailed disease/infection-specific information is not available, suitable values should be chosen based on the guidelines in this chapter.

2. GENERAL PRINCIPLES

Demonstrating freedom from infection involves providing sufficient evidence to demonstrate that infection with a specified agent is not present in a specified population. In practice, it is not possible to definitively prove that a population is free from infection (unless every member of the population is examined simultaneously with a perfect test with both sensitivity and specificity equal to 100%). Instead, the aim is to provide adequate evidence (to an acceptable level of confidence), that infection, if present, is present in less than a specified proportion of the population.

Methodologies to demonstrate freedom from infection should be flexible to deal with the complexity of real life situations. No single method is applicable in all cases. Methodologies must be able to accommodate the variety of aquatic animal species, the multiple diseases of relevance, varying production and surveillance systems, and types and amounts of data and information available.

The methodology used should be based on the best available information that is in accord with current scientific thinking. The methodology should be well documented and supported with references to the scientific literature and other sources, including expert opinion.
Consistency in methodologies should be encouraged and transparency is essential in order to ensure fairness and rationality, consistency in decision making and ease of understanding by all the interested parties. Applications for recognition of infection-free status should document the uncertainties, the assumptions made, and the effect of these on the final estimate.

3. GENERAL REQUIREMENTS FOR DEMONSTRATION OF FREEDOM FROM INFECTION

3.1. Population

The target population to which the demonstration of freedom from infection applies is all individuals of all species susceptible to the infection in a country, zone or aquaculture establishment.

The study population may be the same as the target population or a subset of it. The study population should be (in order of preference):

- The appropriate study population as defined in the relevant disease chapter of the Aquatic Code (if such a definition exists),
- A subset of the target population that defines a group of animals which, if infection were present, would be most likely to have a higher prevalence of infection than the target population. This subset should be defined in terms of:
  - species;
  - time (e.g. season or month of year);
  - stage of life-cycle or growth period;
  - production system and/or management characteristics;
  - location;
  - readily identifiable physical or behavioural characteristics.
- The same as the target population,
- A subset of the target population with the same or lower probability of infection. The nature and impact of any biases on the results of the analysis must be considered, documented and taken into account in the analysis.

3.2. Sources of evidence

Evidence of freedom from infection may be based on a number of different sources, including:

- structured, population-based surveys using one or more tests for the presence of the agent;
- other surveillance, including structured non-random surveillance, such as:
  - sentinel sites;
  - disease notifications and laboratory investigation records;
  - academic and other scientific studies;
- a knowledge of the biology of the agent, including environmental, host population distribution, known geographical distribution, vector distribution and climatic information;
- history of imports of potentially infected material;
• biosecurity measures in place;
• evaluation of the official services; or
• any other sources that provide contributory evidence that infection is not present in the country, zone or aquaculture establishment.

The sources of evidence used to demonstrate freedom from infection must be fully described. In the case of a structured survey, this must include a description of the sampling strategy used for the selection of units for testing. For complex surveillance systems, a full description of the system is required including consideration of any biases that may be inherent in the system.

3.3. Statistical methodology

Analysis of data for evidence of freedom from infection involves estimating the probability \( \alpha \) that the evidence observed (the results of surveillance) could have been produced under the null hypothesis that infection is present in the population at a specified prevalence(s) (the design prevalence[s]). The confidence in (or, equivalently, the sensitivity of) the surveillance system that produced the evidence is equal to 1–\( \alpha \). If the confidence level exceeds a pre-set threshold, the evidence is deemed adequate to demonstrate freedom from infection.

The required level of confidence in the surveillance system (probability that the system would detect infection if infection were present at the specified level) must be greater than or equal to 95%.

The power (probability that the system would report that no infection is present if infection is truly not present) may be set to any value. By convention, this is often set to 80%, but may be adjusted according to the country’s or zone’s requirements.

Different statistical methodologies for the calculation of the probability \( \alpha \), including both quantitative and qualitative approaches, are acceptable as long as they are based on accepted scientific principles.

The methodology used to calculate the confidence in the surveillance system must be scientifically based and clearly documented, including references to published work describing the methodology.

3.4. Clustering of infection

Infection in a country, zone or aquaculture establishment usually clusters rather than being uniformly distributed through a population. Clustering may occur at a number of different levels (e.g. a cluster of moribund fish in a pond, a cluster of ponds in a farm, or a cluster of farms in a zone). Except when dealing with demonstrably homogenous populations, approaches to demonstrating freedom must take this clustering into account in the design and the statistical analysis of the data, at least at what is judged to be the most significant level of clustering for the particular animal population and infection.

3.5. Design prevalence

Calculation of the confidence of a surveillance system is based on the null hypothesis that infection is present in the population. The level of infection is specified by the design prevalence. In the simplest case, this is the prevalence of infection in a homogenous population. More commonly, in the presence of disease clustering, two design prevalence values are required, for instance, the animal-level prevalence (proportion of fish infected in an infected farm) and the group-level prevalence (proportion of infected farms in the country, zone or aquaculture establishment). Further levels of clustering may be considered, requiring further design prevalence values.
The values for design prevalence used in calculations must be those specified in the relevant disease chapter (if present) of this *Aquatic Manual*. If not specified for the particular disease, justification for the selection of design prevalence values must be provided, and should be based on the following guidelines:

- At the individual animal level, the design prevalence is based on the biology of the infection in the *population*. It is equal to the minimum expected prevalence of infection in the *study population*, if the infection had become established in that *population*. It is dependent on the dynamics of infection in the *population* and the definition of the *study population* (which may be defined to maximise the expected prevalence in the presence of infection).

- A suitable design prevalence value at the animal level (e.g. prevalence of infected animals in a cage) may be:
  - between 1% and 5% for infections that are transmitted slowly; and
  - over 5% for more contagious infections.

- At higher levels (e.g. cage, pond, farm, village, etc.) the design prevalence usually reflects the prevalence of infection that is practically and reasonably able to be detected by a *surveillance system*. Detection of infection at the lowest limit (a single infected *unit* in the *population*) is rarely feasible in large *populations*. The expected behaviour of the infection may also play a role. Infections that have the ability to spread rapidly between farms may have a higher farm-level design prevalence than slow-moving infections.

A suitable design prevalence value for the first level of clustering, (e.g. proportion of infected farms in a zone) may be up to 2%.

### 3.6. Test characteristics

All surveillance involves performing one or more *tests* for evidence of the presence of current or past infection, ranging from detailed laboratory examinations to farmer observations. The performance level of a *test* at the *population* level is described in terms of its *sensitivity* and *specificity*. Imperfect sensitivity and/or specificity impact on the interpretation of surveillance results and must be taken into account in the analysis of surveillance data.

All calculations must take the performance level (sensitivity and specificity) of any *tests* used into account. The values of sensitivity and specificity used for calculations must be specified, and the method used to determine or estimate these values must be documented. Where values for sensitivity and/or specificity for a particular *test* are specified in this *Aquatic Manual*, these values may be used without justification.

Where more than one *test* is used in a *surveillance system* (sometimes called using tests in series or parallel), the overall *test system sensitivity* and *specificity* must be calculated using a scientifically valid method.

Pooled testing involves the pooling of specimens from multiple individuals and performing a single *test* on the pool. Pooled testing is an acceptable approach. Where pooled testing is used, the results of testing must be interpreted using sensitivity and specificity values that have been determined or estimated for that particular pooled testing procedure and for the applicable pool sizes being used. Analysis of the results of pooled testing must, where possible, be performed using accepted, statistically based methodologies, which must be fully documented, including published references.

### 3.7. Multiple sources of evidence

Where multiple different data sources providing evidence of freedom from infection exist or are generated, each of these data sources may be analysed according to the provisions of Sections B.3,
Chapter 1.1.4. - Requirements for surveillance for international recognition of freedom from infection

B.4 (for structured surveys) and B.5 (for complex data sources). The resulting estimates of the confidence in each data source may be combined to provide an overall level of confidence for the combined data sources.

The methodology used to combine the estimates from multiple data sources:

- must be scientifically valid, and fully documented, including references to published material; and
- should, where possible, take into account any lack of statistical independence between different data sources.

Surveillance information gathered from the same country, zone or aquaculture establishment at different times may provide cumulative evidence of freedom from infection. Such evidence gathered over time may be combined into an overall level of confidence. For instance, repeated annual surveys may be analysed to provide a cumulative level of confidence. However, a single (larger) survey may be able to achieve the same level of confidence in just 1 year.

Analysis of surveillance information gathered intermittently or continuously over time should, where possible, incorporate the time of collection of the information to take the decreased value of older information into account.

4. SPECIFIC REQUIREMENTS FOR STRUCTURED SURVEY DESIGN AND ANALYSIS

One method of generating evidence for freedom from infection is the use of structured, population-based, targeted surveys. In addition to the requirements specified in Section B.3, the following guidelines should be used when implementing and analysing surveys to demonstrate freedom from infection.

4.1. Survey design

The most important unit of diagnosis is the epidemiological unit. The population of epidemiological units must be clearly defined.

The design of the survey will depend on the size and structure of the population being studied. If the population is relatively small and can be considered to be homogenous with regards to risk of infection, a single-stage survey can be used.

In larger populations where a sampling frame is not available, or when there is a likelihood of clustering of disease, multi-stage sampling is required. In two-stage sampling, at the first stage of sampling, groups of animals (e.g. ponds, farms or villages) are selected. At the second stage, animals are selected for testing from each of the selected groups.

Stratification may be used in survey design.

4.2. Sampling

The objective of sampling from a population is to select a subset of units from the population that is representative of the population with respect to the characteristic of interest (in this case, the presence or absence of infection). Sampling should be carried out in such a way as to provide the best likelihood that the sample will be representative of the population, within the practical constraints imposed by different environments and production systems.

Biased or targeted sampling in this context involves sampling from a defined study population that has a different probability of infection than the target population of which it is a subpopulation. Once the study population has been identified, the objective is still to select a representative sample from this subpopulation.
4.3. Sampling methods

The survey design may involve sampling at several levels.

For sampling at the level of the epidemiological units or higher units, a formal probability sampling (e.g. simple random sampling) method must be used.

When sampling below the level of the epidemiological unit (e.g. individual animal), the sampling method used should provide the best practical chance of generating a sample that is representative of the population of the chosen epidemiological unit. Collecting a truly representative sample of individual animals (whether from a pond, cage or fishery) is often very difficult. To maximise the chance of finding infection, the aim should be to bias the sampling towards infected animals, e.g. selecting moribund animals, life stages with a greater chance of active infection, etc.

The sampling method used at all levels must be fully documented and justified.

4.4. Sample size

The number of units to be sampled from a population should be calculated using a statistically valid technique that takes at least the following factors into account:

- The sensitivity and specificity of the diagnostic test, or test system;
- The design prevalence (or prevalences where a multi-stage design is used);
- The level of confidence that is desired of the survey results.

Additionally, other factors may be considered in sample size calculations, including (but not limited to):

- The size of the population (but it is acceptable to assume that the population is infinitely large);
- The desired power of the survey;
- Uncertainty or variability in estimates of sensitivity and specificity.

The specific sampling requirements will need to be tailor-made for each individual disease, taking into account its characteristics and the specificity and sensitivity of the accepted testing methods for detecting the disease agent in host populations.

Detailed guidelines are to be provided in the next (fifth) edition of the Aquatic Manual. In the meantime, the sampling procedures given in Chapters I.1, I.2 and I.3 may be applied.

4.5. Data analysis

Analysis of test results from a survey shall be in accordance with the provisions of Section B.3 and take at least the following considerations into account:

- The survey design;
- The sensitivity and specificity of the test, or test system;
- The design prevalence (or prevalences where a multi-stage design is used);
- The results of the survey.
4.6. Quality assurance

Surveys should include a documented quality assurance system, to ensure that field and other procedures conform to the specified survey design. Acceptable systems may be quite simple, as long as they provide verifiable documentation of procedures and basic checks to detect significant deviations of procedures from those documented in the survey design.

5. Specific requirements for complex non-survey data sources

Data sources that provide evidence of freedom from infection, but are not based on structured population-based surveys may also be used to demonstrate freedom, either alone or in combination with other data sources. Different methodologies may be used for the analysis of such data sources, but the methodology must comply with the provisions of Section B.3. The approach used should, where possible, also take into account any lack of statistical independence between observations.

Analytical methodologies based on the use of step-wise probability estimates to describe the surveillance system may determine the probability of each step either by:

- the analysis of available data, using a scientifically valid methodology; or where no data are available,
- the use of estimates based on expert opinion, gathered and combined using a formal, documented and scientifically valid methodology.

Where there is significant uncertainty and/or variability in estimates used in the analysis, stochastic modelling or other equivalent techniques should be used to assess the impact of this uncertainty and/or variability on the final estimate of confidence.

PART 2

EXAMPLE SURVEILLANCE SYSTEMS

The following examples describe surveillance systems and approaches to the analysis of evidence that are able to meet the requirements of Part 1 of this chapter. The purpose of these examples is:

- to illustrate the range of approaches that may be acceptable;
- to provide practical guidance and models that may be used for the design of specific surveillance systems; and
- to provide references to available resources that are useful in the development and analysis of surveillance systems.

While these examples demonstrate ways in which freedom from infection may be successfully demonstrated, they are not intended to be prescriptive. Countries are free to use different approaches, as long as they meet the requirements of Part 1 of this chapter.

The examples deal with the use of structured surveys and are designed to illustrate different survey designs, sampling schemes, the calculation of sample size, and analysis of results. It is important to note that alternative approaches to demonstrating freedom using complex non-survey-based data sources are also currently being developed and may soon be published1.

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EXAMPLE 1 – ONE-STAGE STRUCTURED SURVEY (FARM ACCREDITATION)

Context
A freshwater aquaculture industry raising fish in tanks has established a farm accreditation scheme. This involves demonstrating farm-level freedom from a particular (hypothetical) disease (Disease X). The disease does not spread very quickly, and is most common during the winter months, with adult fish at the end of the production cycle being most severely affected. Farms consist of a number of grow-out tanks, ranging from 2 to 20, and each tank holds between 1000 and 5000 fish.

Objective
The objective is to implement surveillance that is capable of providing evidence that an individual farm is free from Disease X. (The issue of national or zone freedom, as opposed to farm freedom, is considered in the next example.)

Approach
The accreditation scheme establishes a set of standard operating procedures and requirements for recognition of freedom, based on the guidelines given in this chapter. These require farms to undertake a structured survey capable of producing 95% confidence that the disease would be detected if it were present. Once farms have been surveyed without detecting disease, they are recognised as free, as long as they maintain a set of minimum biosecurity standards. These standards are designed to prevent the introduction of Disease X into the farm (through the implementation of controls specific to the method of spread of that disease) and to ensure that the disease would be detected rapidly if it were to enter the farm (based on evidence of adequate health record keeping and the prompt investigation of unusual disease events). The effective implementation of these biosecurity measures is evaluated with annual on-farm audits conducted by independent auditors.

Survey standards
Based on the guidelines given in this chapter, a set of standards are established for the conduct of surveys to demonstrate freedom from infection with causative agent of Disease X. These standards include:

- The level of confidence required of the survey is 95% (i.e. Type I error = 5%).
- The power of the survey is arbitrarily set at 95% (i.e. Type II error = 5%, which means that there is a 5% chance of concluding that a non-diseased farm is infected).
- The target population is all the fish on the farm. Due to the patterns of disease in this production system, in which only fish in the final stages of grow-out, and only in winter are affected, the study population is defined as grow-out fish during the winter months.
- The issue of clustering is considered. As fish are grouped into tanks, this is the logical level at which to consider clustering. However, when a farm is infected, the disease often occurs in multiple tanks, so there is little evidence of strong clustering. Also, the small number of tanks on a single farm means that it is difficult to define a design prevalence at the tank level (i.e. the proportion of infected tanks that the survey should be able to detect on the farm). For these reasons, it is decided to treat the entire grow-out population of each farm as a single homogenous population.
- Stratification is also considered. In order to ensure full representation, it is decided to stratify the sample size by tank, proportional to the population of each tank.
- The design prevalence at the animal level is determined based on the epidemiology of the disease. The disease does not spread quickly, however, in the defined target population, it has been reported to affect at least 10% of fish, if the population is infected. In order to take the most conservative approach, an arbitrarily low design prevalence of 2% is used. A prevalence of 10% may have been
used (and would result in a much smaller sample size), but the authorities were not convinced by the thought that the population could still be infected at a level of say 5%, and disease still not be detected.

- The test used involves destructive sampling of the fish, and is based on an antigen-detection enzyme-linked immunosorbent assay (ELISA). Disease X is present in some parts of the country (hence the need for a farm-level accreditation programme). This has provided the opportunity for the sensitivity and the specificity of the ELISA to be evaluated in similar populations to those on farms. A recent study (using a combination of histology and culture as a gold standard) estimated the sensitivity of the ELISA to be 98% (95% confidence interval 96.7–99.2%), and the specificity to be 99.4% (99.2–99.6%). Due to the relatively narrow confidence intervals, it was decided to use the point estimates of the sensitivity and specificity rather than complicate calculations by taking the uncertainty in those estimates into account.

Sample size

The sample size required to meet the objectives of the survey is calculated to take the population size, the test performance, the confidence required and the design prevalence into account. As the population of each farm is relatively large, differences in the total population of each farm have little effect on the calculated sample size. The other parameters for sample size calculation are fixed across all farms. Therefore, a standard sample size (based on the use of this particular ELISA in this population) is calculated. The sample size calculations are performed using the FreeCalc software. Based on the parameters listed above, the sample size required is calculated to be 410 fish per farm. In addition, the program calculates that, given the imperfect specificity, it is still possible for the test to produce up to five false-positive reactors from an uninfected population using this sample size. The authorities are not comfortable with dealing with false-positive reactors, so it is decided to change the test system to include a confirmatory test for any positive reactors. Culture is selected as the most appropriate test, as it has a specificity that is considered to be 100%. However, its sensitivity is only 90% due to the difficulty of growing the organism.

As two tests are now being used, the performance of the test system must be calculated, and the sample size recalculated based on the test system performance.

Using this combination of tests (in which a sample is considered positive only if it tests positive to both tests), the specificity of the combined two tests can be calculated by the formula:

\[ Sp_{\text{Combined}} = Sp_1 + Sp_2 - (Sp_1 \times Sp_2) \]

which produces a combined specificity of \(1 + 0.994 - (1 \times 0.994) = 100\%\)

The sensitivity may be calculated by the formula:

\[ Se_{\text{Combined}} = Se_1 \times Se \]

which produces a combined sensitivity of \(0.9 \times 0.98 = 88.2\%\)

These new values are used to calculate the survey sample size yielding a result of 169 fish. It is worth noting that attempts to improve the performance of a test (in this case increase specificity) generally result in a decrease in the performance of the other aspect of the test performance (sensitivity in this example). However, in this case, the loss of sensitivity is more than compensated for by the decreased sample size due to the improved specificity.

It is also worth noting that, when using a test system with 100% specificity, the effective power of the survey will always be 100%, regardless of the figure used in the design. This is because it is not possible to make a Type II error, and conclude that the farm is infected when it is not.

A check of the impact of population size on the calculated sample size is worthwhile. The calculated sample size is based on an infinitely large population. If the population size is smaller, the impact on sample size is shown in the following table:

<table>
<thead>
<tr>
<th>Population size</th>
<th>Sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>157</td>
</tr>
<tr>
<td>2000</td>
<td>163</td>
</tr>
<tr>
<td>5000</td>
<td>166</td>
</tr>
<tr>
<td>10,000</td>
<td>169</td>
</tr>
</tbody>
</table>

Based on these calculations, it is clear that, for the population sizes under consideration, there is little effect on the sample size. For the sake of simplicity, a standard sample size of 169 is used, regardless of the number of grow-out fish on the farm.

**Sampling**

The selection of individual fish to include in the sample should be done in such a manner as to give the best chance of the sample being representative of the study population. A fuller description of how this may be achieved under different circumstances is provided in *Survey Toolbox*[^3]. An example of a single farm will be used to illustrate some of the issues.

One farm has a total of eight tanks, four of which are used for grow-out. At the time of the survey (during winter), the four grow-out tanks have 1850, 4250, 4270 and 4880 fish, respectively, giving a total population of 15,250 grow-out fish.

Simple random sampling from this entire population is likely to produce sample sizes from each tank roughly in proportion to the number of fish in each tank. However, proportional stratified sampling will guarantee that each tank is represented in proportion. This simply involves dividing the sample size between tanks in proportion to their population. The first tank has 1850 fish out of a total of 15,250, representing 12.13%. Therefore 12.13% of the sample (21 fish) should be taken from the first tank. Using a similar approach the sample size for the other three tanks is 47, 47 and 54 fish, respectively.

Once the sample for each tank is determined, the problem remains as to how to select 21 fish from a tank of 1850 so that they are representative of the population. Several options exist.

- If the fish can be handled individually, random systematic sampling may be used. This is likely to be the case if, for example:
  - fish are harvested during winter and samples can be collected at harvest; or
  - routine management activities involving handling the fish (such as grading or vaccination) are conducted during the winter.

If fish are handled, systematic sampling simply involves selecting a fish at regular intervals. For instance, to select 21 from 1850, the sampling interval should be 1850/21 = 88. This means that every 88th fish from the tank should be sampled. To ensure randomness, it is good practice to use a random number generator to select the starting point.

random number between 1 and 88 (in this case) to select the first fish (e.g. using a random number table), and then select every 88th fish after that.

- If fish cannot be handled individually (by far the most common, and more difficult, circumstance) then the fish to be sampled must be captured from the tanks. Fish should be captured in the most efficient and practical way possible, however every effort should be made to try to ensure that the sample is representative. In this example, a dip net is the normal method used for capturing fish. Using a dip net, convenience sampling would involve capturing 21 fish by repeatedly dipping at one spot and capturing the easiest fish (perhaps the smaller ones). This approach is strongly discouraged. One method of increasing the representativeness is to sample at different locations in the tank – some at one end, some at either side, some at the other end, some in the middle, some close to the edge. Additionally, if there are differences among the fish, an attempt should be made to capture fish in such a way as to give different groups of fish a chance of being caught (i.e. do not just try to catch the small ones, but include big ones as well).

This method of collecting a sample is far from the ideal of random sampling, but due to the practical difficulties of implementing random sampling of individual fish, this approach is acceptable, as long as the efforts made to increase the representativeness of the sample are both genuine and fully documented.

**Testing**

Specimens are collected, processed and tested according to standardised procedures developed under the accreditation programme and designed to meet the requirements of this Aquatic Manual. The testing protocol dictates that any specimens that test positive to ELISA be submitted for culture, and that any positive culture results indicate a true positive specimen (i.e. that the farm is not free from disease). It is important that this protocol be adhered to exactly. If a positive culture is found, then it is not acceptable to retest it, unless further testing is specified in the original testing protocol, and the impact of such testing accounted for in the test system sensitivity and specificity estimates (and therefore the sample size).

**Analysis**

If the calculated sample size of 169 is used, and no positive reactors are found, then the survey will have a confidence of 95%. This can be confirmed by analysing the results using the FreeCalc software mentioned above (which reports a confidence level of 95.06%).

It may happen in some cases that the survey is not conducted exactly as planned, and the actual sample size is less than the target sample size. However, the size of the farm may also be smaller. In these cases, it is advisable to analyse the farm data on a farm-by-farm basis. For example, if only 165 specimens were collected from a farm with only 2520 fish, the resulting confidence would still be 95%. If only 160 fish were collected, the confidence is only 94.5%. If a rigid target of 95% confidence is used, then this survey would fail to meet that target and more evidence would be required.

**EXAMPLE 2 – TWO-STAGE STRUCTURED SURVEY (NATIONAL FREEDOM)**

**Context**

A country aims to declare freedom from Disease Y of crustaceans. The industry in this country is based largely on small-holder ponds, grouped closely together in and around villages. The disease is reasonably highly contagious, and causes mass mortality mid to late in the production cycle, with affected animals becoming moribund and dying in a matter of days. Affected animals show few characteristic signs, but an infected pond will almost invariably break down with mass mortality unless harvested beforehand. It is more common in late summer, but can occur at any time of year. It also occurs occasionally early in the production cycle. In this country, there are some limitations to the availability of laboratory facilities and
the transport infrastructure. However, there is a relatively large government structure, and a comprehensive network of fisheries officers.

**Objective**

The objective is to establish national freedom from Disease Y. The surveillance system must meet the requirements of Part 1 of this chapter, but must also be able to be practically implemented in this small-holder production system.

**Approach**

The aquaculture authorities decide to use a survey to gather evidence of freedom, using a two-stage survey design (sampling villages at the first level, and ponds at the second). Laboratory testing of specimens from a large number of farms is not considered feasible, so a combined test system is developed to minimise the need for expensive laboratory tests.

The unit of observation and analysis is, in this case, the pond, rather than the individual animal. This means that the diagnosis is being made at the pond level (an infected pond or a non-infected pond) rather than at the animal level.

The survey is therefore a survey to demonstrate that no villages are infected (using a random sample of villages and making a village-level diagnosis). The test used to make a village-level diagnosis is, in fact, another survey, this time to demonstrate that no ponds in the village are affected. A test is then performed at the pond level (farmer observation followed, if necessary, by further laboratory testing).

**Survey standards**

- The confidence to be achieved by the survey is 95%. The power is set at 95% (but is likely to be virtually 100% if the test system used achieves nearly 100% specificity, as demonstrated in the previous example).

- The target population is all ponds stocked with shrimp in the country during the study period. The study population is the same, except that those remote areas to which access is not possible are excluded. As outbreaks can occur at any time of year, and at any stage of the production cycle, it is decided not to further refine the definition of the population to target a particular time or age.

- Three tests are used. The first is farmer observation, to determine if mass mortality is occurring in a particular pond. If a pond is positive to the first test (i.e. mass mortality is detected), a second test is applied. The second test used is polymerase chain reaction (PCR). Cases positive to PCR are further tested using transmission experiments.

- Farmer observation can be treated as a test just like any other. In this case, the observation of mass mortality is being used as a test for the presence of Disease Y. As there are a variety of other diseases that are capable of causing mass mortality, the test is not very specific. On the other hand, it is quite unusual for Disease Y to be present, and not result in mass mortality, so the test is quite sensitive. A standard case definition is established for ‘mass mortality’ (for instance, greater than 20% of the pond’s population of shrimp observed dead in the space of less than 1 week). Based on this definition, farmers are able to ‘diagnose’ each pond as having mass mortality. Some farmers may be over-sensitive and decide that mass mortality is occurring when only a small proportion of shrimp are found dead (false positives, leading to a decrease in specificity) while a small number of others fail to recognise the mortalities, decreasing sensitivity.

In order to quantify the sensitivity and specificity of farmer observation of mass mortalities, as a test for Disease Y, a separate study is carried out. This involves both a retrospective study of the number of mass mortality events in a population that is thought to be free from disease, as well as a study of farmers presented with a series of mortality scenarios, to assess their ability to accurately identify a pond with mass mortality. By combining these results, it is estimated that
the sensitivity of farmer-reported mass mortalities as a test for Disease Y is 87% while the specificity is 68%.

- When a farmer detects a pond with mass mortality, specimens are collected from moribund shrimp following a prescribed protocol. Tissue samples from 20 shrimp are collected, and pooled for PCR testing. In the laboratory, the ability of pooled PCR to identify a single infected animal in a pool of 20 has been studied, and the sensitivity of the procedure is 98.6%. A similar study of negative specimens has shown that positive results have occasionally occurred, probably due to laboratory contamination, but maybe also because of the presence of non-viable genetic material from another source (shrimp-based feed stuffs are suspected). The specificity is therefore estimated at 99%.

- Published studies in other countries have shown that the sensitivity of transmission tests, the third type of test to be used, is 95%, partly due to variability in the load of the agent in inoculated material. The specificity is agreed to be 100%.

- Based on these figures, the combined test system sensitivity and specificity are calculated using the formulae presented in Example 1, first with the first two tests, and then with the combined effect of the first two tests and the third test. The result is a sensitivity of 81.5% and a specificity of 100%.

- The design prevalence must be calculated at two levels. First, the pond-level design prevalence (the proportion of ponds in a village that would be infected if disease were present) is determined. In neighbouring infected countries, experience has shown that ponds in close contact with each other are quickly infected. It is unusual to observe an infected village with fewer than 20% of ponds infected. Conservatively, a design prevalence of 5% is used. The second value for design prevalence applies at the village level, or the proportion of infected villages that could be identified by the survey. As it is conceivable that the infection may persist in a local area without rapid spread to other parts of the country, a value of 1% is used. This is considered to be the lowest design prevalence value for which a survey can be practically designed.

- The population of villages in the country is 65,302, according to official government records. Those with shrimp ponds number 12,890, based on records maintained by the aquaculture authorities. These are generated through a five-yearly agricultural census, and updated annually based on reports of fisheries officers. There are no records available of the number of ponds in each of these villages.

**Sample size**

Sample size is calculated for the two levels of sampling, first the number of villages to be sampled and then the number of ponds to be sampled. The number of villages to be sampled depends on the sensitivity and the specificity of the test used to classify villages as infected or not infected. As the ‘test’ used in each village is really just another survey, the sensitivity is equal to the confidence and the specificity is equal to the power of the village-level survey. It is possible to adjust both confidence and power by changing the sample size in the village survey (number of ponds examined), which means that we can determine, within certain limits, what sensitivity and specificity we achieve.

This allows a flexible approach to sample size calculation. If a smaller first-stage sample size is desired (a small number of villages), a high sensitivity and specificity are needed, which means that the number of ponds in each village that need to be examined is larger. A smaller number of ponds will result in lower sensitivity and specificity, requiring a larger number of villages. The approach to determining the optimal (least cost) combination of first- and second-stage sample sizes is described in *Survey Toolbox*.

A further complication is presented by the fact that each village has a different number of ponds. In order to achieve the same (or similar) confidence and power (sensitivity and specificity) for each village, a different sample size may be required. The authorities choose to produce a table of sample sizes for the number of ponds to sample in each village, based on the total ponds in each village.
An example of one possible approach to determining the sample size follows:

The target sensitivity (confidence) achieved by each village-level survey is 95%. The target specificity is 100%. Using the FreeCalc software, with a design prevalence of 1% (the survey is able to detect disease if 1% or more villages are infected), the first-stage sample size is calculated as 314 villages. Within each village, the test used is the combined test system described above with a sensitivity of 81.5% and a specificity of 100%. Based on these figures the following table is developed, listing the number of ponds that need to be sampled in order to achieve 95% sensitivity.

<table>
<thead>
<tr>
<th>Population</th>
<th>Sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>29</td>
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<tr>
<td>40</td>
<td>39</td>
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<tr>
<td>60</td>
<td>47</td>
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<tr>
<td>80</td>
<td>52</td>
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<tr>
<td>100</td>
<td>55</td>
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<tr>
<td>120</td>
<td>57</td>
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<tr>
<td>140</td>
<td>59</td>
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<td>160</td>
<td>61</td>
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<td>180</td>
<td>62</td>
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<td>260</td>
<td>65</td>
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<td>280</td>
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<td>340</td>
<td>67</td>
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<tr>
<td>900</td>
<td>68</td>
</tr>
<tr>
<td>1000</td>
<td>70</td>
</tr>
</tbody>
</table>

**Sampling**

First-stage sampling (selection of villages) is done using random numbers and a sampling frame based on the fisheries authorities list of villages with shrimp ponds. The villages are listed on a spreadsheet with each village numbered from 1 to 12,890. A random number table (such as that included in Survey Toolbox) or software designed for the generation of random numbers (such as EpiCalc) is used.

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4 http://www.myatt.demon.co.uk/epicalc.htm
The second stage of sampling involves random selection of ponds within each village. This requires a sampling frame, or list of each pond in the village. The fisheries authorities use trained local fisheries officers to coordinate the survey. For each selected village, the officer visits the village and convenes a meeting of all shrimp farmers. At the meeting, they are asked how many ponds they have and a list of farmers’ names and the number of ponds is compiled. A simple random sample of the appropriate number of ponds (between 29 and 70, from the table above, depending on the number of ponds in the village) is selected from this list. This is done either using software (such as Survey Toolbox’s RandomAnimal program), or manually with a random number table or decimal dice for random number selection. Details of this process are described in Survey Toolbox. This selection process identifies a particular pond in terms of the name of the owner, and the sequence number amongst the ponds owned (e.g. Mr Smith’s 3rd pond). Identification of the actual pond is based on the owners own numbering system for the ponds.

**Testing**

Once ponds have been identified, the actual survey consists of ‘testing those ponds’. In practice, this involves the farmers observing the ponds during one complete production cycle. The local fisheries officer makes weekly visits to each farmer to check if any of the selected ponds have suffered mass mortality. If any are observed (i.e. the first test is positive), 20 moribund shrimp are collected for laboratory examination (first PCR, and then, if positive, transmission experiments).

**Analysis**

Analysis is performed in two stages. First, the results from each village are analysed to ensure that they meet the required level of confidence. If the target sample size is achieved (and only negative results obtained), the confidence should be 95% or greater in each village. At the second stage, the results from each village are analysed to provide a country level of confidence. Again, if the target sample size (number of villages) is achieved, this should exceed 95%.

**EXAMPLE 3 — SPATIAL SAMPLING AND THE USE OF TESTS WITH IMPERFECT SPECIFICITY**

**Context**

A country has an oyster culture industry, based primarily on rack culture of oysters in 23 estuaries distributed along the coastline. In similar regions in other countries, Disease Z causes mortalities in late summer/early autumn. During an outbreak a high proportion of oysters are affected, however, it is suspected that the agent may be present at relatively low prevalence in the absence of disease outbreaks.

**Objective**

The national authorities wish to demonstrate national freedom from Disease Z. If the disease should be detected, a secondary objective of the survey is to collect adequate evidence to support zoning at the estuary level.

**Approach**

The authorities conclude that clinical surveillance for disease outbreaks is inadequate because of the possibility of low level subclinical infections. It is therefore decided to base surveillance on a structured two-stage survey, in which sampled oysters are subjected to laboratory testing. The first stage of the survey is the selection of estuaries. However, due to the objective of providing evidence for zoning (should disease be found in any of the estuaries), it is decided to use a census approach and sample every estuary. In essence this means that there will be 23 separate surveys, one for each estuary. A range of options for sampling oysters are considered, including sampling at harvest or marketing, or using farms (oyster leases) as a level of sampling or stratification. However the peak time of activity of the agent does not correspond to the harvest period, and the use of farms would exclude the significant numbers of wild oysters present.
in the estuaries. It is therefore decided to attempt to simulate simple random sampling from the entire oyster population in the estuary, using a spatial sampling approach.

**Survey standards**

- The target population is all of the oysters in each of the estuaries. The study population is the oysters present during the peak disease-risk period in late summer early autumn. Wild and cultured oysters are both susceptible to disease, and may have associated with them different (but unknown) risks of infection. They are therefore both included in the study population. As will be described below, sampling is based on mapping. Therefore the study population can more accurately be described as that population falling within those mapped areas identified as oyster habitats.

- A design prevalence value is only required at the oyster level (as a census is being used at the estuary level). While the disease is often recognised with very high prevalence during outbreaks, a low value is used to account for the possibility of persistence of the agent in the absence of clinical signs. A value of 2% is selected.

- The test used is histopathology with immuno-staining techniques. This test is known to produce occasional false-positive results due to nonspecific staining, but is very sensitive. Published studies indicate values of 99.1% for sensitivity and 98.2% for specificity. No other practical tests are available. This means that it is not possible to definitively differentiate false positives from true positives, and that in a survey of any size, a few false positives are expected (i.e. 1.8%).

- The confidence is set at 95% and the power at 80%. In the previous examples, due to the assumed 100% specificity achieved by use of multiple tests, the effective power was 100%. In this case, with imperfect specificity, there will be a risk of falsely concluding that a healthy estuary is infected, so the power is not 100%. The choice of a relatively low figure (80%) means that there is a 1 in 5 chance of falsely calling an estuary infected when it is not infected, but it also dramatically decreases the survey costs, through a lower sample size.

**Sample size**

Based on the assumption that the sampling procedure will mimic simple random sampling, the sample size (number of oysters to sample per estuary) can be calculated with FreeCalc. The population size (number of oysters per estuary) is assumed to be very large. The calculated sample size, using the sensitivity, specificity and design prevalence figures given above, is 450. FreeCalc also reports that, based on this sample size and the specificity of the test, it is possible to get 10 or fewer false-positive test results, and still conclude that the population is free from disease. This is because, if the population were infected at 2% or greater, the anticipated number of positive reactors from a sample of 450 would be greater than 10. In fact, we would expect 9 true positives \((450 \times 2\% \times 99.1\%)\) and 8 false positives \((450 \times 98\% \times 1.8\%)\) or a total of 17 positives if the population were infected at a prevalence of 2%.

This illustrates how probability theory and adequate sample size can help differentiate between true- and false-positive results when there is no alternative but to use a test with imperfect specificity.

**Sampling**

The aim is to collect a sample of 450 oysters that represent an entire estuary. Simple random sampling depends on creating a sampling frame listing every oyster (not possible) and systematic sampling depends on being able to (at least conceptually) line up all the oysters (again, not possible). The authorities decide to use spatial sampling to approximate simple random sampling. Spatial sampling involves selecting random points (defined by coordinates), and then selecting oysters near the selected points. In order to avoid selecting many points with no oysters nearby, the estuary is first mapped (the fisheries authorities already have digital maps defining oyster leases available). To these maps areas with significant concentrations of wild oysters are also added, based on local expertise. Pairs of random numbers are generated such that the defined point falls within the defined oyster areas. Other schemes are considered (including using a rope...
marked at regular intervals, laid out on a lease to define a transect, and collecting an oyster adjacent to each mark on the rope) but the random coordinate approach is adopted.

Survey teams then visit each point by boat (using a GPS [Global Positioning System] unit to pinpoint the location). A range of approaches is available for selecting which oyster to select from a densely populated area, but it should involve some effort at randomness. Survey staff opt for a simple approach: when the GPS receiver indicates that the site has been reached, a pebble is tossed in the air and the oyster closest to the point where it lands is selected. Where oysters are arranged vertically (e.g. wild oysters growing up a post), a systematic approach is used to determine the depth of the oyster to select. First, an oyster at the surface, next, an oyster halfway down, and thirdly, an oyster as deep as can be reached from the boat.

This approach runs the risk of bias towards lightly populated areas, so an estimate of the relative density of oysters at each sampling point is used to weight the results (see Survey Toolbox for more details).

Testing

Specimens are collected, processed, and analysed following a standardised procedure. The results are classified as definitively positive (showing strong staining in a highly characteristic pattern, possibly with associated signs of tissue damage), probably positive (on the balance of probabilities, but less characteristic staining), and negative.

Analysis

The interpretation of the results when using a test with imperfect specificity is based on the assumption that, in order to conclude that the population is free from infection, any positive result identified is really a false positive. With a sample size of 450, up to 10 false positives may be expected while still concluding that the population is free from disease. However, if there is reasonable evidence that there is even a single true positive, then the population cannot be considered free. This is the reason for the classification of positive results into definitive and probable positives. If there are any definitive positives at all, the population in that estuary must be considered infected. The probable positives are consistent with false positives, and therefore up to 10 may be accepted. Using FreeCalc the actual confidence achieved based on the number of (presumed) false positives detected can be calculated. For instance, if 8 'probably positive' results were detected from an estuary, the confidence level for the survey would be 98.76%. On the other hand, if 15 ‘probably positive’ results were detected, the confidence is only 61.9%, indicating that the estuary is likely to be infected.

Discussion

Normally, it may be safely assumed that a surveillance system aimed at demonstrating freedom from disease is 100% specific. This is because any suspected occurrence of disease is investigated until a definitive decision can be made. If the conclusion is that the case is truly a case of disease, then there is no issue of declaring freedom – the disease is known to be present. This example presents a different situation where, due to lack of suitable tests, it is not possible for the surveillance system to be 100% specific. This may represent an unusual situation in practice, but illustrates that methods exist for dealing with this sort of problem. In practice, a conclusion that a country (or estuary) is free from infection, in the face of a small (but statistically acceptable) number of positive results, will usually be backed up by further evidence (such as the absence of clinical disease).
CHAPTER 1.1.5.

METHODS FOR DISINFECTION OF
AQUACULTURE ESTABLISHMENTS

A. METHODS FOR DISINFECTION OF FISH FARMS

1. GENERAL PRINCIPLES

The choice of disinfection procedures depends on the size, type and nature of the materials and sites to
be disinfected. With the exception of the skin of personnel and eggs, which must be disinfected with
non-corrosive products, the surfaces to be disinfected consist of fabric or woven material (clothes,
nets), hard surfaces (plastic, cement) or permeable materials (earth, gravel). Disinfection is more
difficult for permeable surfaces and requires more time. Table 1 indicates the most common
ingredients and the methods to be used on the basis of these criteria.

The use of chemical products entails the implementation of measures to protect personnel and the
cultured animals and to mitigate environmental effects. It is first necessary to protect the skin and eyes
from contact with dangerous substances by using impermeable clothing, boots, eye protection and a
hat. The respiratory tract must be protected by a mask and the operator must not touch any food
without having thoroughly washed his/her hands. Finally, the products must be stored in such a way as
not to present direct or indirect danger to animal/fish or human life or the environment.

The material must be thoroughly cleaned before being disinfected. Organic material generated/
removed during the cleaning process, such as pond sludge, etc., should also be disposed of in an
appropriate manner that prevents spread of disease by such material and is environmentally safe.

Ideally, approved procedures for the use of disinfectants in aquaculture should be established. An
approval scheme should consider disinfection effect against target pathogens, toxicological and
ecotoxicological properties of the products.

Following disinfection or stamping-out, the farm should be restocked from a disease-free source.

Table 1. Disinfection and method of use

<table>
<thead>
<tr>
<th>Processes</th>
<th>Indications</th>
<th>Method of use*</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desiccation, sunlight</td>
<td>Fish pathogens on earthen bottoms</td>
<td>Dry for 3 months at an average temperature of 18°C</td>
<td>Drying period can be reduced by the use of a chemical disinfectant</td>
</tr>
<tr>
<td>Dry heat</td>
<td>Fish pathogens on concrete, stone, iron, ceramic surfaces</td>
<td>Flame-blower, blow-lamp</td>
<td></td>
</tr>
<tr>
<td>Damp heat</td>
<td>Fish pathogens in transportation vehicle tanks</td>
<td>Steam at 100°C or more for 5 minutes</td>
<td></td>
</tr>
<tr>
<td>Ultra-violet rays UV-C (254 nm)</td>
<td>Viruses and bacteria</td>
<td>10 mJ/cm²</td>
<td>Minimum lethal dose</td>
</tr>
</tbody>
</table>
### Table 1 (continued). Disinfection and method of use

<table>
<thead>
<tr>
<th>Processes</th>
<th>Indications</th>
<th>Method of use*</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physical</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultra-violet rays</td>
<td>Infectious pancreatic necrosis (IPN) and nodavirus (VNN/VER) in water</td>
<td>125–200 mJ/cm²</td>
<td></td>
</tr>
<tr>
<td>UV-C (254 nm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chemical</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>Infectious salmon anaemia (ISA)</td>
<td>0.04–0.13%</td>
<td></td>
</tr>
<tr>
<td>Quartenary ammonia</td>
<td>Virus, bacteria, hands, plastic surfaces</td>
<td>0.1–1 g/litre for 1–15 minutes</td>
<td>IPN virus resistant</td>
</tr>
<tr>
<td>Calcium oxide&lt;br&gt;a</td>
<td>Fish pathogens on dried earth-base</td>
<td>0.5 kg/m² for 4 weeks</td>
<td>Replace in water and empty disinfected pools keeping the effluents at pH &lt;8.5</td>
</tr>
<tr>
<td>Calcium hypochlorite&lt;br&gt;a</td>
<td>Bacteria and viruses on all clean surfaces and in water</td>
<td>30 mg available chlorine/litre. Leave to inactivate for several days or neutralise with Na thiosulfate after 3 hours</td>
<td>Can be neutralised with sodium thiosulfate. See special recommendations</td>
</tr>
<tr>
<td>Calcium cyanamide&lt;br&gt;a</td>
<td>Spores on earthen bottoms</td>
<td>3000 kg/ha on dry surfaces; leave in contact for 1 month</td>
<td></td>
</tr>
<tr>
<td>Chloramine T</td>
<td>Destroys ISA</td>
<td>1% for 5 minutes</td>
<td></td>
</tr>
<tr>
<td>Chloramine T</td>
<td>Destroys IPN</td>
<td>1% for 30 minutes</td>
<td></td>
</tr>
<tr>
<td>Chlorine dioxide</td>
<td>ISA</td>
<td>100 ppm for 5 minutes</td>
<td>In water of low organic loading</td>
</tr>
<tr>
<td>Formic acid</td>
<td>Ensile fish waste</td>
<td>pH &lt;4 after at least 24 hours</td>
<td>Destroys bacterial fish pathogens and ISA but not IPN</td>
</tr>
<tr>
<td>Formalin</td>
<td>Fish pathogens in sealed premises</td>
<td>Released from formogenic substances, generally trioxymethylene. Comply with instructions</td>
<td>Nodavirus resistant</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>ISA virus</td>
<td>0.02–0.06%</td>
<td></td>
</tr>
<tr>
<td>Iodine (iodophors)</td>
<td>Bacteria, viruses on nets, boots and clothing</td>
<td>200 mg iodine/litre for a few seconds</td>
<td>See special recommendations</td>
</tr>
<tr>
<td>Iodine (iodophors)</td>
<td>Hands, smooth surfaces</td>
<td>&gt;200 mg iodine/litre a few seconds</td>
<td></td>
</tr>
</tbody>
</table>

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1 Viral nervous necrosis /Viral encephalopathy and retinopathy

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Manual of Diagnostic Tests for Aquatic Animals 2006 51
### Table 1 (continued). Disinfection and method of use

<table>
<thead>
<tr>
<th>Processes</th>
<th>Indications</th>
<th>Method of use*</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ozone</td>
<td>Sterilisation of water, fish</td>
<td>0.2–1 mg/litre for 3 minutes</td>
<td>Costly and very toxic for fish and humans</td>
</tr>
<tr>
<td>Pathogens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ozone in seawater</td>
<td>Surfaces, equipment</td>
<td>0.5–1 mg/litre TRO² for 30–60 minutes</td>
<td></td>
</tr>
<tr>
<td>Peroxy compounds, e.g. Virkon</td>
<td>IPN virus</td>
<td>1% for 1 minute</td>
<td></td>
</tr>
<tr>
<td>Peracetic acid</td>
<td>ISA virus</td>
<td>0.08–0.25%</td>
<td></td>
</tr>
<tr>
<td>Sodium hydroxide¹</td>
<td>Fish pathogens on resistant</td>
<td>Mixture:</td>
<td>The most active disinfectant Ca(OH)₂ stains the surfaces treated; Teepol® is a tensio-active agent.</td>
</tr>
<tr>
<td>surfaces with cracks</td>
<td></td>
<td>Sodium hydroxide, 100 g, Teepol®, 10 g, Calcium hydroxide, 500 g, Water, 10 litres, Spray, 1 litre/10 m². Leave for 48 hours.</td>
<td></td>
</tr>
<tr>
<td>Sodium hypochlorite²</td>
<td>Bacteria and viruses on all clean surfaces and in water</td>
<td>30 mg available chlorine/litre. Leave to inactivate for a few days or neutralise with Na thiosulfate after 3 hours.</td>
<td></td>
</tr>
<tr>
<td>Sodium hypochlorite²</td>
<td>Nets, boots and clothing</td>
<td>200 mg to 1 g available chlorine/litre for several minutes. Leave to inactivate for a few days or neutralise with Na thiosulfate after 3 hours.</td>
<td></td>
</tr>
<tr>
<td>Sodium hypochlorite²</td>
<td>Hands</td>
<td>Rinse with clean water or neutralise with thiosulfate</td>
<td></td>
</tr>
</tbody>
</table>

¹ Dangerous – See precautions indicated in general recommendations
² The concentrations indicated are those for the active substance. NB: The chemicals must be approved for the prescribed use and used according to the manufacturer’s specifications.

### 2. NEUTRALISATION OF HALOGENS

Chlorine and iodine are highly toxic for aquatic animals and, in order to prevent serious accidents that could result from a manipulation error, it is recommended to neutralise these products with sodium thiosulfate – five moles of thiosulfate neutralise four moles of chlorine. The molecular proportions are the same for iodine.

Accordingly, in order to inactivate chlorine, the amount of thiosulfate should be 2.85 times the amount of chlorine (in grams):

\[
\text{Number of grams of thiosulfate} = 2.85 \times \text{number of grams of chlorine.}
\]
For iodine, the amount of thiosulfate should be 0.78 times the amount of iodine in grams:

\[ \text{Number of grams of thiosulfate} = 0.78 \times \text{number of grams of iodine}. \]

It is also possible to prepare a thiosulfate solution at 1% by weight, in which case the neutralising volumes will be as follows (in ml):

1. for chlorine:
   \[ 28.5 \times \frac{\text{[number of litres of the disinfecting solution} \times \text{concentration mg/litre]}}{100} \]
2. for iodine:
   it is necessary to multiply by 7.8 instead of by 28.5.

**B. METHODS FOR DISINFECTION OF MOLLUSCS FARMS**

1. **GENERAL PRINCIPLES**

The general principles pertaining to disinfection of mollusc farms (hatcheries, holding facilities) involve the application of chemical treatments in sufficient concentrations, and for sufficient periods, to kill all pathogenic organisms that would otherwise gain access to surrounding water systems. As the inherent toxicity of disinfectants prohibits safe use in open water, or open water systems, disinfection can only reasonably be applied to hatcheries and tank holding facilities, and, as a rule, all disinfectants must be neutralised before release into the surrounding environment. In addition, as mollusc farms are generally seawater based, compounds produced during seawater disinfection (residual oxidants) must also be disposed of carefully.

Disinfection of eggs and larval stages is not considered practical for most molluscan systems. In addition, there is little information on specific disinfection procedures for pathogens of molluscs (i.e. *Marteilia* spp., *Haplosporidium* spp., *Bonamia* spp., *Perkinsus* spp., iridovirus and pathogenic levels of marine microbes) or seawater. Therefore, disinfectants and concentrations are based on related pathogens or seawater sterilisation. Three stages of disinfection can be applied to hatcheries:

a) pretreatment of influent water, e.g. filters (1.0 and 0.22 µm) or chemical disinfection (see Section B.2.) = protection of stocks of molluscs;

b) treatment within the facilities (especially recycling systems) = protection of stocks of molluscs;

c) treatment of effluent water = protection of the environment.

2. **DISINFECTANTS* – PIPELINES AND TANKS**

Routine disinfection of pipelines and tanks is highly recommended; the frequency of disinfection will vary according to the turnover of stocks of molluscs. High concentrations of molluscs should be rotated between disinfected tanks as often as practical and/or kept in seawater that has been disinfected with ozone (see Section B.3.a) or chlorine (see Section B.3.b) and subsequently neutralised. Each new batch of molluscs introduced into a facility should be placed in predisinfected tanks.

As the presence of organic matter will reduce the disinfection capacity of most disinfectants, filtering influent water (see Section B.1.a) is recommended. In addition, all surfaces must be thoroughly cleaned prior to disinfection. The detergent used must be compatible with the disinfectant and both must be

---

2 **TRO**: Total residual oxidant

* The products specified have proven satisfactory for the purposes indicated; this does not mean that other products may not be equally satisfactory.
compatible with the surface being treated (e.g. iodophor solutions are generally acidic so cannot be used on concrete, which is alkaline). Ensure that the waste produced from washing is disinfected before disposal. Complete coverage of the surfaces is required, e.g. using a high pressure spray or soak. Wear appropriate protective clothing when working with any disinfectant (see Section A.1).

Regular air- or heat-drying of pipelines (daily), tanks and other equipment (e.g. algal culture carboys), in addition to disinfection of their surfaces, is also recommended (especially for disease outbreaks of unidentified aetiology).

a) Chlorine is usually applied as sodium hypochlorite (Chlorox®, household bleach, etc.). Fill all pipelines with 50 mg chlorine/litre (≈ 50 parts per million [ppm]). Allow an exposure time of at least 30 minutes before flushing with clean seawater. This solution is effective against most microbial agents as well as labyrinthulid protozoans. Chlorinated seawater must be neutralised prior to release from the holding facility. Optimal neutralisation is achieved by passage through activated charcoal (removes excess chlorine and chloramines). Reducing agents such as sodium thiosulfate or aeration (which do not remove toxic chloramines) may also be used.

b) Iodophors are generally applied as alkaline solutions (Wescodyne®, Betadine®) at 200–250 mg iodine/litre (ppm) with a contact time of at least 10 minutes.

NOTE: Iodophors are not effective against certain protozoans in suspension, e.g. over 1000 mg iodine/litre is tolerated by Labyrinthuloides haliotidis of abalone. Iodophors may be effective against protozoan parasites following air or heat drying of tank surfaces and pipelines.

3. DISINFECTANTS – EFFLUENT WATER

a) Ozone has been used successfully in controlling the microbial content of effluent water from quarantine facilities. Residual compounds, formed as a result of the interaction of ozone with seawater (residual oxidants), at levels of 0.08–1.0 mg/litre are considered sufficient to significantly reduce live microbes (principally bacteria).

NOTE: The measurement of residual ozone in seawater is problematic due to the rapid and continuous formation of oxidant products in seawater. Residuals formed between ozone and seawater (hypobromite, bromine or hypobromous acid) are toxic to oyster larvae (and possibly other mollusc larvae) and should be removed using a charcoal filter before passing through/out of the mollusc facility. UV treatment of seawater post-ozonation may be required for complete sterilisation, e.g. for quarantine.

b) Chlorine administered as sodium hypochlorite at a concentration of 25 mg chlorine/litre is effective against certain protozoans (L. haliotidis); however, 50 mg chlorine/litre is recommended for complete microbial sterilisation (as for pipelines and tanks – see Section B.2.a). Higher concentrations may be used under certain conditions (e.g. quarantine); however, these require proportionately greater neutralisation treatments and exhaust systems to deal with the toxic fumes produced.

c) Iodophors are not as effective as the above two treatments for killing protozoans.

4. DISINFECTANTS – CLOTHING AND EQUIPMENT

Clean surfaces with detergent and disinfectants prior to proper disinfection.

a) Iodophors (e.g. Wescodyne®, Betadine®) at 200–250 mg iodine/litre can be used as a footbath. NOTE: Iodophors will stain clothing.

b) Chlorine (household bleach solution at 50 mg chlorine/litre) is also an effective footbath or equipment wash.
c) Sodium hydroxide (1% NaOH + 0.1% Teepol® or other detergent) makes an effective footbath for rubber boots. NOTE: Do NOT use for dress shoes/boots.

5. SPECIAL RECOMMENDATIONS

a) Both chlorine and ozone produce long-lived residual oxidant compounds in seawater. Seawater at 35 parts per thousand (ppt) salinity contains 60 ppm bromide ion, which produces hypobromite in the presence of ozone. Disinfected artificial seawater, at the same salinity, produces bromine and hypobromous acid. As these, along with other residual compounds, are toxic to larval oysters (and possibly other molluscs), treated seawater must be passed through an activated charcoal filter before being used for live mollusc larvae.

Alternative protocols for halogen neutralisation involve treatment with sodium or potassium thiosulfate (see Section A.2).

b) Monitoring of residual oxidants must be carried out regularly, especially where temperature fluctuations occur. As residual ozone cannot be measured accurately in seawater, alternative monitoring protocols must be installed, such as a feedback loop.

Exhaust systems should also be in place to remove toxic fumes (produced during disinfection) from enclosed work areas. Ensure compliance with local atmospheric regulations when discharging toxic fumes.

c) The following management practices can be used to reduce opportunistic pathogen proliferation within a mollusc hatchery or holding facility:

i) maintain pathogen-free algal stocks and cultures;

ii) use appropriate water filtration, regular disinfection of tanks, pipes and equipment, and footbaths, and water changes;

iii) isolate infected stocks and associated equipment at the first sign of disease;

iv) discard infected stock and sterilise equipment;

v) identify the source of infection within the holding facility to prevent further infection (algal stocks, seawater influent system, broodstock, larval stock).

C. METHODS FOR DISINFECTION OF CRUSTACEAN FARMS

1. GENERAL PRINCIPLES

The choice of a disinfection method for use in a crustacean farm depends on many factors, which may include: the reason(s) for disinfection, whether the ‘farm’ is a broodstock facility, hatchery, or growout farms; and the type of growout farm. Because the penaeid shrimp are the hosts for all but one of the crustacean diseases currently listed in the Aquatic Code, this Section will focus on penaeid shrimp.

2. REASON(S) FOR DISINFECTION

Disinfection is employed as a common disease management tool in shrimp farming. It may be used as a routine practice in biosecurity programmes designed to exclude specific diseases, as well as a routine sanitary measure employed to reduce disease incidence within farms, or it may be used in disease eradication (stamping out) efforts. The specific reason for disinfection, will determine the disinfection strategy used and how it is applied.
3. **Occurrence of listed diseases**

When an OIE listed disease, or an important but unlisted emerging disease occurs for the first time at a particular farm, at a particular site (i.e. at a quarantine facility), or within a region or country previously believed to be free of that disease, it may be advisable, if not required, to eradicate the disease by depopulating the facility and performing a thorough disinfection of all or part of the facility. Fallowing of the affected facility for a defined period of time may be warranted in some situations (see Chapter 1.7.1, Guidelines for fallowing in aquaculture in the *Aquatic Code*).

4. **Prevention of disease spread to wild populations**

The direct disposal of diseased populations of live shrimp (any life stage; i.e. fertilised or unfertilised eggs, larvae, postlarvae, juveniles, or adults) or waste products derived from them (i.e. processing plant wastes such as shells, broken shrimp pieces, etc.) into receiving waters (i.e. creeks, rivers, estuaries, bays, littoral areas) is a dangerous practice that facilitates the spread of disease from farmed populations to wild crustacean stocks or to neighbouring farms that use the same water supply, and it should not be permitted to occur. With cultured stocks, when the decision is made to discard a population (i.e. that is being cultivated in a hatchery tank or growout pond) due to the presence of disease (or poor culture performance which may be due to an undiagnosed disease), the stock in the tank or pond should be harvested and/or humanely killed in the tank or pond. The water in the tank or pond should be disinfected (see specific subsections on disinfection of tanks and growout ponds in the Section 5) prior to discharge. The emptied tank or pond should be disinfected prior to restocking.

5. **Routine sanitation and biosecurity**

Many crustacean farms, especially those cultivating penaeid shrimp, employ measures that use a number of disinfection methods for disease prevention and control. These measures may be part of a farm’s routine biosecurity programme that may be designed for exclusion of specific diseases as well as serving as general pest and disease exclusion measures.

5.1. **Disinfectants**

The following list comprises the disinfectants recommended for use in shrimp farms (the appropriate disinfectant regimes for each specific application are discussed in the appropriate subsection):  
- chlorine (as calcium hypochlorite, HTH™ or a bleach solution containing a sufficient concentration of hypochlorite);  
- formaldehyde gas (from sublimated paraformaldehyde or concentrated formalin/potassium permanganate reaction);  
- iodine (as contained in iodophors);  
- lime (as calcium oxide or calcium hydroxide);  
- UV light (from natural sunlight);  
- ozone;  
- steam;  
- hot water (60ºC);  
- concentrated acids;  
- desiccation;  
- detergents (for general cleaning, with some degree of disinfection capability for many products).
5.2. Hatcheries and broodstock rearing/holding facilities

Virtually all penaeid shrimp hatcheries and broodstock holding/rearing facilities use seawater that has been disinfected to remove potential pathogens, pests, and disease-carrying agents via mechanical filtration, UV irradiation, and/or chemical disinfection. This may be by passive source water filtration (i.e. by the use of seawater wells or well points) or by mechanical filtration using high pressure pumps and a variety of water filtration devices and pore sizes. Some facilities use filtration coupled with UV light disinfection of source water, while others use chemical disinfection methods, using either chlorination and de-chlorination or high doses of ozone and subsequent removal of residual oxidants. Chemical disinfection of source water typically requires the use of one or more water storage reservoirs in which the water is treated and detoxified before use in the shrimp hatchery or broodstock facility. Numerous manuals are available that provide specifics on hatchery and broodstock facility design and operation for shrimp culture, and in which details on source water disinfection are provided.

a) Disinfection of eggs and larvae in penaeid shrimp hatcheries

Certain penaeid shrimp viral diseases (i.e. Spherical baculovirosis, Tetrahedral baculovirosis, Hepatopancreatic parovirus infections) are transmitted by faecal contamination of spawned eggs. These diseases, as well as infections due to certain other shrimp viruses such as White spot disease virus, and certain bacterial and fungal disease agents, can be eliminated or have their incidence reduced through the routine use of disinfection protocols when used to surface disinfect eggs and/or recently hatched nauplii. A widely used method is given below:

For fertilised eggs

Collect fertilised eggs. Rinse with running seawater for 1–2 minutes. Fully immerse eggs in 100 ppm (parts per million) formalin for 1 minute. Fully immerse eggs in iodophor (0.1 ppm iodine) for 1 minute. Rinse in running seawater 3–5 minutes. Transfer to disinfected larval rearing tanks.

For nauplii

Using phototaxic response to light, collect nauplii with netting or screen. Rinse with running seawater for 1–2 minutes. Fully immerse nauplii in 400 ppm formalin for 30–60 seconds. Fully immerse nauplii in iodophor (0.1 ppm iodine) for 1 minute. Rinse in running seawater 3–5 minutes. Transfer to disinfected larval rearing tanks.

b) Disinfection of tanks, equipment, pipes, air stones, etc.

For routine sanitation, hatchery and broodstock tanks (i.e. tanks for broodstock maturation, matting, spawning, larval rearing and indoor nursery) should be cleaned, disinfected and dried between use. Tanks used for the above-named purposes in crustacean (especially shrimp) hatcheries are typically precast fiberglass tanks or they are constructed of concrete or wood and either coated or painted with resin-based coatings (e.g. epoxy or fiberglass resin) or lined with plastic liners manufactured for that purpose. After harvest of the stock from the tank, all loose objects and large-sized organic debris such as algae, faeces and left-over feed should be removed. With relatively small tanks, it is advisable after harvest of the stock to fill the tank to capacity, immerse all nonporous corrosion resistant equipment (i.e. airlines, air stones, stand pipes, screens, sampling containers, etc.) in the tank, and then add calcium hypochlorite to provide a minimum of 200 ppm of free chlorine. This should be allowed to set overnight. After the proper chlorinated soak-time, the tank can be drained and freshwater rinsed. Before draining the system, the treated water should be dechlorinated (see specific subsections on chlorination described in this Section), unless appropriate effluent collection and treatment systems are in place. After the tank has been rinsed it should be allowed to completely dry. In
the case of large tanks, an initial cleaning to remove loose debris should be followed by disinfection with a concentrated (~1600 ppm as chlorine) solution of calcium hypochlorite. All inside and outside surfaces should then be sprayed with this chlorine solution. The tank should then be allowed to set for several hours and then rinsed, filled and flushed. Surfaces should then be scrubbed free of all remaining material. After disinfection with chlorine, small or large tanks should be rinsed with clean water, then filled and flushed to ensure that no chlorine residues remain before the tank is restocked for another crop.

5.3. Disinfection of growout ponds

Following the routine harvest of a crop from a growout pond (or from a large tank or raceway used for growout of a crop), the pond (tank) bottom should be inspected. Large deposits of organic debris should be treated or removed. This is easily accomplished in lined tanks, raceways, or ponds (i.e. by flushing with a high pressure hose), but poses more of a challenge in large earth bottom ponds. Many methods of pond bottom disinfection and treatment between crops are practiced. These methods are given in detail in a number of shrimp farming manuals, and some will be listed here only with minimal details:

a) Chlorination

This disinfectant may be used for routine treatment of ponds between crops or when disease eradication is the goal. After draining the pond, remove (and dispose of [see section on carcass disposal in Section C.6]) as many animals from the system as is possible (this may be difficult in pond systems where the removal of large numbers of dead shrimp would not be practical). Partially refill the pond (or fill to capacity if required), discontinue the addition of new water, stop the discharge of effluent water, and remove any internal or external sources of aeration or aeration devices, which might be subject to corrosion. Then evenly distribute sufficient granulated calcium hypochlorite (such as Olin HTH™) to provide a minimum residual free chlorine concentration of 10 ppm within the entire system’s water. (NB: The person(s) applying the chlorine should wear waterproof outer ware to protect their skin, an approved chlorine mask, and goggles or a face shield for eye protection.) Redistribute additional calcium hypochlorite as often as required to maintain the residual concentration at near or 10 ppm. Allow the system to set for a minimum of 24–48 hours (especially if applied to large systems) at this minimal chlorine concentration. The chlorine will kill all shrimp and most, if not all, of the other organisms occupying the water column or resident in the pond. After the pond has been treated with chlorine for the required minimum time and before any water is discharged, neutralise the chlorine either passively by exposure to sunlight and air for approximately an additional 48 hours (without the addition of new chlorine) or by the addition of sodium thiosulphate at a rate of five (5) molecules of sodium thiosulphate for each four (4) molecules of chlorine (or the weight of sodium thiosulphate being 2.85 times the weight of chlorine in the water, see example table below).

<table>
<thead>
<tr>
<th>Pond size</th>
<th>Average depth</th>
<th>Volume</th>
<th>Chlorine dose</th>
<th>Chlorine required</th>
<th>HTH (65% active Cl)</th>
<th>Thiosulphate required</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hectare</td>
<td>1 m</td>
<td>10,000 m³</td>
<td>10 ppm</td>
<td>100 kg</td>
<td>154 kg</td>
<td>285 kg</td>
</tr>
</tbody>
</table>

Periodic testing should be done for residual chlorine; water should not be discharged until it has reached 0 ppm. Once the chlorine levels have been ascertained to be at 0 ppm, the system water can be safely dumped into the farm’s effluent system. In some culture systems, in particular raceways, tanks and small lined ponds (i.e. those systems in which the majority of the shrimp were not removed prior to disinfection), the dead shrimp should be collected for proper disposal (see section on carcass disposal in Section C.6).
b) **Liming**

The lime, as calcium oxide or calcium hydroxide, should be applied to a very moist bottom at a rate of 5000 kg/ha or 1500 kg/ha, respectively. Great care should be taken to assure that the lime is spread evenly over the soil surface. The pond should then be allowed to set for at least a week, or at least until the soil has dried to the point of cracking to a depth of approximately 10–20 cm. Additional lime may be applied after ploughing (see below) at a rate of 50% of that normally prescribed. The pond should again be dried for at least a week, depending on the weather.

c) **Drying and ploughing**

Whether or not a pond is treated by chlorination or liming or left to dry untreated, ploughing is a commonly used method of treating a pond bottom to reduce its organic content, improve nutrient recycling, buffer pH, eliminate pests, and achieve disinfection through a combination of microbial degradation, exposure to sunlight, aeration, and desiccation. In some regions, drying and ploughing of dry pond bottoms may only be possible during the ‘dry season’. When pond drying is an option, the pond bottom should be allowed to dry until the surface has cracked to a depth of approximately 10 cm. Once this level of drying has been reached, the soil should be broken up to a depth of approximately 20 cm with a plough, tiller, disk harrow, tine harrow or other similar farm implement. Ponds treated in this manner should be left for at least a week before being refilled and restocked.

5.4. **Disinfection of source water**

Because several of the listed diseases of shrimp listed in the *Aquatic Code*, as well as a number of other important diseases, can be introduced into shrimp farms with source water when it contains vectors or carriers (i.e. wild infected crab or shrimp larvae), most farms operate with biosecurity plans that include provisions for the disinfection of source water. This may be accomplished by a variety of means which may include one or some combination of the following strategies:

a) Filtration of source water – source water is pumped into a supply/settling canal where it first passes through coarse bar screens to remove large aquatic animals and debris. Then the water is passed through a series of progressively finer screens, and final filtration is accomplished by passing source water through a fine mesh (150–250 µm mesh size) bag screen before being introduced into a culture pond or storage reservoir.

b) Instead of using mesh nets, some farms place filtration structures in the supply canal system. A series of compartments within these structures are filled with filter matrixes, beginning with coarse gravel for initial removal of large aquatic animals and debris, an intermediate section which contains a finer matrix of sand and gravel, and the end section which contains fine sand.

c) Chlorination and de-chlorination – source water is pumped to a supply canal or directly into culture ponds or reservoirs (with or without filtration) and treated with sufficient chlorine to kill any potential vectors or carriers in the source water.

d) ‘Zero’ or reduced water exchange: Some farms use supplemental aeration and re-circulation of water in culture ponds and within the supply and discharge systems of the shrimp farm to reduce source water requirements. This reduces the volume of source water that must be disinfected before use, as well as reducing nutrient loss from farms with effluent.

6. **Disease eradication and total facility clean-up**

This action may be necessary for disease control when significant, untreatable diseases occur at sites where eradication is an option. The confirmed diagnosis of an listed diseases, or of an important but unlisted emerging disease occurring for the first time at a particular farm, at a particular site (i.e. at a...
quarantine facility), or within a region or country previously believed to be free of that disease, are events wherein it may be advisable, if not required, to eradicate the disease by depopulating the affected facility and performing a thorough disinfection of all or part of the facility.

Fallowing of the affected facility for a defined period of time may be warranted in some situations (see Chapter 1.7.1, Guidelines for fallowing in aquaculture in the \textit{Aquatic Code}).

The following steps/actions may be used to achieve eradication of a disease through a total facility clean-up (TCU):

6.1. 

**Depopulate all living shrimp stocks from the affected facility**

a) Discontinue stocking of the facility.

b) Harvest and sell (if permitted) marketable stocks through normal market channels. In some circumstances cooking the product before marketing may be advisable. Cooking, either by steam or boiling water, will kill or deactivate all known disease agents of shrimp.

c) For unmarketable stocks the following are options for disposable after harvest:

i) Incineration: burn collected shrimp in a government approved (if required) incinerator. The limitations to this procedure are inherent to the disposal of shrimp. That is, shrimp contain large amounts of water and therefore this procedure may only be feasible for small quantities of shrimp or to larger quantities if the shrimp have been dried prior to incineration.

ii) Burial: although this technique should be applicable to a greater number of instances, it still has its limitations. The shrimp should be placed in a pit of sufficient depth to accommodate all of the shrimp and still provide for at least 50 cm of fill covering the shrimp. The pit should be located some distance from the facility undergoing TCU and a comparable distance from any other facility culturing shrimp. Drainage from the pit area should not be into the aquifer from which the TCU site (or any shrimp culture site) may pump its source water or into the area (estuary or beach) from which source water is drawn. Once a proper site has been selected, then the actual burial can take place. The bottom of the pit should be covered with calcium oxide (quicklime) at a rate of approximately 500 g/m$^2$ (5000 kg/ha) or with calcium hydroxide (slaked or hydrated lime) at a rate of approximately 150 g/m$^2$ (1500 kg/ha). The shrimp should be placed in the pit in layers of approximately 10 cm depth, each covered by a quantity of slaked lime or quicklime sufficient to completely cover the layer (equivalent to approximately 33–100% of the weight of the shrimp). The entire pit, including the top layer of shrimp carcasses, should then be overlaid with a minimum of 50 cm of fill dirt. In some locations, local environmental, public health and zoning officials should be consulted before the shrimp burial pit(s) is (are) dug.

6.2. **Disinfection of culture tanks and ponds**

For methods see appropriate subsections in Section C.5.

6.3. **Clean-up procedures for facility components other than culture areas**

In order for a TCU to be effective, it may be necessary to disinfect the entire facility after all the shrimp have either been harvested or disposed of in some other manner. After depopulation of the facility, every possible animate and inanimate carrier of the disease agent must be identified and either removed from the facility or thoroughly disinfected. The movement of disease agents between live shrimp or dead numerous shrimp can be easily understood, while the same can not be said for their movement via inanimate components. Hence, all areas, units, subunits or components which are contaminated or potentially contaminated must go through a cleaning and
disinfection process. See Table 1 in Section A and Section C.5.1 for a list of disinfectants and their methods of application.

a) Buildings

The disinfection regime used should be building-specific and dependent upon the use-pattern of that particular building.

i) Office buildings: these buildings would most often be subject only to foot traffic from people who have been in contaminated buildings or culture areas. Because of this, the greatest focus of attention should be the floors and cold storage units in the building. Floors should be thoroughly cleaned (if they are non-porous) with standard detergents and cleaning solutions, followed by a thorough drying. If the floors are carpeted, they should be vacuumed and cleaned with a detergent appropriate for carpets, or 'steam' cleaned. All other areas within these buildings, such as walls, bathrooms, desks, refrigerators, freezers, etc. should be examined for possibly contaminated materials (i.e. frozen shrimp in freezers) and any such item found and its container should be cleaned and disinfected or disposed of in a sanitary manner.

ii) Culture buildings: it must be assumed that these buildings have had direct contact with the disease agents and will therefore be handled in a different manner from that of the office buildings. The disinfection regime for these buildings will consist of two steps. First, the building should be thoroughly swept and/or vacuumed (where appropriate) to remove as much large-sized organic and inorganic debris as possible. This should be followed with the second step, treatment with chlorine. Chlorine solution (~1600 ppm) should be applied (by spraying) to all surfaces which are not prone to the corrosive actions of chlorine. Those surfaces which should not be chlorinated, can first be sponged with a iodophor solution minimally providing 200 ppm of free iodine. These can then be covered with plastic or any other protective material. Floor surfaces can be soak-chlorinated to a depth of 5 cm with a 200 ppm chlorine solution. This should be allowed to set for a minimum of 48 hours. If many of the sprayed surfaces are somewhat susceptible to corrosion by chlorine, those surfaces can be freshwater-rinsed after the 48-hour treatment.

In buildings where disinfection with chlorine is not practical, fumigation with formaldehyde gas should be considered. After a general cleaning, fumigation of a sealable building can be initiated. The entire process, from the time the building is first gassed until it can be occupied again, should take a minimum of 36–60 hours. The entire building must be totally sealed off during the actual fumigation; there should be no means by which the gas can escape once it is placed in the building. If possible, the electrical service for the building should be turned off. The required environment for formaldehyde gas disinfection is a minimum temperature of 18°C with a high relative humidity (at saturation is best, i.e. floors should be wet, etc.). Generation of formaldehyde gas is accomplished by the addition of 17.5 g potassium permanganate to each 35 ml of 100% formalin (a 37–39% aqueous solution of formaldehyde gas) for each 2.83 m³ (100 ft³) of space. Ideally, each room in the structure should have its own source of formaldehyde gas to assure that all areas of the building are uniformly treated. The correct amount of each compound (potassium permanganate and formalin) should be weighed out into separate containers, the formalin should be placed in a non-plastic container that is at least 10 times the combined volume of both the formalin and the potassium permanganate. (The person applying a formaldehyde gas fumigation should wear waterproof outer ware to protect their skin, an approved formaldehyde gas mask, and goggles or a face shield for eye protection.) The containers with the proper amounts of the two reagents should then be placed on the floor in the centre of the room, on a large disposable protective (plastic) mat. The formalin and potassium permanganate should not be mixed at this time. Once all rooms have the correct amounts of the two compounds, the building has been completely sealed and the environment modified as necessary, the actual fumigation can
begin. The mixing of the two compounds must be done very rapidly and carefully as the reaction is immediate and somewhat violent as formaldehyde gas is emitted. Starting with the room farthest from the exterior door, add the permanganate to the formalin and proceed to the next room. After all rooms have been completed, lock the exterior door and seal it from the outside with tape. The building should be allowed to set for a minimum of 12 hours. After this disinfection period the building should be flushed with clean air for 24–48 hours. There should be no detectable odour of formaldehyde when people are allowed to reoccupy the building.

An alternate method for the generation of formaldehyde gas is the sublimation of powdered paraformaldehyde. For each 2.83 m³ (100 ft³) of space, approximately 28 g paraformaldehyde should be used. It can be sublimated by being placed in an electric fry pan, which has been set on high. This procedure is somewhat more dangerous, because formaldehyde is flammable and a spark from such a heating device could theoretically ignite the gas. The same procedures noted above for the formalin/permanganate mixture in regards to venting, etc. should also be followed for the use of paraformaldehyde.

iii) Processing buildings: these buildings are typically constructed to permit routine disinfection. For the most part, the procedures followed in the routine operation of such buildings are appropriate for a TCU, provided that the building, its cold rooms, and its freezers are also disinfected and thoroughly dried. If considered necessary, fumigation with formaldehyde gas may be done to insure destruction of the disease agent(s) of concern.

iv) Other buildings: buildings (feed storage, maintenance, tool rooms, etc.) should be treated somewhat like the office building. Care should be taken to remove all the large-sized debris, which would normally be found in relative abundance within these types of buildings. Potentially contaminated surfaces within such buildings should next be spray-chlorinated and allowed to set for 24–48 hours. This should be followed by a freshwater rinse. All equipment, which should not be exposed to the corrosive action of chlorine, should be removed before the spraying, and they should be disinfected by surface disinfection with 200 ppm of iodophor. Once the equipment has been disinfected, it can be brought back into the building. Fumigation with formaldehyde gas is another option for this type of building.

b) Culture support equipment and systems

These are operational units of the shrimp culture facility which may be housed in a building.

i) Artemia systems: all Artemia decapsulation and cyst hatching units and tanks should be treated in the same manner as other tanks. They should be cleaned of all large debris, then filled to the top with clean water and calcium hypochlorite added to achieve a final concentration of 200 ppm (free Cl₂). Chlorination should be allowed to continue for 24–48 hours. The outside of such tanks may be spray-chlorinated (1600 ppm chlorine). Treated tanks can then be dechlorinated with sodium thiosulphate, drained, freshwater rinsed, and allowed to dry for a minimum of one week. Unopened containers of Artemia cysts at the facility can be retained. These should, however, be surface disinfected with chlorine (200 ppm) or iodophor (200 ppm).

ii) Algae systems: containers, tanks, incubators and rooms used to produce algae for feeding the larval stages of shrimp may be handled and disinfected in nearly the same way as other tanks systems. The only major difference being that special care must be taken to assure that all chlorine residues have been rinsed from the units before they are used again. In the case of the culture tubes, flasks, carboys, and flasks used to culture algae, a combination of acid (10% HCl) rinse or steam sterilisation can be used in lieu of disinfection with chlorine or iodophor.
Disinfection of stock cultures of living algae is not possible. The use of disinfection is clearly out of the question; any compound which would kill the disease agent would likewise kill the algae. Hence, there are two basic methods of minimising the chance of a disease agent being present in the stock cultures.

- **Dilution**: all stock cultures can be cloned from the existing stocks. Each culture should be diluted either by means of serial dilutions (for broth cultures) or streaked for single colonies (agar cultures). All dilutions must be performed using strict aseptic techniques with all media being properly autoclaved. Passages from the stock cultures should not occur until the algae culture room has itself been disinfected as per the above building procedures. Once a culture has been diluted and cloned by either of these methods, to the point where there remains only one cell of the original culture, the risk is negligible that a (shrimp) disease agent may be present.

- **New Stock Cultures**: If existing stock culture are discarded in a TCU, new stocks should be purchased from algae supply laboratories, or obtained from other sources where contamination with (shrimp) disease agents is unlikely, such as isolating desired species from wild populations of algae. New stock cultures should not be obtained from any facility that also cultures shrimp and may be contaminated with (shrimp) disease agents of concern.

iii) Farm equipment: nets, seines, porous air-line tubing, etc. which are relatively inexpensive and easily obtainable should be discarded and removed from the facility during a TCU rather than being disinfected as they are not readily disinfected and chlorine is likely to damage them and shorten their useful life.

Non-expendable equipment such as large size flexible plastic tubing, pumps and pipes, transfer tanks, cages, harvest cages, harvest tables, Secchi disks, laboratory glassware, etc. should be soak-chlorinated in 200 ppm solutions for 24–48 hours. This is most easily accomplished by placing these objects in the tanks that are filled with 200 ppm solutions of chlorine. Care should be taken to have all items completely submerged (use heavy items to weigh-down more buoyant objects). A good guide is to place everything (except those that are to be thrown away) that is loose or can be unsecured from its point of attachment, into the 200 ppm chlorine solution in their respective tanks.

In the case of those similar type items which are associated with ponds, they should be placed in a special series of tanks set up near their respective ponds. These tanks should be filled with 200 ppm chlorine solutions. Following soak-chlorination, these items should be allowed to dry and be exposed to natural UV (sunlight) sterilisation. They should be turned at least once to expose all areas of the items to direct sunlight.

Tools and machinery, such as tractors, trucks, portable and stationary power tools, etc., should be thoroughly cleaned with standard cleaning solutions. All traces of mud, shrimp feed, etc. must be removed from these items. Following this, disinfection of surfaces likely to have been contaminated in normal use should be rinsed off with an iodophor solution (at a concentration of 200 ppm) or cleaned with steam.

Small tools and instruments such as, scales and balances, test instruments, small power tools, etc., should be gently sponged off with 200 ppm of chlorine solutions if they are inert plastic or 200 ppm of iodophor if they are otherwise. These should then be placed back in their respective buildings during the formaldehyde fumigation. High precision electronic test equipment should not be subjected to the fumigation, especially if there has been little chance that it was ever contaminated.

iv) ‘New-Water’ Plumbing: all new-water plumbing which is contained within buildings, especially those which have blind ends or terminate in manifolds, should be filled with a
minimum 200 ppm chlorine solution. The chlorine solution should be held in the lines for 24–48 hours minimum, followed by clean water rinsing. Pipes may also be disinfected by recirculating hot water (>60°C) through them for several hours.

v) Uniforms, boots, etc.: all items worn or used by employees should be either disposed of or thoroughly washed and disinfected. In the case of clothing, such as coveralls, normal washing which incorporates a chlorine bleach is sufficient, especially if accompanied by sun drying. Other items, such as boots, gloves and other non-cloth items can be safely soak-chlorinated in a 200 ppm chlorine solution. This should be followed by a freshwater rinse. These items should also be contained within their respective buildings during formaldehyde fumigation.

vi) Feed items: all feed items, such as prepared feeds, fresh feeds (i.e. squid, bloodworms, frozen *Artemia*, bivalve molluscs, etc.) should be removed from the facility and replaced with new feeds from sources known to be free of contamination by shrimp disease-causing agents.

7. **Re-stocking of disinfected farms**

Following a TCU, restocking of the disinfected facilities or farms should be accomplished only with stocks known to be free of the diseases listed in the *Aquatic Code* or other emerging or significant diseases of concern.
SECTION 2.1.

DISEASES OF FISH

CHAPTER 1.1.

GENERAL INFORMATION

A. GENERAL BASIS FOR FISH HEALTH SURVEILLANCE/CONTROL PROGRAMMES

1. TARGET PATHOGENS AND DISEASES

Target pathogens and fish diseases are included in the Aquatic Animal Health Code (the Aquatic Code) according to the following basic considerations: they resist or respond poorly to therapy, have a restricted geographical range, are of high socio-economic importance, and occur in species involved in international trade. For the current OIE list of diseases of fish, please consult the current edition of the Aquatic Code. This Aquatic Manual includes chapters on diseases listed in the Aquatic Code and chapters on certain other diseases of importance to trade.

2. OVERALL APPROACH FOR ANIMAL HEALTH CONTROL IN FISH CULTURE

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

- Assessment of the health status of animals using methods based on the provision in chapter 1.1.4.
- The constraint of restocking open waters and farming facilities only with aquatic animals 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 infected stocks, disinfecting facilities and restocking with fish from approved disease-free sources.
- Notification by every Member Country of its particular requirements, besides those provided by the Aquatic Code, for importation of aquatic animals and aquatic 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 site of origin.

The issue of a health certificate by the appropriate official, based on a health status report and examinations of 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 Aquatic Code and possibly of other specified diseases (see model of international certificate in the Aquatic Code).

The assessment of the health status of fish stocks is based on inspection of fish production sites and further laboratory examination of samples originating from fish specimens taken among the stock of a defined fish population. This endeavour requires the fish sample to be collected according to defined sampling size charts and the samples to be processed according to accepted methods.
Several techniques are applicable for aquatic animal pathogens. For screening and diagnostic purposes, the Aquatic Manual has established two types of examination procedures that will be acceptable for such work; 1) Screening methods, and 2) Diagnostic methods. The accepted methods are listed under each disease chapter.

B. SAMPLING PROCEDURES

1. COLLECTION OF FISH SPECIMENS

1.1. Diagnosis in a disease situation

A minimum number of ten moribund fish or ten fish exhibiting clinical signs of the diseases in question should be collected: fish should be alive when collected, and should be sent to the laboratory alive or killed and packed separately in sealed aseptic refrigerated containers or on ice. The freezing of collected fish should 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. An identification label that includes information on the place, time, date, species, number of samples collected, dead/moribund state on collection, and the name and contact information of the individual collecting the sample(s) should be attached to the sample(s).

1.2. Fish appear to be clinically normal

Fish collection should 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 2), insofar as they are not exposed to possible recontamination by feral fish.

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

• Samples should comprise all susceptible species on the site (see relevant chapters of this Aquatic Manual for the list of species susceptible to each disease), 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 that originates from the same broodfish or spawning population.

The geographical origin of samples should be defined by the name of the sampling site associated with either its geographical co-ordinates or its location along a river course or body of water.

• 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 should comprise randomly selected live fish from all rearing units that represent the lot being examined.

• A general approach to surveillance and sampling is given in chapter 1.1.4 of this Aquatic Manual. The sampling should be designed in order to enable detection, at a 95% confidence level, of infected animals. The following section gives information relevant to sampling finfish. Until disease-specific details are included in the individual disease chapters in this Aquatic Manual, Table 1 can be used to calculate sample size.
• As in the case of clinically infected fish, organ and fluid samples should be taken and processed as soon as possible after fish specimen collection. Sample freezing should be avoided.

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

A general approach to surveillance and sampling is given in chapter 1.1.4 of this Aquatic Manual. The sampling should be designed in order to enable detection, at a 95% confidence level, of infected animals. The following section gives information relevant to sampling finfish. For those diagnostic tests where the sensitivity and specificity have been established, sample size may be determined using methods such as FreeCalc (www.au svet.com.au) or similar programs as outlined in Chapter 1.1.4 Requirements for surveillance for international recognition of freedom from infection. However, for those diagnostic tests where the sensitivity and specificity have not been established, the default sample size should be determined from Table 1 in the present chapter.

Table 1. Random sample size based on assumed pathogen prevalence in lot and assuming 100% sensitivity and specificity of the technique

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

• A fish culture unit should be inspected twice a year for 2 years at the appropriate life stage of the fish and at times of the year when temperature and season offer the best opportunity for observing clinical signs and isolating pathogens. On each occasion, fish of the susceptible species listed in the Aquatic Code for the disease in question should 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. If broodfish are present during one of the two inspections, up to 150 ovarian fluid samples will be taken from broodfish 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, 150 fish specimens should be collected once a year for 2 years. Insofar as it is possible, specimens of the oldest fish and/or salmonid ovarian fluid should be collected as a priority.

• During this 2-year period, the fish production unit may only receive fish from a unit whose health status has already been approved and is equal to or higher than the health status sought for the facility being inspected.
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 from all or certain diseases listed in the *Aquatic Code* after 2 years of surveillance with laboratory tests and in the absence of any suspect clinical signs, twice-yearly inspections should continue. An aquaculture establishment that has been recognised free from infection may maintain its official status as infection-free provided that the basic biosecurity conditions are continuously maintained.

An aquaculture establishment that has been recognised free from infection may discontinue targeted surveillance and maintain its official status as infection-free provided that the basic biosecurity conditions are continuously maintained.

- The fish production unit may only receive fish having a health status higher than or equal to that of those already present.

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 subclinical pathogen carriers.

2.1. Specifications according to fish size

- **Alevin and yolk sac fry**: sample the entire fish but remove the yolk sac if present.

- **Fish 4 to 6 cm**: take the entire viscera including the kidney. A piece of encephalon can be obtained after severing the head at the level of the rear edge of the operculum and pressing it laterally.

- **Fish over 6 cm**: take the kidney, spleen, and heart or encephalon.

- **Broodfish**: take the ovarian fluid and/or tissues.

2.2. Specifications according to clinical status

In the case of clinical infection, besides whole fry or entire viscera, organs to be sampled are anterior kidney, spleen and heart or encephalon for virus tests, kidney and spleen for bacterial tests, and skin or muscle when sampling for tests for epizootic ulcerative syndrome. Samples from ten diseased fish will thus be taken and combined to form pools of a maximum of five fish each.

For detecting subclinical carriers, samples may be combined as pools of no more than five fish/pool. Pools of ovarian fluid from five broodfish should 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 collected following the pooling of ova.

Once aseptically removed from fish, the organs and/or ovarian fluid sampled are each split into two parts if both bacteriological and virological examinations are to be done.

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 carried out within 24 hours after fish sampling, but is still acceptable for up to 48 hours.
Organ samples may also be transported to the laboratory by placing them in vials containing cell culture medium or Hanks’ balanced salt solution (HBSS) with added antibiotics to suppress the growth of bacterial contaminants (one volume of organ in at least five volumes of transportation fluid). Suitable antibiotic concentrations are: gentamycin (1000 µg/ml) or penicillin (800 International Units [IU]/ml) and streptomycin (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

- This procedure is conducted below 15°C and preferably at between 0 and 5°C.
- Decant antibiotic-supplemented medium from organ sample.
- Homogenise organ pools in transport medium at a final dilution of 1/10 using a mortar and pestle or electric homogeniser until a paste is obtained.
- Centrifuge the homogenate in a refrigerated centrifuge at 2–5°C at 2000 to 4000 g for 15 minutes, collect the supernatant and treat for either four hours at 15°C or overnight at 4°C with antibiotics, e.g. gentamicin 1 mg/ml. If shipment of the sample has been made in a transport medium (i.e. with exposure to antibiotics) the treatment of the supernatant with antibiotics may be omitted. The antibiotic treatment makes filtration through membrane filters unnecessary.
- Likewise, ovarian fluid samples may be treated with antibiotics to control microbial contamination. In neither case can homogenates or ovarian fluid samples be diluted more than twofold.
- Ovarian fluid samples should be centrifuged in the same way as organ homogenates, and their supernatants used directly in subsequent steps.

3.3. Treatment to neutralise enzootic viruses

In some countries, fish are often subclinical carriers of enzootic viruses, such as infectious pancreatic necrosis virus (IPNV), which induce a CPE in susceptible cell cultures and thus complicate isolation and identification of target pathogens. In such situations, the infectivity of the enzootic viruses should be neutralised before testing for the viruses listed in the Aquatic Code. However, when it is important to determine whether one of the enzootic viruses is present, samples should be tested with and without the presence of neutralising antibodies (NAbs).

To neutralise aquatic birnaviruses, mix equal volumes (200 µl) of a solution of NAbs against the indigenous birnavirus serotypes with the supernatant to be tested. Allow the mixture to react for 1 hour at 15°C or overnight at 4°C prior to inoculation on to susceptible cell monolayers. The titre of the NAb solution used (it may be a multivalent serum) should be at least 2000 in a 50% plaque reduction test versus the viral serotypes present in the given geographical area.

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

This approach can also be used to neutralise other viruses enzootic to the area being tested.

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 microorganisms is such a disadvantage that
freshly taken tissue is used 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.

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 Aquatic Code:

- Bluegill fry (BF-2)
- Chinook salmon embryo (CHSE-214)
- Epithelioma papulosum cyprini (EPC)
- Rainbow trout gonad (RTG-2)
- Fathead minnow (FHM)
- Grunt fin (GF)
- Salmon head kidney (SHK1)
- Atlantic salmon kidney (ASK)

1.2. Culture media

Traditional Eagle’s minimal essential medium (MEM) with Earle’s salt supplemented with 10% fetal bovine serum (FBS), antibiotics and 2 mM L-glutamine is the most widely used medium for fish cell culture.

Stoker’s medium, however, 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 + 10% tryptose phosphate.

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

Alternatively, Leibovitz medium (L15) supplemented with FBS (5% or 10%), L-glutamine (4 mM) and gentamicin (50 µg/ml) can be used for some cell lines e.g. SHK1.

For cell growth, the FBS content of the medium is usually 10%, whereas for virus isolation or virus production it may be reduced to 2%. Similarly, the pH of the culture medium for cell growth is 7.3–7.4 and is 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 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)
- Spring viraemia of carp virus (SVCV)
• Viral haemorrhagic septicaemia virus (VHSV) (synonym Egtved virus)
• Infectious salmon anaemia virus (ISAV)
• Red sea bream iridovirus (RSIV)
• Infectious pancreatic necrosis virus (IPNV)

b) Virus production

For the production of most of these viruses, the susceptible cell cultures (see relevant sections in this Aquatic Manual) should be inoculated with fairly low multiplicities of infection (m.o.i.), i.e. 10⁻² to 10⁻³ plaque-forming units (PFU) per cell.

The optimal temperatures for virus propagation are:

• 15°C for IHNV, VHSV, ISAV and IPNV
• 20°C for SVCV
• 22°C for EHNV
• 25°C for RSIV

c) Virus preservation and storage

• Centrifuge infected cell cultures at 2–5°C and 2000–4000 g for 15 minutes then dilute the virus-containing supernatants in order to obtain virus titres averaging 1–2 × 10⁶ 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 standard virus stocks at –80°C or liquid nitrogen, and check the titre of each virus stock at regular intervals if it has not been used during that time period.

Lyophilisation: long-term storage (decades) of the seeds of standard virus strains is achievable by lyophilisation. For this purpose, viral suspensions in cell culture medium supplemented with 10% FCS are mixed (v/v) with an equal volume of cryopreservative medium (such as 20% lactalbumine hydrolysate 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 the commonly used isolation peptones (trypsin-soya, brain–heart, etc.) can conveniently be employed. However, the low optimal temperatures of some species result in slow growth, and 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 cultivation. 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. Renibacterium salmoninarum, Flavobacterium psychrophilus and others need 15°C for optimal growth and many of the bacteria isolated from warm water fish may be incubated at 30°C to accelerate the diagnostic steps.
2.2. Storage of cultures

Bacterial strains can be stored for a short term on ordinary media, placing the slants or broths at 4°C. For most strains, the use of commercial media or agar slants with mineral oil overlay 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 phenotype characteristics from changing. When stability of characteristics such as virulence is important, 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 (5–15%) in the former case, and skim milk, lactose, and dextran (5–10%) in the latter. 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 formulae 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. Titre and specificity are influenced, however, by the inoculation programme used. 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 infectious pancreatic necrosis virus

Intravenous injection with 50–100 µg of purified virus on day 0, followed by an identical booster on day 21, and 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 intra-dermal injection with further intravenous boosters:

Day 0: primary injection, 500–1000 µg of purified virus is mixed (v/v) with adjuvant (Freund’s incomplete or other\(^1\)) 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); boost intravenously with the same amount of purified virus as in the primary injection, but without adjuvant.

Prior to the intravenous booster injection, the rabbit should be treated with promothazine (12 mg intramuscularly) to prevent possible anaphylactic response.

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

For rhabdoviruses, this immunisation procedure is well suited to production of antisera to be used in immunofluorescence and enzyme-linked immunosorbent assay. However, a more efficient method for production of neutralising antisera is regular intravenous injection without

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\(^1\) Use of Freund’s complete adjuvants may be restricted on animal welfare grounds. Alternative synthetic adjuvants include trehalose dimycolate and monophosphate lipid A.
adjuvant (0.2 ml) every 3–4 days (twice a week). As many as 15 injections may be necessary; 1 week after the last injection, a serum sample should be collected and tested.

3.2. Antisera to fish bacteria

It is still difficult to obtain antimicrobial sera in large amounts from commercial sources, and it will often be necessary to prepare such antisera. The general methods are the same as for viruses. Bacterial antigens are often used as crude preparations killed by heating or by formalin (0.35% formalin). Rabbit injection at increasing doses can be either intramuscular, each 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-\textit{Renibacterium} sera production, and is also valuable for other bacteria of low antigenicity in rabbits.

The antigen is heat killed (60°C, 45 minutes) 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:

- Day 1: 1 mg + complete Freund’s adjuvant (CFA) (v/v)
- Day 2: 1 mg + incomplete Freund’s adjuvant (IFA) (v/v)
- Day 4: 1 mg + IFA
- Day 6: Collecting of blood samples (about 30 ml)

Withdrawal injections and bleeding for serum collection may be repeated at 1-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 minutes at 56°C. Filter the resulting heat-inactivated serum through a membrane filter (450 nm pore size) and temporarily store it at 4°C for 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 2 months at 4°C without any change in their properties. Dispense (usually as small volumes) and freeze at –20°C or lyophilise.

Immunoglobulins (Ig) 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.

\textit{Presentation of Ig:} Mix a solution of Ig of concentration 2 mg/litre with sterile pure glycerol (v/v) and keep at –20°C. Solutions of Ig with a higher concentration may also be prepared in glycerol.

3.4. Mouse monoclonal antibodies to fish viruses and bacteria

Monoclonal antibodies (MAbs) to most of the fish viruses have been raised over 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 this \textit{Aquatic Manual}. 

The production of MAbs to bacteria has also been described. It has resulted in the development of commercial diagnostic kits for *Renibacterium salmoninarum*, but in most cases remains limited to specialised laboratories.

In theory, mouse monoclonal IgGs can be processed and stored as for polyclonal IgGs. However, the reactivity of certain MAbs may be impaired by processes such as enzymatic- or radio-labelling or lyophilisation. It is thus necessary to test various MAbs for the conditions under which they will be used.

### 3.5. Use of molecular techniques for confirmatory testing and diagnosis

Molecular techniques including nucleic acid probes and the polymerase chain reaction (PCR) have been developed for the identification of many pathogens of aquatic animals. However, as is the case with several other diagnostic techniques, an advantage in sensitivity is frequently offset by problems in interpretation or susceptibility to technical problems. Whereas methods based on direct culture or serology are relatively robust, PCR can be quite dependent on the conditions under which it is run and can be highly subject to laboratory contamination by previous PCR products, yielding false-positive results. Thus, while several nucleic acid probe and PCR protocols are included in this version of this *Aquatic Manual* as diagnostic or confirmatory methods, where possible, well-established techniques (e.g. virus isolation) are specified as standard screening methods. Whenever these newer molecular techniques are used, they should be performed with caution and with special attention to the inclusion of adequate positive and negative controls.

#### 3.5.1. Sample preparation

For these techniques, samples should be prepared to preserve the DNA of the pathogen. Likewise, samples intended for testing with antibody-based methods should be preserved to retain the reactive antigenic sites for the antibodies used.

Samples selected for DNA-based or antibody-based diagnostic tests should be handled and packaged with the greatest care to minimise the potential for cross contamination among the samples or target degradation before the assay can be performed. To prevent contamination, new containers (plastic sample bags or bottles) should be used. A water-resistant label, with the appropriate data filled out, should be placed within each package or container for each sample set.

Some suitable methods for preservation and transport of samples taken for molecular or antibody-based tests are:

- **Live iced specimens or chilled specimens**: for specimens that can be rapidly transported to the laboratory for testing within 24 hours, pack samples in sample bags surrounded by an adequate quantity of wet ice around the bagged samples in an insulated box and ship to the laboratory.

- **Frozen whole specimens**: select live specimens according to the purpose of sampling, quick freeze in the field using crushed dry-ice, or freeze in a field laboratory using a mechanical freezer at −20°C or lower temperature. Prepare and insert the label into the container with the samples, pack samples with an adequate quantity of dry-ice in an insulated box, and ship to the laboratory.

- **Alcohol-preserved samples**: in regions where the storage and shipment of frozen samples is problematic, 90–95% ethanol may be used to preserve, store, and transport certain types of samples. Pack for shipment according to the methods described above.

- **Fixed tissues for in-situ hybridisation and immunohistochemistry**: for this purpose, classic methods for preservation of the tissues are adequate. Davidson’s solution is usually a
good choice for later use of molecular probes. For DNA, specifically, over-fixation (over 24–48 hours) should be avoided.

- **3.5.2 Preservation of RNA and DNA in tissues using RNA\_later**

Tissue is cut to be less than 0.5 cm in one dimension and submerged in 10 volumes of RNA\_later (e.g. a 0.5 g sample requires about 5 ml of RNA\_later). Small organs such as kidney, liver and spleen can be stored whole in RNA\_later. These samples can be stored at 4°C for one month, at 25°C for 1 week or at –20°C indefinitely. Archive RNA\_later-treated tissues at –20°C.

- **3.5.3. DNA extraction**

For DNA extraction, ground the preserved tissues to powder. Around 10 volumes of extraction buffer (NaCl [100 mM], ethylene diamine tetra-acetic acid [EDTA, 25 mM], pH 8, sodium dodecyl sulfate [SDS, 0.5%]) are added with proteinase K (100 µg/ml). Following overnight incubation at 50°C, DNA is extracted using a standard phenol/chloroform protocol, and precipitated with ethanol.

Considering time constraints and risks for laboratory staff, commercially available kits may provide satisfactory technical alternative. Use of commercial kits should be validated by comparison with standard phenol/chloroform protocol prior to its routine use in diagnostic laboratories.

- **3.5.4. RNA extraction**

To isolate RNA from tissues preserved in RNA\_later, simply remove the tissue from RNA\_later and treat it as though it was just harvested. Most tissues can be homogenised directly in lysis or extraction buffer.

- **3.5.5. Preparation of slides for in-situ hybridisation**

For in-situ hybridisation (ISH), tissues are fixed in Davidson’s fixative for approximately 24 hours and then embedded in paraffin according to standard histology methods. Sections are cut at 5 µm thick and placed on aminoalkylsilane-coated slides, which are then baked overnight in an oven at 40°C. The sections are de-waxed by immersing in xylene for 10 minutes. This step is repeated once and then the solvent is eliminated by immersion in two successive absolute ethanol baths for 10 minutes each. The sections are then rehydrated by immersion in an ethanol series. The protocol may require a step of membrane permeabilisation enabling access to the target DNA. For this purpose, sections are treated with proteinase K (100 µg/ml) in TE buffer (Tris [50 mM], EDTA [10 mM]), at 37°C for 30 minutes. For ISH tests, it is essential that both a known positive and a known negative slide be stained to eliminate false positive results due to nonspecific staining/stain dropout, and false negative results due to errors in the staining protocol.

**KEY REFERENCES**


Chapter I.1. - Diseases of fish: General information


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CHAPTER 2.1.1.

EPIZOOTIC HAEMATOPOIETIC NECROSIS

1. CASE DEFINITION

For the purpose of this chapter, epizootic haematopoietic necrosis is considered to be systemic clinical or subclinical infection of finfish with epizootic haematopoietic necrosis virus (EHNV).

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

a) Agent factors

- Aetiological agent, agent strains
  
  EHNV is a member of the genus *Ranavirus* in the family Iridoviridae with the type species Frog virus 3 (FV3) (7). Other species include Bohle virus (BIV), European catfish virus (ECV), European sheatfish virus (ESV) and Santee-Cooper ranavirus. Of these caution should be adhered to in speaking of ECV and ESV as two separate viruses as the scientific literature (17) indicated they are isolates of the same virus. There are many other tentative species in this genus. Ranaviruses have been isolated from healthy or diseased frogs, salamanders and reptiles in America, Europe and Australia (8, 10, 12, 18, 29, 38, 39). Ranaviruses have large (150–180 nm), icosahedral virions, a double-stranded DNA genome 150–170 kb, and replicate in both the nucleus and cytoplasm with cytoplasmic assembly (7). They possess common antigens that can be detected by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence, but no effective neutralising antibodies have been produced to assist identification.

Since the recognition of disease due to EHNV in Australia in 1986, similar systemic necrotising iridovirus syndromes have been reported in farmed fish. These include catfish (*Ictalurus melas*) in France (ECV) (26), sheatfish (*Silurus glanis*) in Germany (ESV) (2, 3), turbot (*Scophthalmus maximus*) in Denmark (5) and others in Finland (4, 31).

EHNV and ECV are distinct viruses that can be differentiated using genomic analysis (1, 17, 23–25). This enables epidemiological separation of disease events in finfish in Australia (EHNV) and Europe (ECV) and differentiation of these from ranaviral occurrences in frogs (FV3 and BIV).

- Survival outside the host (i.e. in the natural environment): EHNV is extremely resistant to drying and in water can survive for months. It can persist in frozen fish tissues for more than 2 years (19) and frozen fish carcases for at least a year (33). For these reasons it is presumed that EHNV would persist for months to years on a fish farm in water and sediment and on plants and equipment.

- Stability of the agent: EHNV is susceptible to 70% ethanol, 200 mg/litre sodium hypochlorite or heating to 60°C for 15 minutes (19).

b) Host factors

- Susceptible host species: natural EHNV infections are known from only two teleost species, redfin perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*) (20–22), however, a number of other finfish species are susceptible to EHNV experimentally. Individuals of the following species died after bath inoculation: Macquarie perch (*Macquaria australasica*), silver
perch (*Bidyanus bidyanus*), mosquito fish (*Gambusia affinis*) and mountain galaxias (*Galaxias olidus*). Based on these results EHNV can be classified as an indiscriminate pathogen.

- **Susceptible stages of the host:** all age classes of rainbow trout and redfin perch.
- **Species or sub-population predilection (probability of detection):** clinical signs are usually more obvious in fingerlings and juvenile fish of both rainbow trout and redfin perch.
- **Target organs and infected tissue:** liver, kidney, spleen and other parenchymal tissues. It is not known if EHNV can be detected in gonadal tissues, ovarian fluid or milt or whether these tissues are suitable for surveillance of broodstock.
- **Persistent infection with lifelong carriers**
  - **Rainbow trout:** the high case fatality rate and low prevalence of EHNV infection in rainbow trout means that the recruitment rate of carriers is likely to be very low (< 2%) (34). Persistent infection with very small numbers of infectious virions was detected in one clinically healthy rainbow trout fingerling 63 days after intraperitoneal inoculation (35), but the significance of this observation is unclear because of the artificial route of infection. EHNV has been detected in grower fish, but as histopathological lesions consistent with EHNV were also present there was active infection rather than a carrier state (36). Too few broodstock samples have been examined to be certain that broodstock are not infected (34). Anti-EHNV antibodies were detected in serum from a low proportion of 1+ to 2+ grower fish, but not in 0+ fingerlings during or after an outbreak; it is however, uncertain whether these were survivors of the outbreak (34, 36) or infected with another, less virulent ranavirus.
  - **Redfin perch:** this species is extremely susceptible to EHNV and it seems unlikely that it is a suitable reservoir host (35). However there is some conflicting evidence. EHNV or a related ranavirus was isolated from 2 of 40 apparently healthy adult redfin perch during epizootics in juveniles in Victoria (20), but as the incubation period extends for up to 28 days (35), these fish may have been in the preclinical phase. Several ranavirus isolates have been obtained from redfin perch in Victoria at times when there was no obvious epizootic and some apparently healthy redfin in Victoria had serum antibodies against EHNV or a related virus (unpublished data).
  - **Murray cod:** this species may be a suitable carrier as infection without disease occurred after bath inoculation (19).
- **Vectors:** being a resistant virus EHNV may be transferred on nets, boats and other equipment, or in fish used for bait by recreational fishers. Birds are potential mechanical vectors for EHNV, carrying it in the gut, on feathers, feet and the bill. Silver gulls (*Larus novaehollandiae*) and great cormorants (*Phalacrocorax carbo*) feed on affected juvenile redfin perch and the gastrointestinal contents of these birds were positive for EHNV by ELISA and polymerase chain reaction (PCR) (33). The virus is likely to become inactivated at typical avian body temperatures (40–44°C), but spread of EHNV by regurgitation of ingested material within a few hours of feeding is possible (33).

c) **Disease pattern**

- **Occurrence and transmission mechanisms**
  - **Rainbow trout:** the disease is generally difficult to identify with very low mortality rates. Natural outbreaks appear to be related to poor husbandry, particularly overcrowding, inadequate water flow and fouling of tanks with feed. Water quality parameters are suboptimal and intercurrent diseases, including skin diseases caused by protozoa and fungi and systemic bacterial infection are common. Damage to skin may provide a route of entry for EHNV. Outbreaks have been seen on farms at water temperatures ranging from 11 to 20°C (34, 36). The incubation period after intraperitoneal inoculation was 3–10 days at 19–21°C compared with 14–32 days at 8–10°C (35).
EHNV has spread between rainbow trout farms by transfer of infected fingerlings and probably transport water (21, 34, 36). It is assumed that consignments of fish contain a low proportion of individuals with progressive subclinical or clinical infection, rather than carrier fish. The low prevalence of infection in rainbow trout means that active infection can easily go unrecognised in a population and be spread by trading fish. There are no data on possible vertical transmission of EHNV on or within ova, and disinfection protocols for ova have not been evaluated. EHNV has not yet been isolated from ovarian tissues or from broodstock, therefore milt and ovarian fluids may not be suitable samples for testing for exclusion of EHNV.

Redfin perch: the disease is recognised by spectacular epizootic mortality in fish of any age, affecting a very large proportion of the population. Typically fingerling and juvenile fish are affected, but in newly infected areas adults have also been affected. When the disease is first recognised in an area there is a dramatic population decline (20, 22, 33). Natural epizootics of EHNV affecting juvenile and adult redfin perch occur mostly in summer (20, 22, 34). It has been assumed that the disease in juvenile fish is related to the annual appearance of large numbers of non-immune young fish and their subsequent exposure to the virus while schooling in shallow waters; adults are uncommonly involved in these outbreaks. It is possible that environmental temperature is the trigger for outbreaks as juvenile fish feed in warm shallow waters on planktonic fauna whereas adults feed on benthic invertebrates and larger prey in deeper cooler water (35). Experimentally the incubation period ranged from 10 to 28 days at 12–18°C compared with 10–11 days at 19–21°C, and adult perch were refractory to infection at temperatures below 12°C (35).

The occurrence of EHNV in redfin perch in widely separated river systems and impoundments and upstream progression indicates that EHNV is spread by means other than water. Redfin perch migrations in Australia are uncertain (see also vectors). Annual recurrence in farmed rainbow trout has been due to reinfection of successive batches of fish from wild redfin perch present in the same catchment.

• Prevalence

**Rainbow trout:** EHNV has most often been reported in young fingerlings <125 mm forklength with daily mortality of less than 0.2% and total mortality up to 4%. However, rainbow trout of all ages may be susceptible although infection has not yet been seen in broodstock (34, 36). During outbreaks, EHNV has been detected by virus isolation in 60–80% of moribund or dead fish, but in only 0–4% of in-contact, clinically healthy fish. The 99% confidence limits for the prevalence of subclinical infection are 0–8% based on samples of 150 fish. The virus could not be found at all in surviving cohorts after an outbreak. It appears therefore that EHNV is poorly infective but has a high case fatality rate. Anti-EHNV antibodies were detected in grower fish at low prevalence (0.7%, 95% confidence limits 0.02% to 3.7%). EHNV may be present on a farm without causing suspicion because the mortality rate may not rise above the usual background rate.

**Redfin perch:** the disease is recognised by epizootic mortality in fish of any age affecting a very large proportion of the population with dramatic population decline (20, 22, 33). Typically fingerling and juvenile fish are affected in endemic areas, but in newly infected areas adults are also affected.

• Geographical distribution

**Rainbow trout:** infection with EHNV is known only from fish farms located in the Murrumbidgee and Shoalhaven river catchments in New South Wales, Australia. Some farms within this region have remained free of the disease (36).

**Redfin perch:** EHNV is endemic in southeastern Australia, but there is a discontinuous distribution. The disease occurs in many small and large impoundments in Victoria and since 1986 has spread progressively upstream in the Murrumbidgee river catchment through New South Wales and the
Australian Capital Territory. Similar spread has been observed in the Murray river in South Australia (33).

- Mortality and morbidity

There are dramatic differences in the susceptibility of redfin perch and rainbow trout to EHNV that are reflected in differences in prevalence of mortality and morbidity. Redfin perch are highly susceptible to EHNV. Experimental bath inoculation with as few as 0.08 TCID\textsubscript{50}(50\% tissue culture infective dose)/ml was lethal, and doses too low to be detected by virus isolation in BF-2 cells were fatal by intraperitoneal inoculation (35). In contrast, and in keeping with the natural pattern of disease, rainbow trout were resistant to bath exposure in $10^{2.2}$ TCID\textsubscript{50}/ml (35) while only 1 of 7 became infected after bath inoculation for 1 hour in $10^{3}$ TCID\textsubscript{50}/ml (21). These findings with field observations suggest low infectivity with a high case fatality rate in rainbow trout and high infectivity and high case fatality rate in redfin perch.

- Economic and/or production impact of the disease

Rainbow trout: there is a low direct economic impact because of the low mortality rate.

Redfin perch: there is spectacular epizootic mortality affecting a very large proportion of the population and leading to loss of the fishery for years (20, 22, 33).

d) Control and prevention

- Vaccination: none available.
- Chemotherapy: none available.
- Immunostimulation: not tested.
- Resistance breeding: not tested.
- Restocking with resistant species: not tested.
- Blocking agents: not tested.
- General husbandry practices: disease control in rainbow trout at the farm level relies on reducing the impact of infection by maintaining low stocking rates and adequate water quality. The mechanism of protection may be through maintenance of healthy integument.

3. DIAGNOSTIC METHODS

a) Field diagnostic methods

- Clinical signs: there are no specific clinical signs. Fish are found dead. Moribund fish may have loss of equilibrium, flared operculae and may be dark in colour. There may be clinical evidence of poor husbandry practices, such as overcrowding and suboptimal water quality manifesting as skin, fin and gill lesions (27).

b) Clinical methods

- Gross pathology: there may be no gross lesions or nonspecific lesions on the skin, fins and gill. A small proportion of fish may have enlargement of kidney, liver or spleen. There may be focal white to yellow lesions in liver corresponding to areas of necrosis (27).
- Microscopic pathology: acute focal, multifocal or locally extensive coagulative or liquefactive necrosis of liver, haematopoietic kidney and spleen are commonly seen in routine haematoxylin and eosin stained sections of formalin-fixed material. A small number of basophilic intracytoplasmic inclusion bodies may be seen, particularly in areas immediately
surrounding necrotic areas in liver and kidney. Necrotic lesions may also be seen in heart, pancreas, gastrointestinal tract, gill and pseudobranch (27).

• Electron microscopy/cytopathology: affected tissues (e.g. kidney, liver and spleen) contain cells exhibiting necrosis. Cells contain conspicuous cytoplasmic inclusions that are rarefied areas of the cytoplasm in which the viruses are assembled. Within the cytoplasm, aggregates (paracrystalline arrays) of large (175 nm ± 6 nm) non-enveloped icosahedral viruses are apparent; single viruses are also present. Complete viruses (containing electron-dense cores) bud/egress from the infected cells through the plasma membrane. The nuclei of infected cells are frequently located peripherally and are distorted in shape.

c) Agent detection and identification methods

• Direct detection methods

  i) Microscopic methods

  • *Light microscopy*

    Fixed sections: routine methods can be used for tissue fixation in 10% buffered neutral formalin, paraffin embedding, preparation of 10 µm sections and staining with haematoxylin and eosin to demonstrate tissue necrosis and basophilic intracytoplasmic inclusion bodies. These inclusion bodies are indicative but not confirmatory for EHN. Formalin-fixed paraffin-embedded sections can also be stained using an immunoperoxidase method (see below) to identify EHNV antigen associated with necrotic lesions.

  • *Electron microscopy*

    Ultrathin sections: routine methods can be used for preparation of tissues and cell cultures (11) to demonstrate tissue necrosis, presence of viruses and virus inclusion bodies. Tissues and cells fixed with an alternative fixation and embedding regime can be used for antigen detection (15).

    Negative contrast electron microscopy: supernatants from dounce homogenised tissues (10% [w/v]) and cell cultures can be used to detect viruses. Ranaviruses have a definitive appearance. They vary in diameter (150–180 nm), have a limiting cell-derived (plasma membrane) envelope that surrounds a capsid of skewed symmetry. Underlying the capsid is a *de novo* membrane that itself surrounds a core containing the dsDNA and minor proteins. These preparations can also be used to confirm ranavirus antigenicity (11).

  ii) Agent isolation and identification

  • *Preparation of fish tissues for virus isolation and ELISA*

    A simple method for preparation of fish tissues for cell culture and ELISA has been validated (37).

    i) Bathe large fish for 30 seconds in 70% ethanol; bathe fingerlings for 5 seconds in 70% ethanol then rinse in sterile water. Dissect fish aseptically in a Class II biosafety cabinet.

    Large fish: remove 0.1 g liver, kidney, spleen (± other organs in specific situations) and place in sterile tubes (ET). ETs suitable for use with pestles for grinding tissues (see below) are available from Edwards Instrument Co., Blue eppendorf tubes #3032-877. In some situations liver, kidney and spleen may be pooled in a single ET.

    Medium fish (30–60 mm forklength): scrape all viscera into tube.

    Small fish (<30 mm forklength): remove head and tail, place rest of fish into tube.
ii) Freeze tubes containing tissues at –80°C until needed.

iii) Add 0.5 ml of homogenising medium (Minimal Essential Medium Eagle, with Earle’s Salts with glutamine [Flow Laboratories] [MEM] with 200 IU/ml penicillin, 200 µg/ml streptomycin and 4 µg/ml amphotericin B) to each tube. Grind tissue with a sterile fitted pestle to a fine mulch (Kontes pellet pestle grinders, Edwards Instrument Co., Cat # K749520-000; pestles can be washed, autoclaved and re-used).

iv) Add another 0.5 ml of homogenising medium to each tube and mix with pestle.

v) Add three sterile glass beads to each tube (3 mm diameter, Selby #219958) and close the lid of the ET.

vi) Vortex the suspension vigorously for 20–30 seconds and place at 4°C for 2 hours.

vii) Vortex the suspension again as above and centrifuge for 10 minutes at 2500 g in a benchtop microcentrifuge.

viii) Transfer the supernatant, now called clarified tissue homogenate, to a fresh sterile ET. Homogenates may be frozen at –80°C until required for virus isolation and ELISA.

- **Cell culture/artificial media**

  Cell culture is the gold-standard test but is costly and time consuming. EHNV grows well in many fish cell lines including BF-2 (bluegill fry cell line; ATCC CCL 91), FHM (fathead minnow; ATCC CCL 42), EPC (epithelioma papulosum cyprini [13]), and CHSE-214 (Chinook salmon embryo cell line; ATCC CRL 1681) at temperatures ranging from 15 to 22°C (9), but BF-2 are preferred by the reference laboratory where an incubation temperature of 22°C both before and after inoculation with virus is used. The procedure for BF-2 cells is provided below. A procedure for CHSE-214 cells is provided under immunoperoxidase staining below.

  The identity of viruses in cell culture is determined by immunostaining, ELISA, immunoelectron microscopy, PCR or other methods.

Samples: tissue homogenates.

Cell culture technical procedure: cells are cultured in tubes with 2 ml growth medium (MEM + 10% fetal calf serum (FCS) with 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml amphotericin B). The cells are incubated until almost confluent at 22°C, which can take up to 4 days depending on the seeding rate. Medium is changed to a maintenance medium (MEM with 2% FCS and 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml amphotericin B) on the day of inoculation. A 1/10 dilution using homogenising medium is made of single or pooled homogenates. Each culture tube is inoculated with 200 µl of sample. One tube is inoculated with undiluted homogenate, and two with 1/10 homogenate. No adsorption step is used. As the culture tubes contain 2 ml of medium, the 1/10 inocula represent a 1/100 dilution of a 0.1 g/ml tissue homogenate. As an alternative, two to three culture tubes can be inoculated directly with 20 µl of undiluted homogenate. Note that a high rate of cell toxicity or contamination often accompanies the use of large (200 µl) undiluted inocula, and the simple method may therefore be preferable. The tubes are incubated at 22°C in an incubator for 6 days. Tubes are read at day 3 and day 6. Tubes are passed at least once to detect samples with low levels of virus. On day 6, the primary tubes (P1) are frozen overnight at −20°C, thawed, gently mixed and then 100 µl of the culture supernatant is inoculated into a fresh tube (P2). Remaining P1 supernatants are transferred to sterile 5 ml tubes and placed at 4°C for testing by ELISA (to confirm the cause of cytopathic effect [CPE] as EHNV). P2 is incubated as above, and a third pass is conducted if necessary.
Interpretation of results:
- CPE is well developed and consists of focal lysis surrounded by rounded granular cells. This change extends rapidly to involve the entire monolayer, which detaches and disintegrates.

- Antibody-based antigen detection methods

It should be noted that antibodies used in all related methods (immunoperoxidase, antigen-capture ELISA and immunoelectron microscopy) cross-react with all known ranaviruses (17).

Detection of EHNV using immunoperoxidase of infected cell cultures

Principle of the test: EHNV replicate within cultured cells. The addition of a mild detergent permeabilises the cells allowing an affinity purified rabbit antibody to bind to intracellular viral proteins. EHNV is detected by a biotinylated anti-species antibody and a streptavidin-peroxidase conjugate. The addition of a substrate results in 'brick-red' staining in areas labelled with antibodies.

Samples: tissue homogenates.

Operating characteristics: when performed as described in this protocol, the staining is conspicuous and specific. The test however has not been validated in respect to sensitivity or reproducibility.

Preparation of cells: the procedure described below is for CHSE-214 cells. Other recommended cell lines can also be used.

i) CHSE-214, 24-well plates are seeded the day before use with 250,000 cells/well (or 4 million cells in 40 ml of growth medium per plate) in 1.5 ml of growth medium (Earle’s MEM with non-essential amino acids [EMEM], 10% FCS, 10 mM HEPES, 2 mM glutamine, 100 IU penicillin and 100 µg streptomycin) and incubated in 5% CO₂ at 22°C overnight. (Note: cultures must be nearly confluent and have healthy dividing cells prior to use.)

ii) Discard the medium, inoculate each well with 150 µl of a 10% suspension of ground tissue (e.g. liver, kidney or spleen), incubate for 1 hour (22°C) then add 1.5 ml of fresh maintenance medium (as for growth medium except 2% FCS) and return to the incubator (22°C).

iii) Observe cultures for CPE. If no CPE by day 10, pass the cultures on to fresh CHSE cells by collecting the cells and medium and adding 150 µl to the cells of the fresh plate; note cells are not freeze–thawed. There is no need to discard the existing medium, just return the new plate to the incubator (22°C). Again observe daily for CPE.

iv) Fix cells (50 µl of a 20% solution of formalin) when CPE is first observed. After incubation (22°C) for 1 hour at room temperature, the medium/formalin mixture is discarded and the wells are rinsed twice with PBS-A (phosphate buffered saline) to remove the formalin. More PBS-A is added if the plates are to be stored, at 4°C.

Protocol

i) Dilute primary anti-EHNV antibody and normal serum to working strength as listed in the data sheet for the relevant agent in 1% skim milk PBSA (SM) solution to the volume required for the test.

ii) Remove PBSA from wells (with fixed cell cultures) and wash wells twice with 0.05% (v/v) PBS/Tween 20 (PBST). Add 50 µl of primary antibody solutions to each well in a 96-well plate well or 200 µl in a 24-well plate well. Incubate on a
plate shaker at 100–200 rpm at room temperature (22–24°C) for 15–30 minutes or without shaking at 37°C for 1 hour.

iii) Dilute biotinylated anti-species serum (secondary antibody) in 0.1% SM solution as listed in the data sheet for the relevant agent to the volume required for the test.

iv) Remove primary antibody solution and wash wells three times with PBST. Add secondary antibody to all wells. Incubate on a plate shaker at 100–200 rpm at room temperature for 15–30 minutes or without shaking at 37°C for 1 hour.

v) Dilute streptavidin-peroxidase conjugate in 0.1% SM solution as listed in the data sheet for the relevant agent to the volume required for the test.

vi) Remove secondary antibodies from wells and wash wells three times with PBST. Add conjugate to each well. Incubate on a plate shaker at 100–200 rpm at room temperature for 15–30 minutes or without shaking at 37°C for 1 hour.

vii) Prepare stock substrate of 3-amino-9-ethylcarbazole (AEC) solution: dissolve one AEC tablet (20 mg) in 2.5 ml of dimethyl formamide. The solution is then diluted 1/10 in 45 ml of 0.05 M acetate buffer (4.1 ml anhydrous sodium acetate in 1 litre of de-ionised water; the pH is adjusted to 5.0 with glacial acetic acid).

viii) Remove conjugate from wells. Wash (three times) with PBST.

ix) Dilute dissolved AEC stock in 47.5 ml of acetate buffer. Just before use, add 25 µl 30% hydrogen peroxide to AEC solution then add to each well. Incubate at room temperature for 20 minutes.

x) Remove substrate solution and wash wells twice with deionised water to stop reaction.

xi) Counterstain with Mayer’s haematoxylin (50 µl/well or 200 µl/well) for 1 minute and rinse with deionised water.

xii) Add 50 µl Scott’s tap water and rinse with deionised water and air dry.

Interpretation of the results
• Positive reaction: granular-like, focal, brick-red staining of cells indicates presence of virus identified by the diagnostic antibody.
• Negative reaction: no red staining apparent – all cells should be stained pale blue due to counterstain.
• Background staining: non-granular, non-focal, more generalised, pale, pinkish staining may occur throughout the culture. This background staining could be due to any number of reasons, e.g. nonspecific antibody reaction with non-viral components, inefficient washing, expiration of other reagents.

Detection of EHNV using antigen-capture ELISA

Antigen-capture ELISA has been validated to detect EHNV in cell cultures and directly in homogenates of fish tissues. The analytical sensitivity is $10^3$ to $10^4$ TCID$_{50}$/ml. Specificity approaches 100% and sensitivity for direct detection in fish tissues is 60% relative to the gold standard of virus isolation in BF-2 cells (16, 37) (unpublished data). (37). ELISA is useful for both diagnosis and certification. Neutralisation tests cannot be used to identify EHNV because neutralising antibodies are not produced following immunisation of mammals or fish. Mouse monoclonal antibodies produced against EHNV are directed against major capsid protein (MCP) epitopes and are non-neutralising (unpublished data). Rabbit-anti-EHNV antibodies have been developed for use in antigen-capture ELISA, immunoperoxidase staining and immunoelectron microscopy (14, 16, 27). Reagents and protocols are available from the reference laboratory.
Samples: tissue homogenates samples prepared using a validated protocol (see below); cell cultures.

Principle of the test: EHNV particles are captured from the sample by an affinity purified rabbit antibody that is coated to the plate. EHNV is detected by a second antibody and a peroxidase-labelled conjugate using the chromogen ABTS (2,2’-azino-di-(3-ethyl-benzthiazoline)-6-sulphonic acid). The enzyme is inactivated after 20 minutes and the resulting optical density (OD) is compared with standards.

Operating characteristics: the protocol is based on published procedures (16, 30, 32, 37). When performed as described in this protocol, the operating characteristics of the test are as given in Table 1. The precision of the assay is <10% CV measured as variation in the OD of the controls between plates and over time when the recommended normalisation procedure is followed.

**Table 1.** EHNV ELISA operating characteristics compared with the gold standard of cell culture in BF-2 cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Positive-negative cut-off**</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissues of fish*</td>
<td>OD 0.5</td>
<td>60</td>
<td>&gt;99</td>
</tr>
<tr>
<td>Tissue culture supernatants with</td>
<td>OD 0.3</td>
<td>&gt;99</td>
<td>&gt;99</td>
</tr>
<tr>
<td>cytopathic effect (BF2 cells)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* redfin perch and rainbow trout only. Higher background OD occurs with golden perch. There are no data for other species.

** these cut-offs are determined by the OIE Reference Laboratory for EHNV and will vary with the batch of control antigen. Values above are for batch 86/8774-4-5-01.

Test components and preparation of reagents
i) Flat bottom microtitre plates (Linbro Cat # 76:381:04, ICN Flow; or Dynatech Immunolon 1 Cat # 011-010-3350; or equivalent) are required.

ii) Affinity purified rabbit anti-EHNV immunoglobulin and sheep anti-EHNV antiserum reagents are supplied in freeze-dried form. Reconstitute using 1 ml of purified water and allow the vial to stand at room temperature for 2 minutes. Mix the vial very gently. These reagents are stable when stored at −20°C for at least 4 years. For routine use in ELISA it is recommended that working stocks of both antibodies be prepared as a 1/10 dilution in TSGM (formula at end of this section). These are stable at −20°C for at least 5 years and do not solidify at this temperature.

iii) The conjugate (commercial reagent, KPL, #14-23-06; 0.5 mg) is supplied as a freeze-dried powder. This reagent has displayed remarkable consistency in activity between different lots over a period of 15 years. The product should be reconstituted in sterile 50% glycerol water, dispensed in 150 µl aliquots and stored at −20°C as undiluted stock. A working stock is prepared by adding 900 µl of TSGM to 100 µl of undiluted stock. The working stock is also stored at −20°C and is stable for at least 1 year. New batches of this conjugate should be titrated against an older batch using standard protocols.

iv) EHNV control antigen, heat-inactivated, is supplied as freeze-dried powder. Reconstitute in 1 ml sterile water and store in small aliquots at −20°C. Prepare dilutions using PBSTG (PBS + Tween + gelatine) on the same day the test is performed. Control EHNV antigen dilutions (A, B, D and F) cover the range of the signal response of the assay and enabling a normalisation procedure to be undertaken.
Chapter 2.1.1. - Epizootic haematopoietic necrosis

Equipment
An automatic plate washer is recommended although plates can be washed by hand. The assay is sensitive to plate washing conditions. If OD of the controls is unexpectedly low, and the conjugate and other reagents are within date, the plate washer should be adjusted so that washing pressure during filling of wells and aspiration of wells is minimised.

An automatic plate reader is recommended although plates can be read by eye.

Precision calibrated pipettes (e.g. Gilson) should be used to prepare dilutions of all reagents and to load reagents into microtitre plate wells.

Protocol
i) Coat a 96-well ELISA plate (100 µl/well) with affinity purified rabbit-anti-EHNV diluted 1/12,800 in borate coating buffer. Incubate overnight at 4°C.

ii) Wash plate five times with wash buffer (milli-Q (MQ) purified water plus 0.05% Tween 20). Note distilled and deionised water can also be used in this and all other steps.

iii) Prepare a blocking solution: warm the solutions in a microwave oven or water bath to dissolve the gelatin, then cool to room temperature.

iv) Block remaining binding sites using blocking solution (100 µl/well) (1% [w/v] gelatin in PBSTG diluent [PBS, 0.05% [v/v] Tween 20, 0.1% [w/v] gelatin]). Incubate at room temperature (RT) for 30 minutes.

v) Wash plate five times as above.

vi) Work in a Class II biological safety cabinet. Dilute the control antigen (see below) in PBSTG and add to the lower right-hand corner of the plate. Add tissue homogenate samples or culture supernatant samples and control antigens at 100 µl/well. All samples and controls are added to duplicate wells. Incubate for 90 minutes at RT.

The control antigens are dilutions of a heat killed cell culture supernatant of EHNV 86/8774. The controls are expected to give the following OD, although there will be some variation from lab to lab and ±10% variation should therefore be allowed:

<table>
<thead>
<tr>
<th>Control</th>
<th>Dilution in PBS*</th>
<th>OD (405 nm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1/5</td>
<td>&gt;2.0</td>
</tr>
<tr>
<td>B</td>
<td>1/40</td>
<td>1.90</td>
</tr>
<tr>
<td>D</td>
<td>1/200</td>
<td>0.68</td>
</tr>
<tr>
<td>F</td>
<td>1/3000</td>
<td>0.16</td>
</tr>
</tbody>
</table>

* These dilutions and OD values are determined by the OIE Reference Laboratory for EHNV and will vary with the batch of control antigen. The values above are for batch 86/8774-4-5-01. The positive-negative cut-off for clarified tissue homogenate samples from redfin perch and rainbow trout in this ELISA is approximated by the OD value of control D on each plate.

vii) Wash plate by hand to avoid contamination of the plate washer. Work in a Class II cabinet. Aspirate wells using a multichannel pipette. Rinse plate twice.

viii) Wash plate five times on the plate washer as above.

ix) Add second antibody sheep-anti-EHNV diluted 1/32,000 in PBSTG (100 µl/well). Incubate for 90 minutes at RT.

x) Wash plate five times on plate washer.

xi) Add conjugate diluted 1/1500 in PBSTG (100 µl/well). Incubate for 90 minutes at RT.
xii) Wash plate five times on plate washer.

xiii) Add ABTS substrate (22 ml ABTS + 10 μl H₂O₂) (100 μl/well) and place the plate on a plate shaker. Time this step from the moment substrate is added to the first wells of plate 1. Incubate for 20 minutes.

xiv) Immediately add ABTS stop solution (50 μl/well), shake plate briefly and read OD at 405 nm. Calculate mean ELISA OD of duplicate wells. Calculate the coefficient of variation of the duplicates: samples with CV >15% should be retested if the mean OD lies near the positive-negative cut-off.

Normalisation of data and decision limit quality control

If it is desired to normalise data from plate to plate and over time, or to undertake decision limit quality control, the following procedure can be followed. Run control antigens in ELISA on at least five occasions over a period of 3 weeks (total 20 separate ELISA plates). Calculate the mean OD for each control antigen. Then for each plate subsequently used, calculate a plate correction factor (PCF) as follows:

$$\text{PCF} = \frac{\text{mean OD control A}}{\text{actual OD}} + \frac{\text{mean OD control B}}{\text{actual OD}} + \frac{\text{mean OD control D}}{\text{actual OD}} + \frac{\text{mean OD control F}}{\text{actual OD}} \times \frac{1}{4}$$

Multiply the actual mean OD of each sample by the PCF for that plate and report these values.

PCF is allowed to vary between 0.8 and 1.2 which approximates a coefficient of variation of 10%. Values outside this range suggest that a plate needs to be retested. Plots of PCF over time provide a ready means for monitoring the stability of reagents, procedural variations and operator errors. This QC method has been validated for antigen capture ELISA.

Buffers and other reagents

**Borate coating buffer**

Boric acid 6.18 g
Disodium tetraborate (Na₂B₄O₇·10H₂O) 9.54 g
NaCl 4.38 g
MQ water to 1 litre
**Autoclave**

**10 × phosphate buffered saline**

NaCl 80.00 g
KCl 2.00 g
Na₂HPO₄ 11.50 g
KH₂PO₄ 2.00 g
MQ water to 900 ml
Adjust pH to 7.2 with HCl or NaOH; make up to 1 litre
**Autoclave**

For working strength dilute 1/10 and recheck pH.
For storage of powder in jars, make up twice the above quantity of powder; store; to make up add 1.8 litres MQW, pH, make up to 2 litres.

**ABTS¹**

Citrate phosphate buffer
Citric acid 21.00 g
Na₂HPO₄ 14.00 g
MQ water to 800 ml; adjust pH to 4.2; make up to 1 litre

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¹ ABTS Cat no. A1888, Sigma Aldrich Pty Ltd, Sydney, Australia; Tel: (+61-2) 9841 6555; Fax: (+61-2) 9841 0500; E-mail: www.sigmaaldrich.com
ABTS 0.55 g
Citrate phosphate buffer to 1 litre
Dispense in 22-ml aliquots and freeze.
Immediately prior to use add 10 µl H₂O₂ per 22-ml aliquot.

ABTS stop solution (0.01% NaN₃ in 0.1 M citric acid)
Citric acid 10.5 g
MQW to 500 ml
Add 50 mg sodium azide or 1 ml of 5% solution.

KPL Conjugate #14-23-06²

TSGM cryoprotectant
10 × Tris/saline, pH 7.4 50 ml
Glycerol 250 ml
Sterile purified water to 500 ml
Autoclave
Add 10% Merthiolate 1 ml
Store in dark bottle at 4°C.

10 × Tris/saline (250 mM Tris, 1.5 M NaCl)
Tris 15.14 g
NaCl 43.83 g
Sterile purified water 500 ml
pH adjust to 7.4

Immunoelectron microscopy

• Gold-labelling of sections containing tissues or cell cultures

Principle of the test: tissues and/or tissue homogenates can be used for examination by electron microscopy. Conventional electron microscopy (examination of ultra-thin sections) will generate data on virus structure and morphogenesis. Negative contrast electron microscopy will produce images that can be used to examine the particulate structure of the virus. The use of antibodies and conjugated gold with these preparations permits both ultrastructure and antigenicity to be examined (15). These collective data enables classification to the genus Ranavirus.

Cells and tissues

i) Fix tissues or cells as described in ref. 15. Briefly, 2.5% (v/v) buffered glutaraldehyde (cacodylate, or phosphate) is used to fix cells for 40 minutes. Following primary fixation the cells are rinsed in the same buffer (3 × 20 minutes), post-fixed in 1% (w/v) buffered osmium tetroxide (1 hour), washed (3 × 5 minutes) in double distilled/reverse osmosis (RO) water, dehydrated through graded alcohol (70–100%) and infiltrated and embedded in an epoxy resin (e.g. Spurrs or epon). For gold labelling of ultra-thin resin sections, attention must be given to fixation and embedding regimes. For example cells should be fixed in 0.25% (v/v) glutaraldehyde with 2–4% paraformaldehyde. No secondary fixation is used and the cells are infiltrated and embedded in an acrylic resin such as LR White.

ii) Following fixation and embedding, cut and transfer ultrathin sections onto filmed nickel grids.

iii) Cut sections from the appropriate LRW blocks.

² Reagent Supplier: Bio-Mediq DPC Australia, P.O. Box 106, Doncaster, Victoria 3108, Australia; Tel.: (+61-3) 9840 2767; Fax: (+61-3) 9840 2767. Visit: www.kpl.com for links to worldwide network distributors
iv) Adhere LRW sections to nickel grids that have been treated with 20% acetic acid, then 90% ethanol and wash in RO water.

v) Block in 2% (w/v) skim milk powder in PBSA (10 minutes).

vi) Block free aldehydes with 0.1 M glycine in PBSA (20 minutes).

vii) Wash in PBSA (3 × 1 minutes). This is an optional step used only if there is an excess of free-aldehydes (a high background may be indicative of this).

viii) If protein A-gold is not being used then block in normal species serum – this serum should be homologous to that complexed to gold. Recommended dilution is approximately 1/40 (10 minutes).

ix) Incubate in primary antibody. If incubation details are unknown then perform initial reactions with 1/100 to 1/2700 dilutions (with three-fold dilutions). Dilute antibodies in 1% (v/v) cold water fish gelatin in PBSA, (60 minutes, RT).

x) Rinse in 1% (v/v) coldwater fish gelatin in PBSA, (6 × 3 minutes).

xi) Incubate in gold-labelled secondary antibody or protein A-gold or protein G-gold. Suggested dilution 1/40 in a PBSA containing 1% (w/v) BSA, 0.1% (v/v) Tween 20 and 0.1% (v/v) Triton X, 60 minutes, RT.

xii) Rinse in PBSA (6 × 3 minutes, RT).

xiii) Post-fix in 2.5% (v/v) glutaraldehyde in PBSA (5 minutes, RT).

xiv) Rinse in water (RO) (3 × 3 minutes, RT).

xv) Dry on filter paper (type not critical).

xvi) Stain in uranyl acetate and lead acetate.

Interpretation of results
• Viruses within the cytoplasm of infected cells will be specifically gold-labelled. Viruses will be located singularly, within assembly bodies (inclusion bodies) and within paracrystalline arrays.

• Gold-labelling of virus particles (viruses adsorbed to grids)
  i) Dounce homogenise 10% (w/v) liver, kidney or spleen and clarify (5 minutes, 2500 g).

  ii) Adsorb supernatant (from homogenate or cell cultures) to grid substrate.

  iii) Use carbon-coated 200 mesh gold grids.

  iv) Fix sample with 0.1% (v/v) glutaraldehyde and 1% (v/v) NP40 in PBS (2 minutes).

  v) Wash in PBS (3 × 3 minutes).

  vi) Block with 5% (v/v) cold water fish gelatin (Sigma) in PBS (10 minutes) followed with incubation buffer (PBS/0.1% cold water fish gelatin).

  vii) Incubate with antibody (affinity purified rabbit anti-EHNV, Lot No. M708; supplied by the OIE Reference Laboratory; suggested dilution 1/500) 1 hour, room temperature.

  viii) Wash grids (6 × 3 minutes) in incubation buffer.

  ix) Incubate with 10 nm protein A-gold (for dilution refer to suppliers recommendation), 1 hour, room temperature.

  x) Wash (6 × 3 minutes).

  xi) Fix with 2.5% glutaraldehyde (5 minutes).
xi) Wash with distilled water (3 × 3 minutes) and stain with 2% phosphotungstic acid (pH 6.8), 1 minute.

Interpretation of results
• The inclusion of NP40 will permit antibodies and protein A-gold to penetrate the outer membrane and react with the underlying capsid. Labelling should be specific for the virus. Non-EHNV affinity purified rabbit serum (1/500) should be included as a negative control.

Immunoperoxidase stain
Samples: formalin-fixed paraffin-embedded tissue sections.
Technical procedure
The following protocol is intended for the qualitative demonstration of EHNV antigens in formalin-fixed paraffin-embedded tissue sections (27). It assumes that antigens may have become cross linked and therefore includes a protease digestion step that may be omitted if unfixed samples are examined. A commercial kit (DAKO® LSAB K0679) with peroxidase-labelled streptavidin and a mixture of biotinylated anti-rabbit/anti-mouse/anti-goat immunoglobulins as link antibodies is used for staining. Other commercially supplied reagents are also used. For convenience these are also supplied by DAKO®. The primary affinity purified rabbit anti-EHNV antibody (Lot No. M708) is supplied freeze-dried by the OIE Reference Laboratory.

i) Cut 5 µm sections and mount on SuperFrost® Plus G/Edge slides (Menzel-Glaser, HD Scientific Cat. No. HD 041300 72P3). Mark around the section with a diamond pencil to limit the spread of reagents.

ii) De-paraffinise the section:
Pre-heat slides in a 60°C incubator for 30 minutes.
Place slides in a xylene bath and incubate for 5 minutes. Repeat once. Note xylene replacements can be used without deleterious effects.
Tap off excess liquid and place slides in absolute ethanol for 3 minutes. Repeat once.
Tap off excess liquid and place slides in 95% ethanol for 3 minutes. Repeat once.
Tap off excess liquid and place slides in distilled or deionised water for 30 seconds.

iii) Expose antigens using a protease treatment. Flood slide with Proteinase K (5–7 µg/ml) and incubate for 20 minutes (ready-to-use solution, DakoCytomation Cat. No. S3020). Rinse slide by immersing three times in water. Place in a PBST bath for 5 minutes (PBS pH 7.2, 0.05% [v/v] Tween 20). Tap off the excess wash solution and carefully wipe around the section.

iv) Perform the immunostaining reaction using the Universal DAKO LSAB®+ Kit, Peroxidase (DakoCytomation Cat No. K0679). Ensuring the tissue section is completely covered, add the following reagents to the slide. Avoid drying out.

v) 3% hydrogen peroxide: cover section and incubate for 5 minutes. Rinse gently with PBST and place in a fresh wash bath.

vi) Primary antibody (affinity purified rabbit anti-EHNV 1:1500 Lot No. M708) and negative control reagent (non-immune rabbit serum at a dilution of 1/1500) on a second slide. Cover the section and incubate for 15 minutes. Rinse slides.
vii) Link: cover the section and incubate for 15 minutes. Rinse slides.

viii) Streptavidin peroxidase: cover the section and incubate for 15 minutes. Rinse slides.

ix) Substrate–chromogen solution: cover the section and incubate for 5 minutes. Rinse slides gently with distilled water.

x) Counterstain by placing slides in a bath of DAKO® Mayer’s Haematoxylin for 1 minute (Lillie’s Modification, Cat. No. S3309). Rinse gently with distilled water. Immerse 10 times into a water bath. Place in distilled or deionised water for 2 minutes.

xi) Mount and coverslip samples with an aqueous based mounting medium (DAKO® Faramount Aqueous Mounting Medium Cat. No. S3025).

Interpretation of results

- EHNV antigen appears as a brown stain in the areas surrounding degenerate and necrotic areas in parenchymal areas. There should be no staining with negative control rabbit serum on the same section.

Availability of test and reagents: antibody reagents and test protocols are available from the OIE Reference Laboratory.

- Molecular techniques

Principal of test: polymerase chain reaction (PCR) implies that there is an enzyme-based amplification reaction in the assay. The term ‘chain reaction’ refers to several cycles of copying a specified stretch of DNA or target nucleic acids (nucleotides), in this case from the genome of an infectious agent. The region to be amplified is defined by two (or more) short nucleotide sequences, termed primer sites, that flank the target sequence. Primers, short oligonucleotides that are complementary to the primer sites, bind to the DNA strand to be copied. Using a polymerase, which is not denatured during heat cycling, it is possible to copy the target sequence by joining free nucleotides to the primers. By repeating the heat-cycling regime 20–40 times, the amount of copied target DNA gained is enough for further operations, such as detection, cloning or sequencing.

Identification of ranavirus at genus and species level is possible using two PCR-based methods based on the major capsid protein (MCP) gene. In the first method two PCR assays using MCP primers are used with restriction analysis to detect and rapidly differentiate EHNV from the European (ECV), North American (FV3) and other Australian ranaviruses (BIV) (25). This can be completed in less than 24 hours at relatively low cost. In the second method a single MCP PCR assay is used to generate a 580 bp product which is then sequenced to identify the type of ranavirus.

Samples: virus from cell culture or direct analysis of tissue homogenate.

Technical procedure

i) PCR and restriction endonuclease analysis (REA)

Amplified product from PCR assay MCP-1 digested with PstI enables differentiation of Australian iridoviruses (EHNV and BIV) from non-Australian iridoviruses (FV3, Americas; and ECV, Europe). Amplified product from PCR assay MCP-2 digested with Hinc II, Acc I and Fnu4HI (individually) enables differentiation of EHNV and BIV (Australia) from each other and from FV3 (Americas) and ECV (Europe).

Preparation of reagents

EHNV-purified DNA and BIV-purified DNA PCR control reagents are supplied by the reference laboratory in freeze-dried form. Reconstitute using 0.5 ml of TE.
buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and allow the vial to stand at room temperature for 2 minutes. Mix the vial very gently. For routine use as a PCR control it is recommended that working stocks be prepared as a 1/10 dilution in TE buffer (pH 8.0). Aliquots of 250 µl should be stored at −20°C. Each aliquot is sufficient for 50 reactions (5 µl added to cocktail) and has a minimum shelf life of 6 months from date of diluting.

Primers M151 and M152 (MCP-1, 321 bp), M153 and M154 (MCP-2, 625 bp) are supplied in working strength and should be stored at −20°C. Primers can also be ordered from commercial suppliers. For primer sequences, refer to Table 2.

**Table 2.** MCP-1 and MCP-2 primer sequences

<table>
<thead>
<tr>
<th>PCR assay</th>
<th>Primer</th>
<th>Sequence</th>
<th>Product size</th>
<th>Gene location</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>M151</td>
<td>AAC-CCG-GCT-TTC-GGG-CAG-CA</td>
<td>321 bp</td>
<td>266–586</td>
</tr>
<tr>
<td></td>
<td>M152</td>
<td>CGG-GGC-GGG-GTT-GAT-GAG-AT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-2</td>
<td>M153</td>
<td>ATG-ACC-GTC-GCC-CTC-ATC-AC</td>
<td>625 bp</td>
<td>842–1466</td>
</tr>
<tr>
<td></td>
<td>M154</td>
<td>CCA-TCG-AGC-CGT-TCA-TGA-TG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**PCR cocktail**

Amplification reactions in a final volume of 50 µl (including 5 µl DNA sample) contain 2.5 µl of each working primer, 200 µM of each of the nucleotides dATP, dTTP, dGTP and dCTP, 10 × PCR buffer (66.6 mM Tris/HCl, 16.6 mM (NH₄)₂SO₄, 2.5 mM MgCl₂, 1.65 mg/ml bovine serum albumin, 10 mM betamercaptoethanol) and 2 U Taq polymerase. Two negative controls are included, one comprising PCR cocktail only and the second containing 5 µl TE buffer. Instructions on preparation of 10 × PCR buffer are included in Table 3.

**Table 3.** 10 × PCR buffer preparation

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount</th>
<th>Final concentration in 50 µl PCR mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>4.050 g</td>
<td>66.6 mM</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>1.100 g</td>
<td>16.6 mM</td>
</tr>
<tr>
<td>BSA (albumin bovine fraction V fatty acid free)</td>
<td>0.825 g</td>
<td>1.65 mg/ml</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>1.25 g</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>TE buffer (sterile)</td>
<td>50 ml</td>
<td></td>
</tr>
</tbody>
</table>

The MCP-1 and MCP-2 reactions have the following profile: 1 cycle of denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 1 minute; a final extension of 72°C for 5 minutes, and cooled to 4°C.

PCR results are assessed by electrophoresis in 2% agarose gels stained with ethidium bromide. EHNV PCR control DNA (1/10 working stock) should give a result similar in intensity to the 10⁻³ band in both cases.

**Restriction endonuclease analysis**

PCR amplicons are subjected to REA with the enzymes described in the following table. All endonucleases should be used according to manufacturers’ instructions. REA reactions are prepared by adding 1–4 µl of PCR product, 2 U of the appropriate restriction endonuclease, 1.6 µl of buffer (supplied with each restriction endonuclease), 1.6 µl of 100 µg/ml bovine serum albumin (for PflMI and HincII) and made up to a final volume of 16 µl with sterile purified water.
Restriction digests are incubated for 2–4 hours at the recommended temperatures and assessed by agarose gel electrophoresis in 3% gels. The predicted band sizes after restriction are given in Table 4.

**Table 4. Restriction endonuclease analysis of ranavirus MCP amplicons**

<table>
<thead>
<tr>
<th>PCR Assay</th>
<th>Restriction enzyme</th>
<th>Predicted band sizes after restriction (bp)</th>
<th>Pattern applies to</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1 (321bp)</td>
<td>PflMI</td>
<td>321, 190</td>
<td>EHNV, BIV</td>
</tr>
<tr>
<td>MCP-1 (321bp)</td>
<td>MI</td>
<td>321, 190</td>
<td>FV3, WV</td>
</tr>
<tr>
<td>MCP-2 (625bp)</td>
<td>Hinc II</td>
<td>100, 138, 387</td>
<td>EHNV</td>
</tr>
<tr>
<td>MCP-2 (625bp)</td>
<td>Hinc II</td>
<td>100, 525</td>
<td>BIV, FV3</td>
</tr>
<tr>
<td>MCP-2 (625bp)</td>
<td>Hinc II</td>
<td>100, 240, 285</td>
<td>WV</td>
</tr>
<tr>
<td>MCH-1</td>
<td>AccI</td>
<td>238, 387</td>
<td>EHNV</td>
</tr>
<tr>
<td>MCH-1</td>
<td>AccI</td>
<td>625</td>
<td>BIV, ESV, ECV, WV</td>
</tr>
<tr>
<td>MCH-1</td>
<td>AccI</td>
<td>164, 461</td>
<td>FV3, GV</td>
</tr>
<tr>
<td>MCH-1</td>
<td>Fnu4HI</td>
<td>33, 38, 44, 239, 271</td>
<td>EHNV</td>
</tr>
<tr>
<td>MCH-1</td>
<td>Fnu4HI</td>
<td>3, 33, 38, 44, 108, 399</td>
<td>BIV</td>
</tr>
<tr>
<td>MCH-1</td>
<td>Fnu4HI</td>
<td>3, 38, 44, 108, 432</td>
<td>FV3, GV</td>
</tr>
<tr>
<td>MCH-1</td>
<td>Fnu4HI</td>
<td>3, 9, 38, 44, 108, 151, 272</td>
<td>ESV, ECV</td>
</tr>
<tr>
<td>MCH-1</td>
<td>Fnu4HI</td>
<td>3, 44, 71, 108, 399</td>
<td>WV</td>
</tr>
</tbody>
</table>

Aliquot into 500 µl volumes and store at −20°C. For working solution, add 3.5 µl of beta-mercaptoethanol per 500 µl 10 × buffer. Any remaining buffer should be discarded after preparing the PCR cocktail.

The sensitivity of PCR in diagnostic applications directly on fish tissues is being evaluated.

Detailed protocols to enable completion of the test, worksheets and purified control EHNV DNA is available from the OIE Reference Laboratory.

**ii) PCR and sequencing**

In this assay two primers, a reverse primer (5'-AAA-GAC-CCG-TTT-TGC-AGC-AGC-AAA-C-3') and a forward primer (5'-CGC-AGT-CAA-GGC-CTT-GAT-GT-3'), are used for amplification of the target MCP sequence (580 base pairs [bp]) of EHNV DNA by PCR. This PCR procedure can be used for the specific detection of ranaviruses from redfin perch, rainbow trout, sheatfish, catfish, guppy fish (*Poecilia reticulata*), doctor fish (*Labroides dimidatus*) and a range of amphibian ranaviruses (17). Nucleic acid (1 µl) is added to Taq polymerase buffer containing 0.1 µM of each primer, 2.5 U Taq polymerase (Promega) and 2.5 mM MgCl₂. The mixture is incubated in an automatic thermal cycler programmed for 35 cycles at 95°C for 60 seconds, 55°C for 60 seconds, and 72°C for 60 seconds, and finally held at 72°C for 15 minutes. Amplified DNA (580 bp) is analysed by agarose gel electrophoresis, excised and sequenced using the Cyclone Sequencing Kit (Bresatec, Australia). Each viral species is identified by its unique DNA sequence available from GenBank. Samples can be submitted to the OIE reference laboratory for specific identification.

- **Agent purification**

Purification of EHNV has been described (16, 30). A protocol is available from the reference laboratory.
• **Serological methods**

Neutralising antibodies have not been detected in fish or mammals exposed to EHNV. Indirect ELISA for detection of antibodies induced following exposure to EHNV have been described for rainbow trout and redfin perch (34, 35). The sensitivity and specificity of these assays in relation to a gold standard test are not known and interpretation of results is currently difficult. Protocols and specific anti-immunoglobulin reagents required to conduct these tests are available from the reference laboratory.

4. **RATING OF TESTS AGAINST PURPOSE OF USE**

The methods currently available for surveillance, detection, and diagnosis of EHNV are listed in Table 5. The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic sensitivity and specificity; C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; D = the method is presently not recommended for this purpose; and NA = not applicable. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation (see Chapter 1.1.2), their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

**Table 5. EHNV surveillance, detection and diagnostic methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance</th>
<th>Presumptive</th>
<th>Confirmatory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ova/milt</td>
<td>Fry/Fingerling</td>
<td>Juveniles</td>
</tr>
<tr>
<td>Gross signs</td>
<td>NA</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Histopathology</td>
<td>NA</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Immunoperoxidase stain</td>
<td>NA</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>Transmission EM</td>
<td>NA</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Immuno-EM</td>
<td>NA</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Cell culture</td>
<td>NA</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Antigen-capture ELISA</td>
<td>NA</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Antibody-capture ELISA</td>
<td>NA</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>PCR-REA</td>
<td>NA</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>PCR - Sequence analysis</td>
<td>NA</td>
<td>D</td>
<td>D</td>
</tr>
</tbody>
</table>

LM = light microscopy; EM = electron microscopy; PCR = polymerase chain reaction.

5. **CORROBORATIVE Diagnostic CRITERIA**

a) **Definition of suspect case**

Finfish, apparently healthy, moribund or dead in which parenchymal tissues contain histological evidence of focal, multifocal or locally extensive liquefactive or coagulative necrosis with or without intracytoplasmic basophilic inclusion bodies.
b) **Definition of confirmed case**

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

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

or

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

And for both 1 and 2,

3. Sequence consistent with EHNV is demonstrated by PCR-REA or PCR-sequencing

6. **Diagnostic/detection methods to declare freedom**

Statistically valid sampling practices need to be used and the correct organs/samples need to be collected; there is no evidence of reproductive tract infection and broodstock are not known to participate in an infection cycle so milt and ovarian fluid are unsuitable samples.

Standardised tests of specified sensitivity and specificity should be used. This restricts certification testing to cell culture, the gold standard test, and antigen-capture ELISA.

The chances of detecting EHNV infection in apparently healthy rainbow trout is extremely low, even where disease is active in the same population, because the prevalence of infection is low and there is a high case fatality rate. For practical purposes, EHNV can only be detected in fish that are clinically affected or that have died with the infection. From a random sample of live rainbow trout it would be possible to misclassify a farm as being free of EHNV even during an outbreak of the disease because the prevalence of infection is generally very low. Consequently the examination of ‘routine’ mortalities is recommended.

During a low-grade outbreak of disease in rainbow trout, the prevalence of EHNV among mortalities may be 60–80% and the contribution of EHNV to ‘background’ mortality is high enough to enable detection of the virus in the absence of overt disease in the population. For EHNV detection and certification purposes the population of interest is ‘the population of mortalities’ and sampling rates can be selected to detect at least one EHNV-infected individual at a given level of confidence given a certain prevalence of infection and test sensitivity (6, 28). During an outbreak of EHNV the virus was detected in at least 2% of dead fish (36). For this reason we assume a prevalence of 2% for sampling of EHNV for certification purposes. The antigen-capture ELISA used to screen tissue homogenates for EHNV has a sensitivity of at least 60% compared to cell culture (37). The sample size required from a very large population of ‘routine’ mortalities to provide 95% confidence in detecting at least one infected individual using a test of 60% sensitivity is approximately 250. In practice, ‘routine’ mortalities should be collected daily and stored in plastic bags at –20°C until a sample of 250 has been gathered. Where possible, young age classes should be selected to simplify dissections and tissue processing. Individual clarified homogenates that are positive in antigen-capture ELISA are then subjected to cell culture to confirm the presence of EHNV. This is an economical approach as it greatly reduces the number of cell cultures required. Alternatively, cell culture could be used and samples from several fish pooled to reduce costs.

Serology might also play a useful role in surveys to identify infected trout populations. Assuming 1% prevalence of seropositive grower fish on an endemically infected farm, a sample of 300 fish would be required to be 95% certain of detecting at least one infected individual (6). Further research is required to confirm the validity of this approach.
REFERENCES


* * *

NB: There is an OIE Reference Laboratory for epizootic haematopoietic necrosis (see Table at the end of this Aquatic Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.1.2.

INFECTIOUS HAEMATOPOIETIC NECROSIS

1. CASE DEFINITION

Infectious haematopoietic necrosis (IHN) is a viral disease affecting several species of salmonid fish. The principal clinical and economic consequences of IHN occur at farms rearing fry or juvenile rainbow trout in freshwater where acute outbreaks can result in very high mortality; however, both Pacific and Atlantic salmon reared in fresh water or sea water can be severely affected. Caused by the rhabdovirus, infectious haematopoietic necrosis virus (IHNV), the disease is typically characterised by gross signs that include lethargy interspersed with bouts of frenzied, abnormal activity, darkening of the skin, pale gills, ascites, distended abdomen, exophthalmia, and petechial haemorrhages internally and externally. Internally, fish appear anaemic and lack food in the gut. Liver, kidney and spleen are pale. Histopathologic findings reveal degenerative necrosis in haematopoietic tissues, kidney, spleen, liver, pancreas, and digestive tract. The blood of affected fry shows reduced haematocrit, leukopenia, degeneration of leukocytes and thrombocytes, and large amounts of cellular debris. For the purpose of this chapter, IHN is considered to be infection with IHNV.

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

For detailed reviews of the disease, see Bootland & Leong (3) or Wolf (20).

a) Agent factors

The fish rhabdovirus, IHNV, has a bullet-shaped virion containing a non-segmented, negative-sense, single-stranded RNA genome of approximately 11,000 nucleotides that encodes six proteins in the following order: a nucleoprotein (N), a phosphoprotein (P, formerly designated M1), a matrix protein (M, formerly designated M2), a glycoprotein (G), a non-virion protein (NV), and a polymerase (L). The presence of the unique NV gene and sequence homology among related fish rhabdoviruses, such as viral haemorrhagic septicaemia virus, has resulted in their placement into the newly created Novirhabdovirus genus of the Rhabdoviridae, with IHNV as the type species. The type strain of IHNV is the Western Regional Aquaculture Center (WRAC) strain available from the American Type Culture Collection (ATCC VR-1392). The GenBank accession number of the entire genomic sequence of the WRAC strain is L40883 (19).

Isolates of IHNV have genetic differences that appear to be most strongly related to geographical location and not to year of isolation or to host species. Sequence analysis of the central region of the G gene has been used to analyse a large number of IHNV isolates from salmonids throughout the range of the virus in western North America (10). Within the large geographical area representing the historic natural range of the virus, the nucleotide diversity remained remarkably low (2–3%). Conversely, isolates of IHNV from commercially reared rainbow trout in Idaho, USA had pairwise nucleotide differences of up to 7% in the central region of the G gene.

On the basis of antigenic studies conducted with neutralising polyclonal rabbit antisera, IHNV isolates form a single serogroup (7). However, mouse monoclonal antibodies have revealed a number of neutralising epitopes on the glycoprotein (8, 15, 18), as well as the existence of a non-neutralising group epitope borne by the nucleoprotein (14). Variations in the virulence and host preference of IHNV strains have been recorded during both natural cases of disease and in experimental infections (11).
Reservoirs of IHNV are clinically infected fish and covert carriers among cultured, feral or wild fish. Virus is shed via faeces, urine, sexual fluids and external mucus, whereas kidney, spleen, encephalon and the digestive tract are the sites in which virus is most abundant during the course of overt infection. The transmission of IHNV between fish is primarily horizontal; however, cases of vertical or ‘egg-associated’ transmission have been recorded. Horizontal transmission is typically by direct exposure, but invertebrate vectors have been proposed to play a role in some cases. Egg-associated transmission is significantly reduced by the now common practice of surface disinfection of eggs with an iodophor solution; it is, however, the only mechanism accounting for the appearance of IHN in new geographical locations among alevins originating from eggs that were incubated and hatched in virus-free water.

IHNV is heat, acid and ether labile. The virus will survive in fresh water for at least 1 month, especially if organic material is present. It is readily inactivated by common disinfectants and drying (20).

b) Host factors

Susceptible species include: rainbow or steelhead trout (Oncorhynchus mykiss), brown trout (Salmo trutta), Pacific salmon including chinook (O. tshawytscha), sockeye (O. nerka), chum (O. keta), masou (O. masou), and coho (O. kisutch), and Atlantic salmon (Salmo salar). Historically, the geographical range of IHN was limited to the western part of North America, but the disease has spread to continental Europe and the Far East via the importation of infected fish and eggs. Once IHN is established in a farmed stock or in a watershed, the disease may become established among carrier fish.

Among individuals of each fish species, there is a high degree of variation in susceptibility to IHN. The age of the fish is extremely important: the younger the fish, the more susceptible to disease. As with viral haemorrhagic septicaemia virus, good overall fish health condition seems to decrease the susceptibility to overt IHN, while co-infections with bacterial diseases (e.g. bacterial coldwater disease), handling and other types of stress frequently cause subclinical infections to become overt. Fish become increasingly resistant to infection with age until spawning, when they once again become highly susceptible and may shed large amounts of virus in sexual products. Survivors of IHN demonstrate a strong protective immunity with the synthesis of circulating antibodies to the virus (13) and, in certain individuals, a covert carrier state (6).

c) Disease pattern

Infection with IHNV often leads to mortality due to the impairment of osmotic balance, and occurs within a clinical context of oedema and haemorrhage. Virus multiplication in endothelial cells of blood capillaries, haematopoietic tissues, and cells of the kidney underlies the clinical signs. High levels of virus are shed from infected juvenile fish. The most important environmental factor affecting the progress of IHN is water temperature. Clinical disease typically occurs between 8°C and 15°C under natural conditions.

Depending on the species of fish, rearing conditions, temperature, and, to some extent, the virus strain, outbreaks of IHN may range from explosive to chronic. Losses in acute outbreaks will exceed several per cent of the population per day and cumulative mortality may reach 90–95% or more. In chronic cases, losses are protracted and fish in various stages of disease can be observed in the pond. The disease is made more severe by co-infection with certain bacterial pathogens, such as Flavobacterium psychrophilum.

d) Control and prevention

Control methods for IHN currently rely on avoidance of exposure to the virus through the implementation of strict control policies and sound hygiene practices (16). The thorough disinfection of fertilised eggs, and the incubation of eggs and rearing of fry and alevins on virus-
free water supplies in premises completely separated from those harbouring possible virus carriers and free from possible contact with inanimate objects, are critical for preventing the occurrence of IHNV in a defined fish production site.

At present, vaccination of salmonids against IHN is at an experimental stage; several new vaccine preparations however, have shown substantial promise in both laboratory and field trials (17). Both autogenous, killed vaccines and a novel DNA vaccine have proven commercially useful in Atlantic salmon net-pen aquaculture on the west coast of North America or for larger salmonids in captive propagation where a vaccine can be delivered economically by injection. However, the application of the available IHN vaccines to millions of very small fish will require additional research on novel methods for mass delivery.

3. DIAGNOSTIC METHODS

The 'Gold Standard' for detection of IHNV is the isolation of the virus in cell culture followed by its immunological or molecular identification. While the other diagnostic methods listed below can be used for confirmation of the identity of virus isolated in cell culture or for confirmation of overt infections in fish, they are not approved for use as primary surveillance methods for obtaining or maintaining approved IHN-free status.

Due to substantial variation in the strength and duration of the serological responses of fish to virus infections, the detection of fish antibodies to viruses has not thus far been accepted as a routine diagnostic method for assessing the viral status of fish populations. In the future, validation of serological techniques for diagnosis of fish virus infections could render the use of fish serology more widely acceptable for diagnostic purposes.

a) Field diagnostic methods

IHNV occurs among several species of salmonids with fry being the most highly susceptible stage. Older fish are typically more resistant to clinical disease. Under natural conditions, most clinical IHNV is seen in fry when the water temperature is between 8 and 15°C. During outbreaks, fish are typically lethargic with bouts of frenzied, abnormal activity such as spiral swimming and flashing. Affected fish exhibit darkening of the skin, pale gills, ascites, distended abdomen, exophthalmia, and petechial haemorrhages internally and externally. A trailing faecal cast is observed in some species. Spinal deformities are present among some of the surviving fish.

b) Clinical methods

Internally, fish appear anaemic and lack food in the gut. Ascitic fluid and petechiae are observed in the organs of the body cavity. The liver, kidney and spleen are pale. Histopathological findings reveal degenerative necrosis in haematopoietic tissues, kidney, spleen, liver, pancreas and digestive tract. The blood of affected fry shows reduced haematocrit, leukopenia, degeneration of leukocytes and thrombocytes, and large amounts of cellular debris. The cellular debris, termed necrobiotic bodies, can be seen in stained tissue imprints from the anterior kidney and has diagnostic value. As with other haemorrhagic viremias of fish, blood chemistry is altered in severe cases.

Electron microscopy of virus-infected cells reveals bullet-shaped virions of approximately 150–190 nm in length and 65–75 nm in width (20). The virions are visible at the cell surface or within vacuoles or intracellular spaces after budding through cellular membranes. The virion possesses an outer envelope containing host lipids and the viral glycoprotein spikes that react with immunogold staining to decorate the virion surface.

c) Agent detection and identification methods

The traditional procedure for detection of IHNV is based on virus isolation in cell culture. Confirmatory identification may be achieved by use of immunological (neutralisation, indirect fluorescent antibody test or enzyme-linked immunosorbent assay), or molecular (DNA probe or polymerase chain reaction) methods (1, 2, 4, 5, 9, 12, 19).
i) Isolation of IHNV in cell culture

a) Cell lines to be used: EPC and BF-2

b) Inoculation of cell monolayers

i) Following the virus extraction procedure described in Section B.3.2 of Chapter I.1, make an additional tenfold dilution of the 1/10 organ homogenate supernatants and transfer an appropriate volume of each of the two dilutions on to 24-hour-old cell monolayers. Inoculate at least 2 cm² of drained cell monolayer with 100 µl of each dilution.

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

iii) If required, the inoculum may be pre-incubated with neutralising antiserum against infectious pancreatic necrosis virus (IPNV) or other endemic viruses as previously described.

c) Monitoring incubation

i) Follow the course of infection in positive controls and other inoculated cell cultures by daily microscopic examination at ×40–100 magnification 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 during incubation. This can be achieved, especially in open plates, by using cell culture medium containing sodium bicarbonate that is further buffered by addition of Tris or HEPES buffer (N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid).

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

iv) If no CPE develops in the inoculated cultures (despite normal progression of CPE in the virus controls), the inoculated cultures should be subcultured for a further 7 days. Should the virus control fail to develop CPE, the process should be repeated with fresh cell cultures and new batches of samples.

d) Subcultivation procedures

i) Collect aliquots of cell culture medium from all monolayers inoculated with various dilutions of fluid or organ homogenate samples.

ii) If required, repeat the neutralisation test to IPNV or other endemic viruses as previously described.

iii) Inoculate cell monolayers as described above.

iv) Incubate and monitor as described above.

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

ii) Identification of IHNV isolated in cell culture

• Neutralisation test

i) Collect the medium from cultures exhibiting CPE that involves at least 25% of the cell monolayer and centrifuge it at 2000 g for 15 minutes at 4°C to remove cell debris.

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

• a known isolate of IHNV (positive neutralisation test).
• a heterologous virus (negative neutralisation test).
iii) If required, a similar neutralisation test may be performed using antibodies to IPNV or other enzootic viruses to ensure that no contaminant has escaped the first assay.

\textit{a) The neutralisation test procedure}

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

ii) Mix aliquots (for example 200 µl) of each virus dilution with equal volumes of an appropriate dilution of rabbit polyclonal or mouse monoclonal antibody (MAb) against IHNV, and similarly treat aliquots of each virus dilution with cell culture medium. (The neutralising antibody solution must have a 50% plaque reduction titre of at least 2000).

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

iv) Transfer aliquots of each of the above mixtures on to drained cell monolayers (inoculate two cell cultures per dilution) and allow adsorption to occur for 0.5–1 hour at 15°C; 24- or 12-well cell culture plates are suitable for this purpose, using a 50 µl inoculum.

v) When adsorption is complete, add cell culture medium, supplemented with 2% FCS and buffered at pH 7.4–7.6, to each well and 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 the cell culture medium and staining the cell monolayers with a solution of 1% crystal violet in 10% buffered formalin.

vii) The tested virus is identified as IHNV when CPE is prevented or noticeably delayed in the cell cultures that received the virus suspension treated with the IHNV-specific antibody, whereas CPE is present in the untreated sample.

In the absence of significant neutralisation when IHNV is suspected, it is advisable to conduct an indirect fluorescent antibody test (IFAT) as antigenic drift has been observed in the neutralising epitopes on the IHNV glycoprotein, resulting in occasional failures of the neutralisation test for certain strains of the virus.

\textbf{• Indirect fluorescent antibody test}

\textit{a) Preparation of monolayers}

i) Prepare monolayers of cells in 2 cm$^2$ wells of plastic cell culture plates or on glass coverslips to reach around 80% confluency within 24 hours of incubation at 22°C. Seed six cell monolayers per virus isolate to be tested, plus two for positive and two for negative controls. The FCS content of the cell culture medium can be reduced to 2–4%.

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 tenfold 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 5000–10,000 infectious units/ml in the cell culture medium.

iv) Incubate at 15°C for 24 hours.

v) Remove the cell culture medium, rinse once with 0.01 M phosphate buffered saline (PBS), pH 7.2, then three times briefly with cold acetone (stored at $-20^\circ$C) for glass cover-slips or a mixture of 30% acetone and 70% ethanol, also at $-20^\circ$C, for plastic wells.

vi) Let the fixative act for 15 minutes. 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 minutes and process immediately or freeze at $-20^\circ$C.
b) *The indirect fluorescent antibody procedure*

i) Prepare a solution of antibody against IHNV in PBS containing 0.05% Tween 80 (PBST) at the appropriate dilution (established previously or given by the reagent supplier). The antibody used should be able to bind to all isolates of the virus.

ii) Rehydrate the dried cell monolayers by four rinsing steps with the PBST solution, and remove this buffer completely after the last rinsing.

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

iv) Rinse four times with PBST as above.

v) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate (FITC)-conjugated 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.

vi) Rinse four times with PBST.

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

viii) Examine under incident UV light using a microscope with a ×20–40 objective lens having a high numerical aperture. Positive and negative controls must be found to give the expected results prior to any other observation.

• **Enzyme-linked immunosorbent assay**

  a) *Preparation of microplates*

i) Coat the wells of microplates designed for enzyme-linked immunosorbent assays (ELISAs) with appropriate dilutions of immunoglobulins (Ig) specific for IHNV, in 0.01 M PBS, pH 7.2 (200 µl/well). The Ig may be polyclonal or monoclonal, most often from rabbit or mouse. For the identification of IHNV, MAbs specific for certain domains of the nucleocapsid (N) protein are suitable.

ii) Incubate overnight at 4°C.

iii) Rinse four times with 0.01 M PBS 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 four times with PBST.

  b) *The enzyme-linked immunosorbent assay procedure*

i) Add 2% Triton X-100 to the virus suspension to be identified.

ii) Dispense 100 µl/well of two- or four-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.

iii) Rinse four times with PBST.

iv) Add to the wells either biotinylated polyclonal rabbit antiserum to IHNV or biotinylated mouse MAb to an N protein epitope different from the one recognised by the coating MAb.

v) Incubate for 1 hour at 37°C.

vi) Rinse four times with PBST.

vii) Add streptavidin-conjugated horseradish peroxidase to those wells that have received the biotin-conjugated antibody, and incubate for 1 hour at 20°C.
viii) Rinse four times with PBST.

ix) Add the substrate and chromogen. Stop the course of the test when positive controls react, and read the results.

**DNA probe**

*a) Infect cells with virus isolates*

i) Trypsinise EPC (or other) cells and resuspend to 1.0 × 10^6 cells per ml with MEM-5-T (5% FBS [fetal bovine serum], Tris buffer). Transfer 1 ml of cell suspension to each well of a 24-well tissue culture plate and incubate the plate at an appropriate temperature.

ii) On the day after seeding cells, remove the fluid from each cell monolayer. Infect cells with positive control (IHNV) and with the unknown fish virus isolates in 1 ml total volume to provide a multiplicity of infection (m.o.i.) of 10 PFU per cell. Negative control cells receive 1 ml MEM-5-T. Incubate for 2 hours at 15–20°C on a rocker platform.

iii) Incubate infected and control cells at 15°C overnight. The desired product is the N gene mRNA, and maximum production occurs at about 12 hours post-infection when inoculated at high m.o.i. Thus, mRNA production is optimum when infected cells are at an early stage of virus replication, and RNA can be extracted from cells at the first sign of any CPE. Alternatively, use recently infected cells from the virus isolation procedure.

*b) Make or purchase the following solutions*

i) Prehybridisation buffer

- Deionised water (DEPC [diethyl pyrocarbonate]-treated; autoclaved) 69.5 ml
- 10 × Denhardt’s solution 10 ml of 100 × stock (step iv)
- 2 × SSC (standard saline citrate) 10 ml of 20 × stock (step vi)
- 1% SDS (sodium dodecyl sulfate) 10 ml of 10% stock (step viii)
- 0.1 mg/ml SSS (sonicated salmon sperm) DNA 0.5 ml stock (step v)

ii) Hybridisation solution

- Prehybridisation buffer 10 ml (step i)
- Biotinylated DNA probe 100 ng/ml (Store at –20°C; may re-use up to five times)

iii) Post-hybridisation solution

- 2 × SSC 50 ml of 20 × stock (step vi)
- 0.1% SDS 5 ml of 10% stock (step viii)
- Deionised water (DEPC-treated; autoclaved) up to 500 ml

iv) 100 × Denhardt’s solution

- Bovine serum albumin 50 g
- Polyvinylpyrrolidone 360 50 g
- Ficoll 400 50 g
- Deionised water (DEPC-treated; autoclaved) up to 500 ml

v) Sonicated salmon sperm DNA (SSS DNA at 20 mg/ml)

Transfer 0.5 ml of SSS DNA (20 mg/ml) into vials and place in boiling water for 10 minutes. Cool vials in crushed ice and store at –20°C. When needed, add 0.5 ml to prehybridisation buffer (see step i).
vi) 20 × standard saline citrate (20 × SSC)

NaCl 87.65 g
Citric acid 44.11 g
Deionised water (DEPC-treated; autoclaved) up to 500 ml
(Adjust to pH 7.0 with HCl, autoclaved)

vii) 10 × standard saline citrate (10 × SSC)

NaCl 43.82 g
Citric acid 22.05 g
Deionised water (DEPC-treated; autoclaved) up to 500 ml
(Adjust to pH 7.0 with HCl, autoclaved OR dilute 1/2 from 20 × SSC using deionised water, autoclaved)

viii) 10% sodium dodecyl sulfate (10% SDS)

Lauryl sulfate sodium salt 10.0 g
Deionised water (DEPC-treated; autoclaved) up to 100 ml
(Adjust to pH 7.2. Do not autoclave this solution!)

ix) Streptavidin/alkaline phosphate conjugate (SA/AP)

0.1 µg/ml streptavidin/alkaline phosphatase conjugate. Prepare by diluting SA/AP 1/1000 in buffer A (step x). (May re-use this solution up to five times, store at 4°C.)

x) Buffer A

0.1 M Tris, pH 7.5 50 ml of 1 M stock (step xiv)
0.1 M NaCl 10 ml of 5 M stock (step xii)
2 mM MgCl₂ 1 ml of 1 M stock
0.05% Triton X-100 0.25 ml
Deionised water (DEPC-treated; autoclaved) up to 500 ml

x) Buffer B

0.1 M Tris, pH 9.5 50 ml of 1 M stock (step xiii)
0.1 M NaCl 10 ml of 5 M stock (step xii)
50 mM MgCl₂ 25 ml of 1 M stock
Deionised water (DEPC-treated; autoclaved) up to 500 ml

xiii) 5 M NaCl

NaCl 146.1 g
Deionised water (DEPC-treated; autoclaved) up to 500 ml
(autoclave this solution)

xiii) 1 M Tris buffer, pH 9.5

Tris base 54.7 g
Tris HCl 7.6 g
Deionised water (DEPC-treated; autoclaved) up to 500 ml
(Adjust to pH 9.5, then autoclave)

xiv) 1 M Tris buffer, pH 7.5

Tris base 11.8 g
Tris HCl 63.5 g
Deionised water (DEPC-treated; autoclaved) up to 500 ml
(Adjust to pH 7.5, then autoclave)

xv) Chloroform

Chloroform (Store at –20°C until needed)
xvi) Isopropyl alcohol

2-propanol (isopropyl alcohol). Use undiluted for precipitation of RNA.

xvii) Trizol


xviii) Alkaline phosphatase conjugate substrate kit

(NOTE: This product contains dimethylformamide. Use in area with good ventilation.) Dissolve alkaline phosphatase (AP) colour development buffer in 1 litre volume of distilled deionised water. Filter-sterilise then store at 4°C. Immediately before use, add 0.4 ml of AP colour reagent A and 0.4 ml AP colour reagent B to 39.2 ml colour development buffer at room temperature.

c) Prepare biotinylated oligonucleotide probe

i) The biotinylated probe (5’-CTT-GTT-TTG-GCA-GTA-TGT-GGC-CAT-CTT-GTC-3’) is made using the 30-nucleotide sequence identified by Deering et al. (4); however, three nucleotides containing biotin can be conveniently added to the 5’ end during DNA synthesis rather than by a subsequent terminal transferase reaction at the 3’ end. This antisense probe is complementary to a conserved region in the middle of the IHNV nucleoprotein gene mRNA. It should react only with IHNV and should recognise all isolates of the virus. The final concentration of the biotinylated probe will be 0.1 µg/ml in hybridisation solution.

d) Extraction of mRNA from infected cells

i) Always wear protective gloves to avoid contaminating solutions with RNase from skin and to avoid injury. Provide adequate ventilation, especially for RNA extraction steps with Trizol and for dimethylformamide in colour development solutions.

ii) Remove culture medium from infected cells and add 1.0 ml Trizol to each well. Replace lid and place plates on a rocker platform for 5–10 minutes at room temperature to digest the cells. During incubation, load 100 µl chloroform into siliconised 1.7 ml centrifuge tubes, keep on ice.

iii) Triturate cell debris by pipetting with a sterile 1 ml pipette (5×), then transfer solution from each well into a separate tube. Vortex the tubes (about 3 seconds each), store on crushed ice for 5 minutes to allow phase separation.

iv) Centrifuge the suspension at 12,500 g for 15 minutes at 4°C. The RNA will remain in the clear aqueous phase while DNA and protein will be left in the lower red phenol phase.

v) During step iv, load another set of tubes with 0.5 ml of absolute isopropyl alcohol, store on ice.

vi) Carefully transfer the upper aqueous phase, which contains the RNA (about 0.5 ml), to a tube that contains an equal volume (0.5 ml) of absolute isopropyl alcohol. Vortex tubes for 1 second, and chill tubes on ice for 15 minutes to precipitate RNA.

vii) Centrifuge the mixture at 12,500 g for 15 minutes at 4°C. Remove as much fluid from the pellet as possible. Partially dry the pellet following the manufacturer's instructions.

viii) Prepare a nitrocellulose membrane (0.45 µm pore size): wet in distilled deionised water for 1 minute, pour water off, then soak for at least 5 minutes in 10 × SSC.

ix) Warm the prehybridisation buffer to 55°C in a water bath.

x) Add 170 µl distilled deionised, ribonuclease-free (DEPC-treated and autoclaved) or molecular-biology grade water to the RNA pellets. Mix the contents and warm the tubes in a heat block at 65°C for 15–20 minutes (RNA pellets should dissolve).
xi) Install the membrane in 96-well vacuum blotting device, attach vacuum pump hoses and add 200 µl of 10 × SSC to each well to insure that the membrane is not dry when the RNA is added.

xii) Heat IHNV PCR products (if used as positive control instead of RNA from IHNV-infected cells) to 95°C for 5 minutes to denature the double-stranded DNA, then place tubes directly on ice.

xiii) Add 170 µl of 20 × SSC into microcentrifuge tubes containing the dissolved pellets of RNA in 170 µl of water (tubes now contain 340 µl of 10 × SSC). Store on ice.

xiv) Add 100 µl of each RNA solution to wells of a blotting device that contain 200 µl of 10 × SSC. Blot positive controls last. Apply vacuum for about 1 minute until fluid is pulled through the membrane. Remove membrane and transfer to thick filter paper wetted with 10 × SSC.

xv) Cut the membrane into sections if required and label the membranes in one corner.

xvi) Place the membrane(s) between dry sheets of blotting paper and microwave for 60 seconds on high power to attach nucleic acids to the membrane. Include a beaker of water in the microwave. (Ultraviolet radiation or other suitable methods may be used.)

e) Hybridisation of probe to RNA on nitrocellulose membrane

i) Place the membranes (spots up) in a hybridisation pouch, bottle or plastic bag. Add 10 ml prehybridisation buffer to each membrane. Prehybridise for 1 hour at 55°C in a shaker water bath.

ii) Thaw the probe solution and add 100 µl to prehybridisation buffer. React the membranes in probe solution for 1 hour in shaker water bath at 55°C.

iii) Remove probe solutions and store in tubes at –20°C for re-use up to five times.

iv) Rinse the membranes with 40 ml of post-hybridisation solution. Discard the solution then add a fresh 40 ml of post-hybridisation solution. Wash for 15 minutes on a rocker platform at room temperature. Wash two more times (40 ml) for 15 minutes each at room temperature on a rocker platform. Put the container with the membranes into a 55°C water bath with pre-warmed post-hybridisation solution (55°C) for 15 minutes.

v) Rinse the membranes briefly with 40 ml of buffer A.

f) Colour development of biotinylated probe

i) Incubate the membranes in a solution containing 40 µl streptavidin/alkaline phosphatase in 40 ml buffer A for 30 minutes at room temperature on a rocker platform. This solution can be re-used at least five times.

ii) Rinse the membranes briefly (40 ml) then wash twice using 40 ml buffer A incubated for 7 minutes at room temperature on a rocker platform.

iii) Wash twice in 40 ml buffer B incubated for 7 minutes at room temperature on a rocker platform.

iv) Warm the colour development buffer prepared according to the manufacturer’s protocol. Buffer should be filter-sterilised through a 0.2 µm membrane and stored at 4°C. Immediately before use, add 0.4 ml alkaline phosphatase (AP) colour reagent A and 0.4 ml AP colour reagent B to 39.2 ml AP colour development buffer at room temperature. Add 40 ml colour development solution containing nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) to each container with the membranes. Incubate at room temperature on a rocker platform and observe for colour development (up to 15 minutes).

v) Wash the membranes in distilled deionised water for 10 minutes, change the water at least once to remove excess colour development solution. Store membranes in distilled deionised water until photographs can be taken (if desired).
• Polymerase chain reaction

a) Viral RNA preparation

i) Collect aliquots of culture medium from cell monolayers exhibiting CPE and centrifuge at 2000 \( g \) for 15 minutes at 4°C to remove cell debris.

ii) Release RNA from virus solution by diluting the sample 1/20 in sterile deionised water, heating the tube at 95°C for 2 minutes, then store on ice. This simple procedure is especially suitable for virus grown in cell culture where few PCR-inhibiting substances are present. Alternatively, RNA can be extracted from a pellet of infected cells using methods described above or by use of other commercially available kits (19).

b) Reverse-transcription and first round PCR protocol

i) Prepare a master mix for the number of samples to be analysed. Work under a hood and wear gloves.

ii) The master mix for one 50 µl reverse-transcription PCR is prepared as follows: 23.75 µl ribonuclease-free (DEPC-treated) or molecular-biology grade water; 5 µl 10 × buffer; 5 µl 25 mM MgCl\(_2\); 5 µl 2 mM dNTP; 2.5 µl (20 pmoles/µl) Upstream Primer 5'–TCA-AGG-GGG-GAG-TCC-TCG-A-3'; 2.5 µl (20 pmoles/µl) Downstream Primer 5'–CAC-CGT-ACT-TTG-CTG-CTA-C-3'; 0.5 µl Taq polymerase (5 U/µl); 0.5 µl AMV reverse transcriptase (9 U/µl); 0.25 µl RNasin (39 U/µl).

iii) Centrifuge the tubes briefly (10 seconds) to make sure the contents are at the bottom.

iv) Place the tubes in the thermal cycler and start the following cycles – 1 cycle: 50°C for 15 minutes; 1 cycle: 95°C for 2 minutes; 25 cycles: 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds; 1 cycle: 72°C for 7 minutes and soak at 4°C.

vi) Visualise the 786 bp PCR amplicon by electrophoresis of the product in 1.5% agarose gel with ethidium bromide and observe using UV transillumination.

c) Second round PCR protocol

i) If the first round PCR provides insufficient amplified product, an internally nested set of primers is used for additional DNA amplification. Prepare a master mix for the number of samples to be analysed. Work under a hood and wear gloves.

ii) The master mix for one 50 µl second round PCR is prepared as follows: 27.5 µl molecular-biology grade water; 5 µl 10 × buffer; 5 µl 25 mM MgCl\(_2\); 5 µl 2 mM dNTP; 2.5 µl (20 pmoles/µl) Upstream Primer 5'–TTC-GCA-GAT-CCC-AAC-AAC-AA-3'; 2.5 µl (20 pmoles/µl) Downstream Primer 5'–GCG-CAC-AGT-GCC-TTG-GCT-3'; 0.5 µl Taq polymerase (5 U/µl).

iii) Dispense 48 µl of master mix into each tube and add 2 µl of first round PCR template.

iv) Centrifuge the tubes briefly (10 seconds) to make sure the contents are at the bottom.

v) Place the tubes in a thermal cycler and start the following cycles – 1 cycle: 95°C for 2 minutes; 25 cycles: 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds; 1 cycle: 72°C for 7 minutes and soak at 4°C.

vi) Visualise the 323 bp PCR amplicon by electrophoresis of the product in 1.5% agarose gel with ethidium bromide and observe using UV transillumination.

The DNA probe procedure (Section 3.c.ii – DNA probe) can be used to further confirm that the amplicon is from IHNV. Alternatively, the amplicon can be sequenced (19).

NOTE: Other primer sets have been used for the successful amplification of a portion of the N gene of IHNV (19); however, the primer sequences used here have been shown to be conserved among all known isolates of IHNV and are not present in the N gene of the related fish rhabdoviruses, viral hemorrhagic septicemia virus or hirame rhabdovirus.
iii) **Diagnostic Methods for IHN in fish infected with IHNV**

- **Virus isolation with subsequent identification**
  
  a) *Sampling procedures*
  
  See the following sections in Chapter I.1:
  
  B.1. for the selection of fish specimens
  
  B.2. for the selection of materials sampled.

  b) *Processing of organ samples*
  
  See the following sections in Chapter I.1:
  
  B.3.1. for transportation
  
  B.3.2. for virus extraction and obtaining organ homogenates.

  c) *Virus isolation and identification*
  
  As outlined above

- **Indirect fluorescent antibody test**
  
  a) *Processing of organ samples*
  
  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 Chapter I.1. Section B.3.1.) together with the other organs required for virus isolation in case this becomes necessary later.
  
  iv) Allow the imprint to air-dry for 20 minutes.

  b) *Indirect fluorescent antibody procedure*
  
  i) Fix with acetone or ethanol/acetone and dry as indicated in Section 3.c.ii – indirect fluorescent antibody.
  
  ii) Rehydrate the above preparations and block with 5% skim milk or 1% bovine serum albumin, in phosphate buffered saline containing 0.05% Tween 80 (PBST) for 30 minutes at 37°C.
  
  iii) Rinse four times with PBST.
  
  iv) Treat the imprints with the solution of antibody to IHNV and rinse as indicated in Section 3.c.ii – indirect fluorescent antibody.
  
  v) Block and rinse as described previously.
  
  vi) Reveal the reaction with suitable FITC-conjugated specific antibody, rinse and observe as indicated above.

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

**Enzyme-linked immunosorbent assay**

a) *Sampling procedures*

See the following sections in Chapter I.1:

B.1. for the selection of fish specimens

B.2. for the selection of materials sampled.
b) Processing of organ samples

See the following sections in Chapter I.1.:
B.3.1. for transportation
B.3.2. for virus extraction and obtaining organ homogenates.

c) Microplate processing

As described in Section 3.c.ii – enzyme-linked immunosorbent assay.

d) The enzyme-linked immunosorbent assay procedure

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 the homogenate with 2% Triton X-100 as above and 2 mM of phenyl methyl sulfonide fluoride; mix gently.

iii) Complete the other steps of the procedure described in Section 3.c.ii – enzyme-linked immunosorbent assay.

If the test is negative, process the organ samples stored at 4°C for virus isolation in cell culture as described in Section 3.c.i.

• Polymerase chain reaction

a) Sampling procedures

See the following sections in Chapter I.1.:
B.1. for the selection of fish specimens
B.2. for the selection of materials sampled.

b) Processing of organ samples

See the following sections in Chapter I.1.:
B.3.1. for transportation
B.3.2. for virus extraction and obtaining organ homogenates.

c) Viral RNA preparation

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

ii) Release RNA from tissue homogenate by diluting sample 1/20 in sterile deionised water, heating the tube at 95°C for 2 minutes, then storing on ice. Alternatively, RNA can be extracted from tissue homogenates using methods described above or by use of other commercially available kits (19).

d) The polymerase chain reaction procedure

Perform the remaining steps of the PCR procedure described above.

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

4. RATING OF TESTS AGAINST PURPOSE OF USE

The methods currently available for surveillance, detection, and diagnosis of IHNV are listed in Table 1. The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic sensitivity and specificity; C = the method has application in
Chapter 2.1.2. - Infectious haematopoietic necrosis

some situations, but cost, accuracy, or other factors severely limits its application; and D = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation (see Chapter 1.1.2), their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

### Table 1. IHNV surveillance, detection and diagnostic methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Targeted surveillance to declare freedom from infection</th>
<th>Presumptive diagnosis of infection or disease</th>
<th>Confirmatory diagnosis of infection or disease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gametes</td>
<td>Fry</td>
<td>Juveniles</td>
</tr>
<tr>
<td>Gross signs</td>
<td>D</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>Virus isolation in cells with confirmatory ID</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Direct light microscopy on tissue imprints</td>
<td>D</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>Histopathology of tissues and organs</td>
<td>D</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>Transmission electron microscopy of tissues</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Antibody-based assays to detect IHNV antigens</td>
<td>D</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>DNA probes to detect IHNV nucleic acids</td>
<td>D</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>Polymerase chain reaction of tissue extracts</td>
<td>B</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>Direct sequencing of nucleic acids from tissues</td>
<td>D</td>
<td>D</td>
<td>D</td>
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</tbody>
</table>

5. CORROBORATIVE DIAGNOSTIC CRITERIA

a) Definition of suspect case

A suspect case is defined as the presence of typical, gross clinical signs of the disease in a population of susceptible fish, OR a typical internal histopathological presentation among susceptible species, OR typical cytopathic effect in cell culture without identification of the agent, OR a single positive result from one of the diagnostic assays ranked as ‘A’ or ‘B’ in Table 1.

b) Definition of confirmed case

A confirmed case is defined as a suspect case that has EITHER: 1) produced typical cytopathic effect in cell culture with subsequent identification of the agent by one of the antibody-based or molecular tests listed in Table 1 OR: 2) a second positive result from a different diagnostic assay ranked as ‘A’ or ‘B’ in the last column of Table 1.

6. DIAGNOSTIC/DETECTION METHODS TO DECLARE FREEDOM

The method for targeted surveillance to declare freedom from IHN is isolation of virus in cell culture. For this purpose, the most susceptible stages of the most susceptible species should be examined.
Reproductive fluids and tissues collected from adult fish of a susceptible species at spawning should be included in at least one of the sampling periods each year.

**REFERENCES**


nucleoprotein of infectious hematopoietic necrosis virus (IHNV) reveal differences among isolates of


and viral hemorrhagic septicemia. *In: Molecular Diagnosis of Salmonid Diseases*. Cunningham C.O.,


**NB:** There is an OIE Reference Laboratory for infectious haematopoietic necrosis (see Table at the end
of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: [www.oie.int](http://www.oie.int)).
CHAPTER 2.1.3.

ONCORHYNCHUS MASOU VIRUS DISEASE

SUMMARY

Oncorhynchus masou virus disease (OMVD) is an oncogenic and skin ulcerative condition among salmonid fish in Japan, and probably in the coastal rivers of eastern Asia that harbour Pacific salmon. Oncorhynchus masou virus (OMV), the causative virus, is also known as Yamame tumour virus (YTV), Nerka virus Towada Lake, Akita and Amori prefecture (NeVTA), coho salmon tumour virus (CSTV), Oncorhynchus kisutch virus (OKV), coho salmon herpesvirus (CHSV), rainbow trout kidney virus (RKV), or rainbow trout herpesvirus (RHV). For a recent and more detailed review of the condition, see refs 4, 12 and 13.

Fish species that are susceptible to OMV include: kokanee (sockeye) salmon (Oncorhynchus nerka), masou salmon (O. masou), chum salmon (O. keta), coho salmon (O. kisutch) and rainbow trout (O. mykiss) (6).

Clinically, the initial infection by OMV (taxonomically known as Salmonid herpesvirus 2; SalHV-2) appears as a systemic and frequently lethal infection that is associated with oedema and haemorrhages. Virus multiplication in endothelial cells of blood capillaries, haematopoietic tissue and hepatocytes underlies the clinical signs (7, 11). 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 1 year post-infection. In the case of coho salmon, 1-year-old infected fish in particular show ulcers on the skin, white spots on the liver and neoplastic tissues around the mouth parts or body surface. In rainbow trout, the diseased fish exhibit almost no external signs, although some fish manifest ulcerative lesions on the skin. Internally, intestinal haemorrhage and white spots on the liver are observed (5, 15, 16).

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

On the basis of antigenic studies conducted with neutralising polyclonal rabbit antisera, OMV differs from Salmonid herpesvirus 1 (SalHV-1), which is present in the western United States of America and is only weakly pathogenic (3, 9, 12).

The reservoirs of OMV are clinically infected fish and covert carriers among groups of cultured, feral or wild fish. Infectious virus is shed via faeces, urine, sexual products and probably skin mucus, while the kidney, spleen, liver and tumours are the sites where virus is the most abundant during the course of overt infection. The transmission of OMV is horizontal and possibly ‘egg-surface associated’. Horizontal transmission may be direct or vectorial, water being the major abiotic factor. Animate vectors and inanimate objects also act in OMV transmission. Disinfection of the eggs just after fertilisation and eyed stage is effective in preventing OMV infection. OMV disease was not reported in alevins originating from disinfected eggs that had been incubated and hatched in virus-free water (14).

Salmonids are the only fish species susceptible to OMV infection; the order of the fish species from the most to the least susceptible is: kokanee salmon, chum salmon, masou salmon, coho salmon and rainbow trout. The age of the fish is critical and 1-month-old alevins are the most susceptible target for virus infection (6). The main environmental factor favouring OMV infection is low water temperature (below 14°C) (10).
The screening procedures for OMV are based on direct isolation of the virus in cell culture and co-culture of neoplastic tissues with salmonid cell lines (8). Confirmatory testing is by immunological identification using neutralisation or immunofluorescence tests, and virus-specific gene detection using polymerase chain reaction.

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 inanimate objects are the key measures needed to decrease contamination of OMV in a defined fish production site (14).

**DIAGNOSTIC PROCEDURES**

The screening for and diagnosis of *Oncorhynchus masou* virus disease (OMVD) is based on direct methods, which are either the isolation of the *Oncorhynchus masou* virus (OMV) in cell culture followed by its immunological identification (conventional approach), or the immunological demonstration of OMV antigen in infected fish tissues (2, 5).

Due to insufficient knowledge of the serological responses of fish to virus infections, the detection of fish antibodies to viruses has not thus far been recognised as a valuable diagnostic method for assessing the viral 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:

- **Clinically affected fish:** Whole alevin (body length ≤4 cm), viscera including kidney (4 cm ≤ body length ≤6 cm) or, for larger size fish, skin ulcerative lesions or neoplastic tissues, and kidney, spleen, liver and encephalon.
- **Asymptomatic fish** (apparently healthy fish): Kidney, spleen and encephalon (any size fish) and/or ovarian fluid from broodfish at spawning time.

**Sampling procedures:** see Chapter I.1. Section B.

1. **STANDARD SCREENING METHOD FOR OMV**

1.1. Isolation of OMV in cell culture

Cell line to be used: RTG-2 or CHSE-214

a) **Inoculation of cell monolayers**

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

ii) Allow to adsorb for 0.5–1 hour at 10–15°C and, without withdrawing the inoculate, add cell culture medium buffered at pH 7.6 and supplemented with 2% fetal calf serum (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 ×40–100 magnification for 14 days. The use of a phase-contrast microscope is recommended.
ii) Maintain the pH of the cell culture medium at between 7.3 and 7.6 during incubation. This can be achieved by the addition to the inoculated cell culture medium of sterile bicarbonate buffer (for tightly closed cell culture flasks) or Tris buffer solution (for cell culture plates) or, even better, by using HEPES-buffered medium (HEPES = N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid).

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 Section 1.2. below).

If a fish health surveillance/control programme is being implemented, provisions may 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 develops in the inoculated cultures (despite normal progression of CPE in the virus controls), the inoculated cultures should be subcultured for a further 7 days. Should the virus controls fail to develop CPE, the process should be repeated with fresh susceptible cells and new batches of samples.

c) Subcultivation procedures

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

ii) If required, repeat the neutralisation test to infectious pancreatic necrosis virus (IPNV) and/or infectious haematopoietic necrosis virus (IHNV) as previously described (see Chapter I.1. Section B.3.3.), with dilution of the above supernatant (1/1 to 1/100).

iii) Inoculate cell monolayers as described above in Section 1.1.a.

iv) Incubate and monitor as described above in Section 1.1.b.

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

d) Isolation of OMV from cultures of neoplastic cells

i) Collect neoplastic tissues, disinfect with iodophore, 50 parts per million for 20 minutes, and wash three times with Hanks’ balanced salt solution.

ii) The tissues are left overnight in 0.25% trypsin in phosphate buffered saline (PBS) at 5°C. Then, 3.5 × 10^5 neoplastic cells/ml are seeded in a tissue culture flask and incubated with culture medium containing 20% fetal bovine serum (FBS).

iii) Harvest the primary neoplastic cell culture and co-cultivate with RTG-2 or CHSE-214 cells.

iv) Incubate and monitor as described in Section 1.1.b.

1.2. Identification of OMV

a) Neutralisation test

i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge at 2000 g for 15 minutes at 4°C to remove cell debris.

ii) Dilute the 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 neutralising antibody [NAb] solution must have a 50% plaque reduction titre of at least 2000.)
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) If required, a similar neutralisation test may be performed using antibodies to IPNV, to ensure that no IPNV contaminant has 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 on to cell monolayers (inoculate two cell cultures per dilution) and allow adsorption to occur for 0.5–1 hour at 15°C; 24- 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 10–15°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 the cell culture medium and staining the cell monolayers with a solution of 1% crystal violet in 20% ethanol.

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

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

b) Indirect fluorescent antibody test

i) Prepare monolayers of cells in 2 cm² wells of cell culture plastic plates or on cover-slips in order to reach around 80% confluency, which is usually achieved within 4 hours of incubation at 22°C (seed six cell monolayers per virus isolate to be identified, plus two for positive and two 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 tenfold 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 5000–10,000 plaque-forming units (PFU)/ml in the cell culture medium.

iv) Incubate at 15°C for 48 hours.

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

vi) Let the fixative act for 15 minutes. 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 minutes and process immediately or freeze at −20°C.

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

ix) Rehydrate the cell monolayers by four rinsing steps with the PBST solution, and remove this buffer completely after the last rinsing.
x) Treat the cell monolayers with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur. The volume of solution to be used is 0.25 ml/2 cm² well.

xi) Rinse four times with PBST as above.

xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate (FITC)-conjugated 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 four times with PBST.

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

xv) Examine under incident UV light using a microscope with ×10 eye pieces and ×20–40 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

i) Coat the wells of microplates designed for enzyme-linked immunosorbent assays (ELISAs) with appropriate dilutions of monoclonal antibody or purified immunoglobulins (Ig) specific for OMV, in 0.01 M PBS, pH 7.2 (200 µl/well).

ii) Incubate overnight at 4°C.

iii) Rinse four times with 0.01 M PBS 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 four times with PBST.

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

vii) Dispense 100 µl/well of a two- or four-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 four times with PBST.

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

x) Incubate for 1 hour at 37°C.

xi) Rinse four times with PBST.

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

xiii) Rinse four times with PBST.

xiv) Add the substrate and chromogen. Stop the course of the test when positive controls react, and monitor the results.

d) Polymerase chain reaction method

i) Extract nucleic acid from cells infected with OMV strains and SalHV-1 using the InstGene Matrix (Biorad).

ii) Pellet the virus-infected tissues or infected cultured cells by centrifugation at 19,000 g (14,800 rpm) for 15 minutes.

iii) Wash the pellets twice with 1 ml PBS and mix with 200 µl of chelating resin (Sigma).
iv) Incubate the mixture at 56°C for 20 minutes in a water bath, vortex it, and then place it in
a boiling water bath for 8 minutes.

v) Vortex the samples and centrifuge at 8200 \( g \) (10,000 rpm) for 90 seconds.

vi) Subject the supernatant to PCR.

vii) The forward primer (F10) is 5'-GTA-CCG-AAA-CTC-CCG-AGT-C-3', and the reverse
primer (R5) is 5'-AAC-TTG-AAC-TAC-TCC-GGG-G-3'.

viii) Incubate the specimens, primer sets and reaction mixtures for 30 cycles in an automatic
thermal cycler (GeneAmp PCR 9700, Applied Biosystems), with each cycle consisting of
denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension at
72°C for 30 seconds.

ix) Analyse the amplified product for size and purity by electrophoresis (100 V for
30 minutes) in 2% agarose gel and stain with ethidium bromide.

x) A PCR using these primer sets amplified a 439 base-pair segment of DNA from OMV
strains isolated from masu salmon, coho salmon and rainbow trout, and liver, kidney,
brain and nervous tissues, and an 800 base-pair segment of DNA from SalHV-1. SalHV-1
and SalHV-2 could be distinguished by agarose gel profile of this amplified DNA (1).

2. DIAGNOSTIC METHODS FOR OMV

2.1. Virus isolation with subsequent identification

As in Sections 1.1. and 1.2.

2.2. Indirect fluorescent antibody test

a) Indirect fluorescent antibody test method

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 Section B.3.1. in Chapter I.1.) together with the
other organs required for virus isolation in case this becomes necessary later.

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

v) Fix with acetone or ethanol/acetone and dry as indicated in Section 1.2.b. steps v–vii.

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

vii) Rinse four times with PBST.

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

ix) Block and rinse as described previously in steps vi and vii.

x) Reveal the reaction with suitable FITC-conjugated specific antibody, rinse and observe as
indicated in Section 1.2.b. steps xii–xv.

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

2.3. Enzyme-linked immunosorbent assay

a) Microplate processing

As described in Section 1.2.c. of this chapter up to step iv (inclusive).
b) **Sampling procedures**

   See the following sections in Chapter I.1.:
   B.1. for the selection of fish specimens
   B.2. for the selection of materials sampled.

c) **Processing of organ samples**

   See the following sections in Chapter I.1.:
   B.3.1. for transportation
   B.3.2. for virus extraction and obtaining organ homogenates.

d) **The enzyme-linked immunosorbent assay procedure**

   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, as in Section 1.2.c. step vi, and 2 mM of phenyl methyl sulfonide fluoride; mix gently.

   iii) Complete the other steps of the procedure described in Section 1.2.c.

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

2.4. **Polymerase chain reaction method**

   a) **DNA extraction**

      As described in Section 1.2.d. of this chapter up to step v (inclusive).

   b) **Primer set and PCR condition**

      As described in Section 1.2.d of this chapter, steps vi–viii. PCR using these primer sets amplified a 439 base-pair segment of DNA from OMV. SalHV-1 and SalHV-2 could be distinguished by agarose gel profile of this amplified DNA (1).

**REFERENCES**


Chapter 2.1.3. - *Oncorhynchus masou* virus disease


**NB:** There is an OIE Reference Laboratory for *Oncorhynchus masou* virus disease (see Table at the end of this Aquatic Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.1.4.

SPRING VIRAEMIA OF CARP

1. CASE DEFINITION

Spring viraemia of carp (SVC) is a rhabdovirus infection (13) capable of inducing an acute haemorrhagic and contagious viraemia in several carp species and of some other cyprinid and ictalurid fish species (11).

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

a) Agent factors

The aetiological agent is spring viraemia of carp virus (SVCV) in the family Rhabdoviridae, and tentatively placed in the genus *Vesiculovirus* (24). The virus genome is a non-segmented, negative sense, single strand of RNA. The genome contains 11,019 nucleotides encoding five proteins in the following order: a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a glycoprotein (G) and an RNA-dependent, RNA polymerase (L). The genome does not contain a non-virion (NV) gene between the G and L genes as is found in fish rhabdoviruses of the genus *Novirhabdovirus* (3). The type strain of SVCV is available from the American Type Culture Collection (ATCC VR-1390, ref. 12). Two complete genome sequences of the type strain have been submitted to Genbank (Genbank accession U18101 by Bjorklund et al. [7] and Genbank accession AJ318079 by Hoffmann et al. [17]).

Antibodies directed against SVCV cross-react to various degrees with pike fry rhabdovirus (PFRV) indicating that the two viruses are closely related. The viruses have been shown to share common antigenic determinants on the G, N and M proteins, but can be differentiated by neutralisation assays (23). Ahne et al. (5) showed that the two viruses could also be differentiated by a ribonuclease protection assay using a G-gene probe, suggesting that genetic differences exist between the two viruses. Stone et al. (22) used sequence analysis of a 550 nucleotide region of the G-gene to compare 36 putative SVCV and PFRV isolates from different fish species and geographical locations. The analysis showed that the isolates could be separated into four distinct genogroups and that all of the SVCV isolates could be assigned to group 1, sharing <61% nucleotide identity with viruses in the other three genogroups. Further analysis also showed that the SVCV genogroup 1 could be further sub-divided into at least four sub-genogroups.

The virus has been shown to remain viable outside the host for more than 4 weeks in water at 10°C and for more than 6 weeks in pond mud at 4°C.

The virus is inactivated at 60°C for 30 minutes and at pH 12 for 10 minutes and pH 3 for 3 hours. Oxidising agents, sodium dodecyl sulphate, non-ionic detergents and lipid solvents are all effective for inactivation of SVCV. The following disinfectants are also effective for inactivation: formalin 3% for 5 minutes; sodium hydroxide 2% for 10 minutes; chlorine 540 mg/litre for 20 minutes and iodine compounds 250 ppm for 30 minutes (12).

b) Host factors

Naturally occurring SVC infections have been recorded from common carp (*Cyprinus carpio carpio*) and koi carp (*Cyprinus carpio koi*), crucian carp (*Carassius carassius*), sheatfish, (also known as European catfish or wels) (*Silurus glanis*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*), grass carp (white amur) (*Ctenopharyngodon idella*), goldfish (*Carassius auratus*), orfe (*Leuciscus idus*), and tench (*Tinca tinca*). Other cyprinid species have been shown to be susceptible to SVCV by experimental bath infection, including roach (*Rutilus rutilus*) (16) and
zebrafish (Danio rerio) (21). So, it is reasonable to assume that other cyprinid species in temperate waters may be susceptible to infection. Some other species can also be infected experimentally, e.g. Northern pike (Esox lucius), guppy (Lebistes reticulatus) and pumpkinseed (Lepomis gibbosus) (3).

Generally, young fish up to 1 year are those most susceptible to clinical disease, but all age groups can be affected. Moreover, 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 or age-related status of innate immunity appears to be extremely important: the younger the fish, the higher the susceptibility to overt disease, but even adult broodfish can be susceptible to infection. It is clear that the environmental factor that is critical for virulence of an SVC infection is water temperature (11): in yearling or older fish overt infection is not often observed above 17°C whereas fry can be affected at temperatures as high as 22–23°C. Apart from the formerly cited cyprinid and ictalurid species, it seems that very young fish of various pond fish species are susceptible to SVC under experimental conditions over a wide temperature range. The most striking example is that of the European pike (Esox lucius), which can be easily infected via the water route (2). Poor physiological condition of overwintered fish is a contributory factor to disease susceptibility.

Common carp (Cyprinus carpio) strains are the principal host of SVCV and are considered to be most susceptible to SVCV infection followed, in order of susceptibility, by other carp species (including hybrids), other susceptible cyprinid species and finally susceptible non-cyprinid fish species. When sampling during surveillance programmes for SVC, common carp or strains such as koi or ghost (koi × common) carp are preferentially selected, followed by carp hybrids (e.g. common carp × crucian carp) then other carp species such as crucian carp, goldfish, grass carp, bighead carp and silver carp. Should these species not be available then other known susceptible species should be sampled in the following preferential order: tench, orfe, wels catfish and finally any other cyprinid species present. For disease surveillance purposes all cyprinid species should be considered as potential covert carriers of SVCV. Cyprinid species are commonly mixed together in polyculture systems and the risk of transmission of SVCV between species, during disease outbreaks, is high.

The reservoirs of SVCV are clinically infected fish and covert virus carriers among cultured, feral or wild fish. Virulent virus is shed via faeces, urine, gill and skin mucus and exudate of skin blisters or oedematous scale pockets. However, liver, kidney, spleen, gill and encephalon are the organs in which SVCV is most abundant during the course of overt infection (10).

The mode of transmission for SVCV is horizontal, but ‘egg-associated’ transmission (usually called ‘vertical’ transmission) cannot be ruled out. 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 geometra (Annelida, Hirudinea) are able to transfer SVCV from diseased to healthy fish (1). Regurgitated infected fish from Heron (Ardea cinerea) fed SVCV contained virus up to 120 minutes after feeding (20). Aquatic arthropods may also be able to function as SVCV vectors (3). An SVC-like virus has been isolated from penaeid shrimp in Hawaii; it was replicated and produced histological changes in blue shrimp (19). Once SVCV is established in pond stock or pond farm stock, it may be very difficult to eradicate without destroying all kinds of life in the fish production site.

c) Disease pattern

Disease patterns are influenced by water temperature, age and condition of the fish, population density and stress factors. The immune status of the fish is also an important factor with both non-specific (interferon) and specific immunity (serum antibodies, cellular immunity) having important roles. Clinical disease dominates at low water temperatures below 10°C when the host immune response is suppressed or delayed. Secondary and concomitant bacterial and/or parasitic infections can affect the mortality rate and display of signs (11).
The geographical range of SVC was for a long time limited to countries of the European continent that experience low water temperatures during winter. The disease has been recorded from most European countries and from certain of the western Independent States of the former Soviet Union (Belarus, Georgia, Lithuania, Moldova, Russia and the Ukraine). However, in 2002, SVC was reported for the first time in two separate sites in the USA (8, 15), and detection of the virus in carp in the People’s Republic of China was confirmed in 2004 (18). The disease has also been recorded in goldfish imported into Brazil (6).

d) Control and prevention

Methods to control SVC disease mainly rely on avoiding exposure to the virus coupled with good hygiene practices. This is feasible on small farms supplied by spring or borehole water and a secure system to prevent fish entering the farm via the discharge water. Hygiene measures should include disinfection of eggs by iodophore treatment (4), regular disinfection of ponds, chemical disinfection of farm equipment, careful handling of fish to avoid stress and safe disposal of dead fish. Reducing fish stocking density during winter and early spring will reduce the spread of the virus. In rearing facilities with a controlled environment, elevation of water temperature above 19–20°C can stop or prevent SVC outbreaks (12). A safe and effective vaccine is not currently available. However, a number of experimental inactivated preparations and live attenuated vaccines have given encouraging results (12).

Detailed reviews of SVC disease have been published by Wolf (26), Fijan (12) and Ahne et al. (3).

3. DIAGNOSTIC METHODS

Diagnosis of SVC in clinically affected fish may be achieved by virus isolation or more rapidly by direct immunofluorescence (IF) tests (10) or enzyme-linked immunosorbent assays (ELISAs) (25) on infected tissues. Ideally, direct diagnosis by IF or ELISA should be confirmed by virus isolation followed by a virus neutralisation (VN) test or PCR and sequence analysis. However, virus isolation may not be possible from decomposed clinical samples (25), so the presence of signs of SVC disease and a positive direct IF test or ELISA may be considered sufficient to initiate control measures.

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

Fish material suitable for virological examination is:

- **Asymptomatic fish** (apparently healthy fish): kidney, spleen, gill and encephalon (any size fish).

- **Clinically affected fish**: whole alevin (body length < or = 4 cm), entire viscera including kidney and encephalon (> 4 cm body length < and = 6 cm) or, for larger size fish, liver, kidney, spleen and encephalon.

a) Field diagnostic methods

During an SVC disease outbreak there will be a noticeable increase in mortality in the population. Generally, young fish up to 1 year are most susceptible to clinical disease, but all age groups can be affected. Fish become lethargic, separate from the shoal and gather at the water inlet or sides of a pond and some may experience loss of equilibrium. Diseased fish will appear darker in coloration. On closer examination of individual fish, typical clinical signs include exophthalmia, pale gills, haemorrhages on the skin, base of the fins and the vent, abdominal distension or dropsy and a protruding vent (anus) often with trailing mucoid faecal casts.
b) **Clinical methods**

There are no pathognomonic gross lesions. Final diagnosis must await direct detection of viral antigen in tissues or virus isolation and identification. Lesions may be absent in cases of sudden mortality. Gross pathologies may include excess ascitic fluid in the abdominal cavity usually containing blood, degeneration of the gill lamellae and inflammation of the intestines, which contain mucus instead of food. Oedema and haemorrhage of the visceral organs is commonly observed. Petechial or focal haemorrhages may be seen in the muscle and fat tissue and may also be seen in the swim bladder.

Histopathological changes can be observed in all major organs. In the liver, blood vessels show oedematous perivasculitis progressing to necrosis. Liver parenchyma shows hyperaemia with multiple focal necroses and degeneration. The heart shows pericarditis and infiltration of the myocardium progressing to focal degeneration and necrosis. The spleen shows hyperaemia with hyperplasia of the reticuloendothelium and enlarged melanomacrophage centres and the pancreas is inflamed with multifocal necrosis. In the kidney, damage is seen to excretory and haematopoietic tissue. Renal tubules are clogged with casts and the cells undergo hyaline degeneration and vacuolation. The intestine shows perivascular inflammation, desquamation of the epithelium and atrophy of the villi. The peritoneum is inflamed and lymph vessels are filled with detritus and macrophages. In the swim bladder the epithelial lamina changes from a monolayer to a discontinuous multi-layer and vessels in the sub-mucosa are dilated with nearby lymphocyte infiltration.

c) **Agent detection and identification methods**

- **Direct detection methods**

  i) **Isolation of SVCV in cell culture**

     a) **Virus extraction**

        Use the procedure described in Chapter I.1 (Section B.3.2.).

     b) **Inoculation of cell monolayers**

        i) Make two serial tenfold dilutions of the 1/10 organ homogenate supernatants and transfer an appropriate volume of each of these two dilutions on to 24-hour-old cell monolayers. Inoculate at least 2 cm\(^2\) of drained cell monolayer with 100 µl of each dilution.

        ii) Inoculate directly or allow to adsorb for 0.5–1 hour at 10–15°C and, without withdrawing inoculate, add the cell culture medium buffered at pH 7.6 and supplemented with 2% fetal calf serum (FCS) (1 ml/well for 24-well cell culture plates), and incubate at 20°C.

  c) **Monitoring incubation**

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

     ii) Maintain the pH of the cell culture medium at between 7.3 and 7.6 during incubation. This can be achieved by the addition to the inoculated cell culture medium of sterile bicarbonate buffer (for tightly closed cell culture flasks) or HEPES-buffered medium (HEPES = N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid) or 2 M Tris/HCl buffer solution (for cell culture plates).
iii) If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of the tested homogenate supernatants, identification procedures must be undertaken immediately (see Section 3.c.ii below).

iv) If no CPE develops in the inoculated cultures (despite normal progression of CPE in the virus controls), the inoculated cultures should be subcultured for a further 7 days. Should the virus control fail to develop CPE, the process should be repeated with fresh susceptible cells and new batches of samples.

d) Subcultivation procedures

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

ii) Inoculate cell monolayers as described above in Section 3.c.i.a.

iii) Incubate and monitor as described above in Section 3.c.i.b.

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

ii) Identification of virus isolated in cell culture

- Rapid presumptive methods

a) Indirect fluorescent antibody test

i) Prepare monolayers of cells in 2 cm² wells of cell culture plastic plates or on cover-slips in order to reach around 80% confluency within 24 hours of incubation at 30°C (seed six cell monolayers per virus isolate to be identified, plus two for positive and two 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 tenfold 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 5000–10,000 plaque-forming units (PFU)/ml in the cell culture medium.

iv) Incubate at 20°C for 24 hours.

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

vi) Let the fixative act for 15 minutes. 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 minutes and process immediately or freeze at –20°C.

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

ix) Rehydrate the dried cell monolayers by four rinsing steps with the PBST solution, and remove this buffer completely after the last rinsing.

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

xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate (FITC)-conjugated 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 four times with PBST.

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

xv) Examine under incident UV light using a microscope with ×10 eye pieces and ×20–40 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.

b) Enzyme-linked immunosorbent assay

i) Coat the wells of microplates designed for ELISAs with appropriate dilutions of purified immunoglobulins (Ig) specific for SVCV, in 0.02 M carbonate buffer, 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 SVCV, monoclonal antibodies (MAbs) specific for certain domains of the nucleocapsid (N) protein are suitable.

ii) Incubate overnight at 4°C.

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

iv) Block with skim milk (5% in carbonate buffer) or other blocking solution for 1 hour at 37°C (300 µl/well).

v) Rinse four times with PBST.

vi) Add 2% non-ionic detergent (Triton X-100 or Nonidet P-40) to the virus suspension to be identified.

vii) Dispense 100 µl/well of two- or four-step dilutions of the virus to be identified, and of the non-infected cell culture harvest (negative control), and the SVCV control virus (positive control); allow to react with the coated antibody to SVCV for 1 hour at 20°C.

viii) Rinse four times with PBST.

ix) Add to the wells, 200 µl of horseradish peroxidase (HRPO)-conjugated MAb or polyclonal antibody to SVCV; or polyclonal IgG 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 for 1 hour at 37°C.

xi) Rinse four times with PBST.

xii) Add 200 µl of HRPO-conjugated streptavidin or ExtrAvidin (Sigma) to those wells that have received the biotin-conjugated antibody and incubate for 1 hour at 37°C.

xiii) Rinse four times with PBST.

xiv) Add 200 µl of the substrate and chromogen. Stop the course of the test when positive controls react, and read the results.

xv) Alternatively, add substrate and chromogen to those wells containing the peroxidase-conjugated antibody and proceed as above.
• Confirmatory identification methods

a) Neutralisation test

i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge at 2000 g for 15 minutes at 4°C, or filter through a 0.45 µm pore membrane to remove cell debris.

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

iii) Mix aliquots of each dilution with equal volumes of an antibody solution against SVCV, and similarly treat aliquots of each virus dilution with cell culture medium.

(The neutralising antibody [NAb] solution must have a 50% plaque reduction titre of at least 2000 based on neutralisation of 50–100 PFU of SVC virus.)

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 20°C for 1 hour.

vi) Transfer aliquots of each of the above mixtures on to cell monolayers (inoculate two cell cultures per dilution) and allow adsorption to occur for 0.5–1 hour at 15–20°C; 24- 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, to each well and incubate at 20°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 the cell culture medium and staining the cell monolayers with a solution of 1% crystal violet in 20% ethanol.

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

NOTE: Presumptive SVCV isolates identified by ELISA or IFAT may not be neutralised by NAb to SVCV. Also, some SVCV sub-genogroups may not be completely neutralised by NAb. Where neutralisation by NAb to SVCV is absent or incomplete, confirmation by PCR and nucleotide sequence analysis of PCR products is recommended to confirm the presence of SVCV.

b) Polymerase chain reaction (PCR)

i) The genome of SVCV consists of a single strand of RNA of approximately 11 kb, with negative polarity. Amplification of a 714 bp fragment of SVCV cDNA is performed using primers derived from sequences of the region coding for the glycoprotein gene: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' (SVCV F1) and 5'-AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH*-ACN*-CAY*-3' SVCV R2), using a modification of the method of Stone et al. (22).

ii) Total RNA is extracted from 100 µl of viral supernatant from infected EPC cells and dissolthed in 40 µl molecular biology grade DNase- and RNase-free water (BHD). (A number of total RNA extraction kits are available commercially that will produce high quality RNA suitable for reverse transcription (RT) PCR. Examples are Trizol Reagent™ [BRL, Life
Technologies, Paisley, UK], SV Total RNA isolation system [Promega] and Nucleospin® RNA [AB gene].

iii) For cDNA synthesis, a reverse transcription reaction is performed at 37°C for 1 hour in a 20 µl volume consisting of 1 × M-MLV RT reaction buffer (50 mM Tris, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM MgCl₂ containing 1 mM dNTP, 100 pmol SVCV R2 primer, 20 units M-MLV reverse transcriptase (Promega, Southampton, UK) or equivalent reverse transcriptase and 1/10 of the total RNA extracted above.

iv) PCR is performed in a 50 µl reaction volume 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, and 0.1% Triton X-100) containing 2.5 mM MgCl₂, 200 µM dNTPs, 50 pmol each of the SVCV R2 and SVCV F1 primers, 1.25 units of RedHot DNA polymerase (AB Gene, Epsom, UK) or equivalent Taq DNA polymerase, and 2.5 µl reverse transcription reaction mix. The reaction mix is overlaid with mineral oil and subjected to 35 temperature cycles of: 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C followed by a final extension step of 10 minutes at 72°C. Amplified DNA (714 bp) is analysed by agarose gel electrophoresis.

v) If the cytopathic effects in culture are not extensive it is possible that a product will not be generated using a single round of amplification. To avoid such problems use the semi-nested assay using primers: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' (SVCV F1) and 5'-CTG-GGG-TTT-CCN*-CCT-CAA-AGY*-TGY*-3' (SVC R4) according to Stone et al. (22).

vi) The second round of PCR is performed in a 50 µl reaction volume 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, and 0.1% Triton X-100) containing 2.5 mM MgCl₂, 200 µM dNTPs, 50 pmol each of the SVCV R4 and SVCV F1 primers, 1.25 units of RedHot DNA polymerase (AB Gene, Epsom, UK) or equivalent Taq DNA polymerase, and 2.5 µl of the first round product. The reaction mix is overlaid with mineral oil and subjected to 35 temperature cycles of: 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C followed by a final extension step of 10 minutes at 72°C. Amplified DNA (606 bp) is analysed by agarose gel electrophoresis.

vii) All amplified products are confirmed as SVCV in origin by cloning and sequencing, and the SVCV subtype (Ia–Id) is identified using a BLAST search (http://www.ebi.ac.uk/blastall/index.html) or by phylogenetic analysis using the SVCV sequences available in the fishpathogens.net database. Phylogenetic analysis is undertaken using a 550 bp region corresponding to nucleotides 405–954 of the glycoprotein gene.

viii) In some cases where the cytopathic effect is extensive and the virus replicates to a high titre, sufficient PCR amplicon will be available for direct sequencing. Where the amplified product is weak it is recommended that the product be inserted into an appropriate sequencing vector (e.g. pGEM-T, pCR® 4-TOPO®) prior to undertaking the sequencing. At least two independent amplification and sequencing events should be undertaken to eliminate potential sequence errors introduced by the Taq polymerase.

NOTE: SVCV primer-annealing sites were identified by the alignment of the published amino acid sequences for the glycoprotein of SVCV (ref. 7; Genbank accession U18101), and the vesicular stomatitis virus (VSV) New Jersey (ref. 14; Genbank accession V01214), and Piry strains (Genbank accession D26175). Primers were then designed to anneal to the regions encoding the conserved amino acids using the published sequence for SVCV (7) as a skeleton, and introducing degenerate bases at the 3' termini to allow for potential differences in codon usage. The appropriate IUB codes have been used where appropriate and indicated (*).
iii) Diagnostic methods for clinically diseased fish

- **Direct detection in fish tissues**
  
  **a) 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 Chapter I.1. Section B.3.1) together with the other organs required for virus isolation in case this becomes necessary later.
  
  iv) Allow the imprint to air-dry for 20 minutes.
  
  v) Fix with acetone or ethanol/acetone and dry as indicated in Section 3.c.ii.a steps v–vii.
  
  vi) Rehydrate the above preparations (see Section 3.c.ii.a step ix) and block with 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C.
  
  vii) Rinse four times with PBST.
  
  viii) Treat the imprints with the solution of antibody to SVCV and rinse as indicated in Section 3.c.ii.a steps viii–xi.
  
  ix) Block and rinse as previously in steps vi and vii.
  
  x) Reveal the reaction with suitable FITC-conjugated specific antibody, rinse and observe as indicated in Section 3.c.ii.a steps xii–xv.
  
  If the test is negative, process the organ samples stored at 4°C, for virus isolation in cell culture as described in Section 3.c.i.

**b) Enzyme-linked immunosorbent assay**

i) Microplate processing

As described in Section 3.c.ii.b of this chapter up to step iv (inclusive).

ii) Sampling procedures

See the following sections in Chapter I.1:
  
  B.1 for the selection of fish specimens
  
  B.2 for the selection of materials sampled.

iii) Processing of organ samples

See the following sections in Chapter I.1:
  
  B.3.1 for transportation
  
  B.3.2 for virus extraction and obtaining organ homogenates.

iv) The enzyme-linked immunosorbent assay procedure

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

- Treat the remaining part of the homogenate with 2% Triton X-100 or Nonidet P-40 and 2 mM of phenyl methyl sulfonide fluoride; mix gently.

- Complete the other steps of the procedure described in Section 3.c.ii.b.

- If the test is negative, process the organ samples stored at 4°C, for virus isolation in cell culture as described in Section 3.c.i.
• *Virus isolation in cell culture*

As described in Section 3.c.i of this chapter.

4. **RATING OF TESTS AGAINST PURPOSE OF USE**

The methods currently available for surveillance, detection and diagnosis of SVC are listed in Table 1. The designations used in the table indicate: A = the method is currently the recommended method for reasons of availability, utility and diagnostic sensitivity and specificity; B = the method is a standard method with good diagnostic sensitivity and specificity; C = the method has application in some situations, but cost, accuracy or other factors severely limits its application; D = the method is currently not recommended for this purpose. Although not all of the tests listed as category A or B have undergone formal standardisation and validation (see Chapter 1.1.2), their routine nature and the fact that they have been used widely without dubious results makes them acceptable.

**Table 1. SVC surveillance, detection and diagnostic methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance to declare freedom from infection</th>
<th>Presumptive diagnosis of infection or disease</th>
<th>Confirmatory diagnosis of infection or disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross signs</td>
<td>D</td>
<td>B</td>
<td>D</td>
</tr>
<tr>
<td>Histopathology of tissues and organs</td>
<td>D</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Isolation of SVCV in cell culture</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Antibody-based assays to detect SVCV antigen (IFAT, ELISA)</td>
<td>D</td>
<td>A</td>
<td>C</td>
</tr>
<tr>
<td>Transmission EM of tissues</td>
<td>D</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>PCR of tissue extracts</td>
<td>C</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>PCR – sequence analysis</td>
<td>NA</td>
<td>C</td>
<td>A</td>
</tr>
</tbody>
</table>

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

NOTE: Four genogroups of piscine rhabdoviruses have recently been described (22): Genogroup I (SVCV), Genogroup II (Grass carp rhabdovirus), Genogroup III (Pike fry rhabdovirus) and Genogroup IV (Tench rhabdovirus). Antibodies directed against SVCV cross-react to various degrees with all of the rhabdoviruses in the other three groups. Further analysis also showed that the SVCV genogroup could be further sub-divided into at least four sub-genogroups. The ability to confirm SVCV based on results from serological tests, such as ELISA, IFAT and serum neutralisation, relies on the specificity of the antibodies used in the tests. Antibodies directed against SVCV can cross-react to various degrees with all of the rhabdoviruses in the other, recently described, piscine rhabdovirus genogroups (22).

Many diagnostic laboratories have encountered difficulties in obtaining antibodies against SVCV that are suitable for use in serological tests and have turned to commercially available test kits. Two commercial test kits are available for identification of SVCV, the Testline ELISA kit (TestLine, Brno, Czech Republic) and the Bio-X IFAT kit (Bio-X Diagnostics, Jemelle, Belgium). Recently the tests have been assessed for their specificity against virus isolates from Genogroups I, II, III and IV by Dixon & Longshaw (9) who found that the TestLine ELISA, which uses a polyclonal rabbit antibody, was non-specific and could not distinguish SVCV from viruses in the other three genogroups. Conversely, the Bio-X IFAT, which uses a monoclonal mouse antibody, was too specific and could only detect SVCV isolates from 1 of the 4 SVCV sub-groups.
These commercial test kits can be applied for presumptive diagnosis of SVC but the problems of specificity severely limits their application for confirmatory diagnosis. It is recommended that the PCR test and nucleotide sequence analysis of PCR products are used for confirmatory identification of SVCV.

5. CORROBORATIVE DIAGNOSTIC CRITERIA

a) Definition of suspect case

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

b) Definition of confirmed case

A confirmed case is defined as a suspect case that has produced a typical CPE in cell culture with subsequent identification of the causative agent by one of the serological or molecular assays described above OR a second positive result from a separate and different diagnostic assay described above.

6. DIAGNOSTIC/DETECTION METHODS TO DECLARE FREEDOM

The method for surveillance of susceptible fish populations for declaration of freedom from SVC is inoculation of cell culture with tissue extracts (as described in Section 3.c above) to demonstrate absence of the virus.

REFERENCES


* *
* *

**NB:** There is an OIE Reference Laboratory for Spring viraemia of carp (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: [www.oie.int](http://www.oie.int)).
CHAPTER 2.1.5.

VIRAL HAEMORRHAGIC SEPTICAEMIA

1. CASE DEFINITION

For the purpose of this chapter, viral haemorrhagic septicaemia (VHS) is a disease of farmed rainbow trout (51, 60), farmed turbot (47), farmed Japanese flounder (17) as well as several wild marine species (14, 32, 33, 56) caused by viral haemorrhagic septicaemia virus (VHSV, synonym: Egtved virus), a virus belonging to the genus Novirhabdovirus, within the family Rhabdoviridae (57).

In rainbow trout, the acute form of the disease occurs during the early stages of infection (up to 30 days post-infection) during which period sick fish display clear clinical signs: rapid onset of mortality (can reach up to 100% in fry), lethargy, darkening of skin, exophthalmia, anaemia (pale gills), haemorrhages at the base of fins, gills, eyes and skin, abnormal swimming such as flashing and spiralling, distended abdomen due to oedema in the peritoneal cavity. In addition, following the acute phase, the disease can occur in a subclinical, chronic state in which affected fish do not exhibit external signs. Due to the tropism of the virus for the brain, VHS can also occur in a nervous form, characterised by severe abnormal swimming behaviour such as constant flashing and/or spiralling. Corroborative diagnostic criteria are summarised in Section 5 of this chapter.

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

a) Agent factors

The aetiological agent of VHS is a rhabdovirus (VHSV; Egtved virus) belonging to the genus, Novirhabdovirus, within the family Rhabdoviridae, which also includes infectious haematopoietic necrosis virus (IHNV). Virions are bullet-shaped (approximately 70 nm in diameter and 180 nm in length), contain a negative-sense, single-stranded RNA genome (ca. 11,000 nucleotides) and possess an envelope that contains the membrane glycoprotein, which is the major neutralising surface antigen. The genome encodes six proteins (a nucleoprotein, N; a phosphoprotein, P; a matrix protein, M; the envelope glycoprotein, G; a non-virion protein, NV; a virus-associated polymerase, L).

Neutralisation by polyclonal and monoclonal antibodies suggests a single serotype consisting of three subtypes of VHSV (43):

Subtype I – includes strains F1 (Denmark) and Hededam
Subtype II – includes strains 23/75, DK-5131 and DK-5276
Subtype III – includes strains DK-5151 and DK-5422

Apart from the above variation, VHSV seems to share a VHS-group neutralising epitope, and several non-neutralising epitopes located on the viral glycoprotein (G protein) and it is not possible to distinguish marine isolates from freshwater isolates using routine antibody-based tests. Moreover, sero-grouping does not appear to correlate with genotypes identified using nucleic acid sequence analysis and, with the ever-growing host range, serotyping is likely to have a minor impact on understanding the epidemiology of VHS. Based on nucleic acid sequence analysis, four major genotypes have been identified (52), as shown in Table 1 (discussed later).

The genetic differences appear to be more strongly related to geographical location than to year of isolation or host species (52). The genetic characteristics of VHSV isolates highly pathogenic for
rainbow trout have not yet been defined. All examined isolates from wild marine fish have shown low or no mortality in infection trials by immersion with rainbow trout (7, 12, 34, 36, 49, 53). Significant mortality in farmed turbot has been reported (47) and isolates from European marine fish species are pathogenic for turbot (21) and Atlantic cod (53). While Japanese flounder are highly susceptible to Japanese VHSV isolates (17), it has been shown that rainbow trout are not susceptible to disease when injected experimentally with each of two Japanese isolates of VHSV (18). Mortality from natural infection in free-living marine fish species has been observed along the Pacific coast of North America (14, 32). In addition, isolates from Pacific herring have been shown to be pathogenic for Pacific herring under experimental conditions (23).

Evidence indicates that VHSV survival outside the host is dependent on the virus strain and the physico-chemical conditions of the aqueous medium (45). North American marine strains appear to be more sensitive to freeze–thaw cycles than European freshwater strains (24). Addition of a protein source (e.g. ovarian fluids or serum) provides a protective effect prolonging survival. A marine isolate from maricultured rainbow trout, when diluted in salt water and maintained at 4°C with 1% serum remained infectious for more than 10 months (16). Independent of viral strain, survival is temperature dependent showing an inverse correlation; virus survives for longer periods at 4°C compared with 20°C (45). Temperatures above 20°C are most harmful.

With respect to agent inactivation, VHSV is very sensitive to UVC (280–200 nm wavelength) irradiation, exhibiting a 3-log reduction in infectivity in freshwater at 7.9 ± 1.5 Jm−2. Use of UVC to inactivate VHSV may be useful for treatment of inflow water for hatcheries, but due to the interference from suspended organics treatment of effluent water would not be effective (44).

For disinfection, VHSV is sensitive to a number of common disinfectants (50, 60). Virucidal activity of some of these agents is reduced when diluted in seawater compared with phosphate buffered saline (25).

b) **Host factors**

The number of VHSV-susceptible host species is steadily increasing and includes rainbow trout and other species of trout, Atlantic salmon, several fish species inhabiting freshwater streams, rivers and lakes, and several farmed and wild marine fish species. The number of wild marine species from which VHSV has been isolated is still growing and has led some to conclude that VHSV is endemic in marine or anadromous fish species. The range of fish species from which VHSV has been isolated as well as those shown to be susceptible to VHSV by experimental infection has been reviewed recently (48) and will not be listed here.

The reservoirs of VHSV are clinically infected fish as well as covert carriers among cultured, feral or wild fish. Virulent virus is shed primarily in the urine, whereas kidney, spleen, encephalon and heart are the sites in which virus is most abundant. Survivors of epizootics become lifelong carriers of the virus. The finding that a broad range of wild fish species, in both the marine and freshwater environments can carry the virus provides strong evidence that viral populations can be maintained in the wild and act as a source of infection for farmed fish. Conversely, naïve, wild fish are susceptible to infection with virus derived from farmed fish (9, 20). Thus the bi-directional transfer of virus between farmed and wild fish populations plays a significant role in maintaining viral populations (30).

Several factors influence susceptibility to VHS. Among each fish species, there is individual variability in susceptibility, and the age of the fish appears to be of some importance – the younger the fish the higher the susceptibility. All ages of fish are susceptible to infection, but younger fish are more susceptible than older fish, probably due to maturation of the innate immune system and/or the development of at least some immunity in older fish following prior exposure. In highly susceptible fish stocks, however, overt infection is seen in all sizes of fish.
c) **Disease pattern**

Knowledge of the mechanism of virus transmission has come mainly from studies on European freshwater VHSV isolates that have shown transmission to be horizontal through contact with other infected fish or contaminated water, fomites etc. Virus is shed from infected fish via the urine (39) and reproductive fluids primarily and can also be transferred by piscivorous birds (46). Transmission occurs in the temperature range 1–12°C and incubation time is 1–2 weeks at the higher temperatures and 3–4 weeks at the lower temperatures within this range.

During and immediately following an epizootic (which can involve mass mortality) in wild (32) or farmed populations in freshwater (31) or marine environments (14, 17), virus can be isolated readily in cell culture. Kidney and spleen tissues yield the highest viral titres. In carrier (clinically healthy) fish (30), detection of VHSV is more problematical. While VHSV will grow in a range of fish cell lines, the BF-2 cell line appears to be highly sensitive to infection by freshwater European strains (42). However, susceptibility of a cell line to infection will be dependent on a range of parameters including cell line lineage and viral strain differences (14, 17).

The carrier status of VHSV in freshwater fish species is well established (9, 20). The virological status of such carriers will be dependent on a range of parameters including the length of time following initial exposure and geographical proximity to fish-farm outlets. Reported prevalence of carriers, based on detection of anti-VHSV antibodies, is in the range of 6–67% (10, 32). More recently, since the discovery of VHSV strains in marine species, there have been a number of studies involving extensive sampling of a broad range of fish species from the coastal waters of continental Europe (3), the UK (22), North America (14, 36) and Japan (55). In these studies, samples were analysed for the presence of virus by inoculation on to fish cell lines. In some studies, fish samples were pooled and therefore determination of precise prevalence is difficult. Nevertheless, based on virus isolation in cell culture and irrespective of fish species examined, the prevalence of VHSV in the marine fish species sampled in these studies was in the range of 0.02–4.7%.

Until the late 1980s, VHS was considered to be restricted to farmed rainbow trout in continental Europe, with the occasional isolation from a restricted number of other freshwater fish species (e.g. brown trout, pike [19, 29]) with Great Britain, Northern Ireland and Ireland free of VHS. With the detection and isolation of VHSV from Pacific salmon off the Pacific North American coast in the late 1980s (4, 15, 58), subsequent studies have demonstrated that VHSV occurs in numerous farmed and wild fish species along the Pacific and Atlantic North American coast (8, 14, 32, 33, 56), the seas around the UK (7, 22, 51), the Baltic Sea, Skagerrak and Kattegat (36), and in the waters around Japan (55).

A recent study (52) has demonstrated that there are four main genotypes of VHSV that correlate with geographical distribution (Table 1). Ability to distinguish these four genotypes will be important to future disease management.

*Table 1. Viral haemorrhagic septicaemia virus genotypes*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>Mainly isolates from farmed rainbow trout in continental Europe</td>
</tr>
<tr>
<td>Ib</td>
<td>Mainly isolates from marine hosts in the Baltic Sea, Skagerrak and Kattegat, related to Ia</td>
</tr>
<tr>
<td>II</td>
<td>Isolates from marine hosts in the Baltic Sea, unlike Ib, with no strong link to Ia</td>
</tr>
<tr>
<td>III</td>
<td>Mainly isolates from around the British Isles</td>
</tr>
<tr>
<td>IV</td>
<td>Isolates from the Pacific Northwest (North American) and Japan</td>
</tr>
</tbody>
</table>

Water temperature is an important environmental factor. Disease generally occurs at temperatures between 4°C and 14°C. Temperatures above 15°C are inhibitory to virus growth (6). At higher temperatures, disease rarely occurs (60). Low water temperatures (1–5°C) generally result in an extended disease course with low daily mortality but high accumulated mortality. VHS outbreaks
occur during all seasons, but are most common in spring when water temperatures are rising or fluctuating. For more detailed descriptions of the condition, see reviews by Wolf (60) and Smail (50).

As with other viral diseases of fish, other predisposing factors include stress and fish age. VHSV can remain covert and disease can be precipitated following a stress event (16). Severity of disease is inversely proportional to fish age; young fish (e.g. rainbow trout fry) are more susceptible to disease and associated mortality than older fish (e.g. fingerlings and growers).

d) Control and prevention

In the absence of anti-viral agents, control methods for VHS currently lie in official health surveillance schemes coupled with control policy measures. Examples of practices that have been successful in reducing the number of infected farms in an endemic area and preventing reinfection, such as stamping-out and falling procedures, have been reviewed previously (40). Methods to reduce the impact of the disease rely on the prevention of contact between the virus and host species (hatchery disinfection, use of specific-pathogen free [SPF] stock, use of spring or bore water) as well as reduction in physiological stressors.

While mechanisms of immunity in fish are not fully understood (28), immune resistance to VHSV due to prior exposure of fish to virus has been well-established (60). However, although research on vaccine development for VHS has been ongoing for three decades or more, a commercial vaccine is not yet available.

3. DIAGNOSTIC METHODS

a) Field diagnostic methods

• Clinical signs

While there are no VHS-specific gross clinical signs, diseased fish exhibit those signs typical of systemic infection and such signs will vary depending on the severity and stage of infection.

Acute phase: rapid onset of mortality, lethargy, darkening of skin, exophthalmia, anaemia (pale gills), haemorrhages at the base of fins, gills, eyes and skin, abnormal swimming such as flashing and spiralling, distended abdomen due to oedema in peritoneal cavity.

Chronic phase: no obvious external signs

Nervous Form: due to the tropism of the virus for the brain, the nervous form is characterised by severe abnormal swimming behaviour such as constant flashing and spiralling.

Latent infections: no external signs.

b) Clinical methods

• Gross pathology: the kidney and liver are markedly affected. The kidney is dark red in the acute phase but can demonstrate severe necrosis in moribund fish. The liver is pale and mottled. There can be a generalised petechial haemorrhaging in the skin, muscle tissue and internal organs. Gastrointestinal tract is devoid of food.

• Microscopic pathology: the kidney, liver and spleen show extensive focal necrosis and degeneration – cytoplasmic vacuoles, pyknosis, karyolysis, lymphocytic invasion. While the skeletal muscle does not appear to be a site of infection, erythrocytes can accumulate in the skeletal muscle bundles and fibres without causing damage to the muscle per se (50).
• **Electron microscopy/cytopathology:** VHSV is a typical bullet-shaped rhabdovirus, 60–75 nm in diameter and 180–240 nm in length. Ultrastructural aspects of the development of viral infection in cell culture have been described previously (2).

c) **Agent detection and identification methods**

The standard surveillance method (to detect carrier fish) for VHS is based on direct methods, i.e. the isolation of VHSV in cell culture followed by identification using antibody-based methods (indirect fluorescent antibody test [IFAT], enzyme-linked immunosorbent assay [ELISA]) or nucleic acid-based methods (e.g. reverse-transcription polymerase chain reaction [RT-PCR]). Due to low sensitivity, the direct immunological (by antibody-binding assay) demonstration of VHSV antigen in infected fish tissues can only be used when VHS infection is suspected (based on clinical signs, epidemiological data and histopathology). PCR-based technology using direct detection of VHSV-specific nucleotide sequences in fish tissue is under development (5, 13). The technique can be used for confirmation of overt infection in fish, but has not yet been validated for use in direct surveillance programmes for obtaining approved VHS-free status.

• **Direct detection methods**

  i) **Microscopic methods**

  The kidney and liver are prime targets and examination of histological sections from diseased fish reveals degeneration and necrosis of haematopoietic tissues of the kidney (and the spleen) and focal degeneration and necrosis of the liver. Sections of the skeletal muscle may show many foci of red blood cells, while the muscle fibres remain undamaged.

  ii) **Agent isolation and identification**

    • **Cell culture**

    Isolation of VHS virus in cultures of a number of established fish cell lines is well-documented (27, 42). While inoculation of fish cell lines with fish tissues processed for virus isolation is considered the ‘Gold’ Standard for surveillance programmes (to detect carrier fish) with respect to sensitivity, the precise sensitivity of the procedure is unknown. Infected fish material suitable for virological examination is dependent on fish size. Thus whole alevin (body length <4 cm), viscera including kidney (4 cm < body length < 6 cm) or, for larger size fish, kidney, spleen, heart and encephalon, and ovarian fluid from broodfish at time of spawning are suitable samples.

    The fish cell lines BF-2 and RTG-2 are recommended. Alternatively, EPC or FHM cells may be used, but are in general less susceptible than BF-2 and RTG-2 (27, 42).

    Detection of virus through the development of viral cytopathic effect (CPE) in cell culture would be followed by virus identification through either antibody-based tests or nucleic acid-based tests. Any antibody-based tests would require the use of antibodies validated for their sensitivity and specificity (1). With the proliferation of VHS viral isolates from marine fish species there is a potential risk that some antibody preparations will not detect all isolates.

    a) **Virus extraction**

    Use the procedure described in Chapter I.1 (Section B.3.2).

    b) **Inoculation of cell monolayers**

    i) Prior to inoculation of cells, the supernatant is mixed with equal parts of a suitably diluted pool of antisera to the indigenous serotypes of infectious
pancreatic necrosis virus (IPNV) and incubated for a minimum of 1 hour at 15°C or for 12–18 hours at 4°C. The titre of the antiserum must be at least 1/2000 in a 50% plaque neutralisation test.

ii) Treatment of all inocula with antiserum to IPNV (a virus that in some parts of the world occurs in 50% of fish samples) aims at preventing CPE due to IPNV from developing in inoculated cell cultures. This will reduce the duration of virological examinations as well as the number of cases in which the occurrence of CPE would have to be considered potentially indicative of VHS.

iii) When samples come from production units that are considered to be free from IPNV, treatment of inocula with antiserum to IPNV may be omitted.

iv) Transfer organ homogenate supernatant on to 24-hour-old monolayers overlaid with cell culture medium containing 2–10% fetal calf serum (FCS) and suitable buffer (e.g. Tris or HEPES [N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid]) in two dilutions, i.e. the primary dilution and, in addition, a 1/10 dilution thereof, resulting in final dilutions of tissue material in cell culture medium of 1/100 and 1/1000, respectively (in order to prevent homologous interference). The ratio of inoculum size to volume of cell culture medium should be about 1:10.

v) For each dilution a minimum of about 2 cm² cell area, corresponding to one well in a 24-well cell culture tray, must be used. Use of cell culture trays is recommended, but other units of similar or bigger growth area may be used instead.

c) Monitoring incubation

i) Follow the course of infection in positive controls and other inoculated cell cultures by daily microscopic examination at ×40–100 magnification for 7 days. The use of a phase-contrast microscope is recommended. If no positive controls are included or if decreased cell susceptibility is suspected, titration of frozen stocks of VHSV shall be performed at least every 6 months to verify the susceptibility of the cell cultures to infection.

ii) Inoculated cell cultures shall be incubated at 15°C for 7–10 days and be inspected regularly (at least three times a week) for the occurrence of CPE at ×40–100 magnification.

iii) It is important to maintain the pH of the cell culture medium at between 7.3 and 7.6 during incubation. This is achieved by the addition of sterile bicarbonate buffer (for tightly closed cell culture flasks or plates in an atmosphere of 5% CO₂/95% air) or 2 M Tris buffer solution (for cell culture plates) to the cell culture medium or, preferably, by using HEPES-buffered medium.

iv) If CPE appears in cell cultures inoculated with dilutions of the homogenate, identification procedures must be undertaken immediately (see virus identification below).

v) If no CPE has developed after the primary incubation for 7–10 days, subcultivation is performed to fresh cell cultures using a cell area similar to that of the primary culture.

d) Subcultivation procedures

i) Aliquots of medium (supernatant) from all cultures/wells constituting the primary culture are pooled according to cell line 7–10 days after inoculation.
ii) The pools are then inoculated into homologous cell cultures undiluted and
diluted 1/10 (resulting in final dilutions of 1/10 and 1/100, respectively, of the
supernatant) as previously described.

iii) The inoculation may be preceded by preincubation of the dilutions with IPN
antiserum at appropriate dilution as described in Chapter I.1 Section B.1.

iv) Alternatively, aliquots of 10% of the medium constituting the primary culture
are inoculated directly into a well with fresh cell culture (well-to-well
subcultivation).

v) The inoculated cultures are then incubated for 7 days with observation as
described for the primary inoculation.

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

• Virus identification

  a) Neutralisation test

  i) Collect the culture medium of the cell monolayers exhibiting CPE and
  centrifuge it at 2000 g for 15 minutes at 4°C, or filter through a 45-µm pore
  membrane to remove cell debris.

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

  iii) Mix aliquots (for example 200 µl) of each dilution with equal volumes of a
  VHSV antibody solution, and likewise treat aliquots of each virus dilution with
  cell culture medium. (The neutralising antibody [NAb] solution must have a
  50% plaque reduction titre of at least 2000.)

  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) If required, a similar neutralisation test may be performed using antibodies to
  IPNV.

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

  vii) Transfer aliquots of each of the above mixtures on to 24-hour-old monolayers
  overlaid with cell culture medium containing 10% FCS (inoculate two wells per
  dilution) and incubate at 15°C; 24- or 12-well cell culture plates are suitable for
  this purpose, using a 50 µl inoculum.

  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 the cell culture
  medium and staining cell monolayers with a solution of 1% crystal violet in
  20% ethanol.

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

  x) In the absence of any neutralisation by NAb to VHSV, it is mandatory to
  conduct an indirect fluorescent antibody test (IFAT), an immunoperoxidase
test, an enzyme-linked immunosorbent assay (ELISA) or RT-PCR using the
suspect sample. Some cases of antigenic drift of surface antigen have been
observed, resulting in occasional failure of the neutralisation test using NAb to
VHSV.
Other neutralisation tests of proven efficiency may be used alternatively.

**b) Indirect fluorescent antibody test**

i) Prepare monolayers of cells in 2 cm$^2$ wells of cell culture plastic plates or on cover-slips in order to reach around 80% confluency, which is usually achieved within 24 hours of incubation at 22°C (seed six cell monolayers per virus isolate to be identified, plus two for positive and two 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 tenfold 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 5000–10,000 plaque-forming units (PFU)/ml in the cell culture medium.

iv) Incubate at 15°C for 24 hours.

v) Remove the cell culture medium, rinse once with 0.01 M phosphate buffered saline (PBS), pH 7.2, then three times briefly with a cold mixture of acetone 30%/ethanol 70% (v/v) (stored at −20°C).

vi) Let the fixative act for 15 minutes. 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 minutes and process immediately or freeze at −20°C.

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

ix) Rehydrate the dried cell monolayers by four rinsing steps with the PBST solution, and remove this buffer completely after the last rinsing.

x) Treat the cell monolayers with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur, e.g. by adding a piece of wet cotton to the humid chamber. The volume of solution to be used is 0.25 ml/2 cm$^2$ well.

xi) Rinse four times with PBST as above.

xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate (FITC)- or tetramethylrhodamine-5-(and-6-)isothiocyanate (TRITC)-conjugated antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These conjugated antibodies are most often rabbit or goat antibodies.

xiii) Rinse four times with PBST.

xiv) Examine the treated cell monolayers on plastic plates immediately, or mount the cover-slips using, for example glycerol saline, pH 8.5 prior to microscopic observation.

xv) Examine under incident UV light using a microscope with × 10 eye pieces and × 20–40 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.
Other IFAT or immunocytochemical (alkaline phosphatase or peroxidase) techniques of proven efficiency may be used alternatively.

c) Enzyme-linked immunosorbent assay
   i) Coat the wells of microtitre plates designed for ELISAs with appropriate dilutions of purified immunoglobulins (Ig) or serum specific for VHSV, in 0.01 M PBS, pH 7.2 (200 µl/well).
   ii) Incubate overnight at 4°C.
   iii) Rinse four times with 0.01 M PBS 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 four times with PBST.
   vi) Add 2% Triton X-100 to the virus suspension to be identified.
   vii) Dispense 100 µl/well of two- or four-step dilutions of the virus to be identified and of VHSV control virus, and negative control (e.g. infectious haematopoietic necrosis virus) and allow to react with the coated antibody to VHSV for 1 hour at 20°C.
   viii) Rinse four times with PBST.
   ix) Add to the wells either biotinylated polyclonal VHSV antiserum or MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin.
   x) Incubate for 1 hour at 37°C.
   xi) Rinse four times with PBST.
   xii) Add streptavidin-conjugated horseradish peroxidase to those wells that have received the biotin-conjugated antibody, and incubate for 1 hour at 20°C.
   xiii) Rinse four times with PBST. Add the substrate and chromogen. Stop the course of the test when positive controls react, and read the results.
   xiv) Interpretations of the results is according to the optical absorbencies achieved by negative and positive controls and must follow the guidelines for each test, e.g. absorbency at 450 nm of positive control must be minimum 5–10 × A450 of negative control. Absorbencies > 3 × A450 of negative controls are regarded as positives. Alternatively, two standard deviations above the mean of the control tissues can be used.

The above biotin–avidin-based ELISA version is given as an example (37). Other ELISA versions of proven efficiency may be used instead (38, 41).

d) Reverse-transcription polymerase chain reaction
   To avoid cross-contamination and exposure to RNAses, all work with RNA should be performed under an appropriate hood and using gloves.
   i) Isolation of RNA: Total RNA from infected cells in suspension (cell culture medium) is extracted using the phenol-chloroform method or by RNA affinity spin columns, e.g. RNeasy Total RNA kit (Qiagen, Germany), according to the manufacturer’s instructions. RNA must be resuspended in distilled RNase-free water (e.g. water treated with 0.1% diethyl pyrocarbonate.
   ii) RT-PCR: The RT-PCR amplification can be performed in one or two step(s) using a primer set that binds to a conserved region within the nucleocapsid (N): 5'-GGG-GAC-CCC-AGA-CTG-T-3’ (forward primer) and 5'-TCT-CTG-
TCA-CCT-TGA-TCC-3’ (reverse primer). The resulting amplicon is 811 base pairs (bp).

iii) One-step RT-PCR: 50 µl single tube RT-PCR can be performed using Titan One Tube RT-PCR System (Roche, Germany) according to the manufacturer’s instructions. Briefly, the PCR reaction mixture consists of: 5 µl of extracted viral RNA (approximately 0.5–2 µg), 50 pmol of each primer, 1 µl 10 mM dNTP, 10 µl 5 × RT-PCR reaction buffer (with 7.5 mM MgCl2 and dimethyl sulfoxide), 2.5 µl 100 mM DTT, and 1 µl enzyme mix. The following cycles are recommended: 50°C for 30 minutes, 94°C for 2 minutes, 35 cycles at 94°C for 30 seconds, 52°C for 30 seconds, 68°C for 60 seconds; the RNA reaction is finally held at 68°C for 7 minutes.

iv) Two-step RT-PCR: The cDNA mixture consists of 5 µl of the extracted viral RNA (approximately 0.5–2 µg), 50 pmol forward primer, 1 × reverse transcript buffer (with 50 mM Tris/HCl, pH 8.3, 3 mM MgCl2, 75 mM KCl, 10 mM), 1 mM mixed dNTP, 5 U reverse transcriptase (StrataScript RNase H-reverse transcriptase, Stratagene, USA) adjusted with H2O to a final volume of 20 µl. Reverse transcription should be performed at 42°C for 30 minutes, 94°C for 5 minutes and cooled at 4–10°C for 3 minutes.

v) The master mixture for subsequent PCR amplification is prepared on 5 µl of the cDNA reaction, 50 pmol of each primer, 1 × Taq polymerase buffer (with 10 mM Tris/HCl, pH 9, 1.5 mM MgCl2, 50 mM KCl, 0.01% gelatin, 0.1% Triton X-100), 1 mM mixed dNTP, 1.5 U Taq polymerase and adjusted with H2O up to final volume of 50 µl. The subsequent PCR is performed by the following cycles: 94°C for 2 minutes, 35 cycles at 94°C for 30 seconds, 52°C for 30 seconds, 68°C for 60 seconds, and is finally held at 68°C for 7 minutes.

vi) Quantity and specificity of the RT-PCR reactions can be evaluated by 1% agarose gel electrophoresis of 1/10 reaction in 1.5% agarose gel with ethidium bromide and observed using UV transillumination.

Please note: The thermal protocols may need optimisation, depending on the thermal cycler in use.

There are a number of effective PCR methods available (52, 59) of which this is one. Newer diagnostic PCR tests, some with specific improvements, are being developed on a fairly regular basis but few, if any, undergo systematic validation as recommended in Chapter 1.1.3 of this Aquatic Manual, before being superseded. Other PCR tests of proven efficiency may be used instead

- Antibody-based antigen detection methods

Antibody-based antigen detection methods such as IFAT, ELISA and various immunohistochromical procedures for the detection of VHSV have been developed over the years (38). It is generally accepted that the prime target organs are kidney and liver and the use of tissue imprints or tissue sections can play an important diagnostic role during the acute phase of the disease (11). These techniques can provide detection and identification relatively quickly compared with virus isolation in cell culture. However, various parameters such as antibody sensitivity and specificity and sample preparation can influence the results; a negative result should be viewed with caution. These techniques should not be used in attempts to detect carrier fish.

- 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 Chapter I.1. Section B.3.1) together with the other organs required for virus isolation in case this becomes necessary later.

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

v) Fix with acetone or ethanol/acetone and dry as indicated in Section 3.c.ii Virus identification, b, steps v–vii.

vi) Rehydrate the above preparations (see Section 3.c.ii Virus identification, b, step ix) and block with 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C.

vii) Rinse four times with PBST.

viii) Treat the imprints with the solution of antibody to VHSV and rinse as indicated in Section 3.c.ii Virus identification, b.

ix) Block and rinse as described previously in steps vi and vii.

x) Reveal the reaction with suitable FITC-conjugated specific antibody, rinse and observe as indicated in Section 3.c.ii Virus identification, b, steps xii–xv.

xi) If the test is negative, process the organ samples stored at 4°C for virus isolation in cell culture as described above.

• **Enzyme-linked immunosorbent assay (38)**

  a) **Sampling procedures**

  See the following sections in Chapter I.1:
  B.1. for the selection of fish specimens
  B.2. for the selection of materials sampled.

  b) **Processing of organ samples**

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

  c) **The enzyme-linked immunosorbent assay procedure**

    i) Microtitre plate processing is described in Section 3.c.ii Virus identification, c of this chapter up to step iv (inclusive).

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

    iii) Treat the remaining part of the homogenate with 2% Triton X-100 as in Section 3.c.ii Virus identification, c, step vi, and 2 mM of phenyl methyl sulfonide fluoride; mix gently.

    iv) Complete the other steps (vii–xiv) of the procedure described in Section 3.c.ii Virus identification, c.

    v) If the test is negative, process the organ samples stored at 4°C for virus isolation in cell culture as described above.

The above biotin–avidin-based ELISA version is given as an example. Other ELISA versions of proven efficiency may be used instead (41).

• **Molecular techniques**

Use of molecular tests (RT-PCR and real-time PCR) is becoming more common due to their rapidity and sensitivity (35). In addition, with the discovery of the Pacific North
American marine strain and, subsequently, European and Atlantic marine strains, sequencing of PCR products can provide important epidemiological data (52, 54). While the use of these tests for virus detection and identification during the acute stage of disease may be justified, their sensitivity compared with virus isolation by culture needs to be established by further research to determine whether application to surveillance programmes could be developed.

- **Indirect detection methods**

- **Serological methods**

  Due to insufficient knowledge of the serology of virus infections of fish, the detection of fish antibodies to viruses has thus far not been accepted as a routine diagnostic method for assessing the viral status of fish populations (26, 28).

### 4. RATING OF TESTS AGAINST PURPOSE OF USE

The methods currently available for surveillance, detection, and diagnosis of VHSV are listed in Table 2. The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic sensitivity and specificity; C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and D = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation (see Chapter 1.1.2), their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

#### Table 2. *Viral haemorrhagic septicaemia* virus surveillance, detection and diagnostic methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance</th>
<th>Presumptive</th>
<th>Confirmatory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fry</td>
<td>Growers</td>
<td>Broodstock</td>
</tr>
<tr>
<td>1. Gross signs</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>2. Histopathology</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>3. Transmission EM</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>4. Isolation in cell culture followed by identification by method 5 or 6 below</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>5. Antibody-based assays</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>6. PCR followed by sequencing</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
</tbody>
</table>

EM = electron microscopy; PCR = polymerase chain reaction.

### 5. CORROBORATIVE DIAGNOSTIC CRITERIA

a) **Definition of suspect case**

The presence of VHSV shall be suspected if:

i) The diseased fish is a susceptible host species (realising that it is likely that not all susceptible species are known. The known marine host range is rapidly expanding).
Water temperature is in the 1–18°C range (disease outbreaks at temperatures above 18°C are unknown). For farmed rainbow trout, VHS outbreaks appear to be more common during the spring when temperatures are low but on the rise or fluctuating.

Increased mortality rate: mortality rates can be high in all age groups. Severity of disease is inversely proportional to fish age and mortality rates in fry can approach 100% (lower in older fish).

Fish are demonstrating common clinical signs for systemic infection, one or more of the following: lethargy, darkening of skin, exophthalmia, anaemia (pale gills), haemorrhages at base of fins, gills, eyes and skin, abnormal swimming such as flashing and spiralling, distended abdomen due to oedema in peritoneal cavity.

Gross pathology: kidney is dark red in the acute phase but can demonstrate severe necrosis in moribund fish. Liver is pale and mottled. There can be a generalised petechial haemorrhaging in the skin, muscle tissue and internal organs. Gastrointestinal tract is devoid of food.

Histopathology: kidney, liver and spleen show extensive focal necrosis and degeneration – cytoplasmic vacuoles, pyknosis, karyolysis, lymphocytic invasion. While skeletal muscle does not appear to be a site of infection, erythrocytes can accumulate in skeletal muscle bundles and fibres without causing damage to the muscle per se.

**OR**

ii) Isolation and identification of VHSV in cell culture from a single sample of fish tissues as described in Section 3.c).

**OR**

iii) Presence of VHSV is indicated by results from two independent laboratory tests such as RT-PCR and IFAT as described in Section 3.c).

**b) Definition of confirmed case**

The presence of VHSV shall be considered as confirmed if, in addition to the criteria in Section 5.a.i, one or more of the following criteria are met:

i) VHS virus isolation in cell culture as evident by viral cytopathic effect typical of VHSV infection followed by virus identification by either antibody-based test (IFAT, ELISA, immunocytochemistry) and/or PCR followed by sequencing of the amplicon,

ii) VHSV is detected in tissues or tissue preparations by immunoassay using specific anti-VHSV antibodies,

iii) Detection of VHSV by RT-PCR followed by sequencing of the amplicon.

Alternatively, in the absence of clinical signs and post-mortem findings consistent with VHS, VHSV presence shall be considered as confirmed if one of the following criteria is met:

i) VHSV is isolated and identified, as described in Section 3.c, in two samples from one or more fish tested on separate occasions;

ii) VHSV is isolated and identified, as described in Section 3.c, from a fish and VHS virus is detected in fish tissues from any fish by immunoassay or RT-PCR followed by sequencing of the amplicon.

**6. DIAGNOSTIC/DETECTION METHODS TO DECLARE FREEDOM**

Using appropriate procedures as described in Chapter 1.1.4:

No VHS virus isolated on cell culture.
REFERENCES


*  
*  *

**NB:** There is an OIE Reference Laboratory for Viral haemorrhagic septicaemia (see Table at the end of this Aquatic Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
NB: This disease is no longer listed in chapter 1.2.3 of the Aquatic Code. This chapter has not been updated since 2003

CHAPTER 2.1.6.

CHANNEL CATFISH VIRUS DISEASE

SUMMARY

Channel catfish virus disease (CCVD) is caused by a herpesvirus designated Ictalurid herpesvirus 1 by the International Committee on Taxonomy of Viruses, but the commonly used name is channel catfish virus (CCV). CCV affects channel catfish (Ictalurus punctatus) in the United States of America. For more detailed reviews of the condition, see Wolf (22) or Plumb (15).

CCVD is of importance because of its clinical and economic consequences in channel catfish farming. CCVD results in high mortality rates in populations of fry and juvenile catfish. Diseased fish demonstrate ascites, exophthalmia and haemorrhage in fins and musculature. Histologically the most remarkable damage occurs in the kidney with extensive necrosis of renal tubules and interstitial tissue.

In survivors, CCVD results in a strong protective immunity, the synthesis of circulating antibodies to the virus and, a covert latent carrier state. During this latent carrier state the virus is undetectable by traditional culture or antigen-detection means, even when adults are immunosuppressed during spawning.

On the basis of antigenic studies conducted with polyclonal rabbit antibodies, CCV isolates form a homogeneous group. However, the use of monoclonal antibodies shows some variation in antigenic determinants between isolates (1). Some variation in the virulence of CCV strains has been recorded during natural outbreaks of disease and has been demonstrated experimentally. Additionally, molecular data indicate genetic variation within this species (7, 18).

Reservoirs of CCV are clinically infected fish and covert carriers. Infectious CCV can be detected in the water from tanks of experimentally infected fish, but the route of shedding has not been determined. The sites where the virus is most abundant during the course of overt infection are posterior kidney, skin, gills, spleen and intestine, respectively, in decreasing magnitude (12, 13). The transmission of CCV is horizontal and vertical. Horizontal transmission may be direct or vectorial with water being the main abiotic vector. The virus has been shown to readily adsorb to pond sediments (6) and interaction with suspended clay particles in pond water may influence horizontal transmission. Animate vectors and inanimate objects could also act in CCV transmission. Vertical transmission is thought to be common, but the mechanism of vertical transmission is not known, as infectious virus has not been detected on the skin or in the sexual products of spawning adults. Once CCVD occurs in a fish population, survivors of the disease become covert carrier fish.

Channel catfish and the closely related blue catfish (Ictalurus furcatus) have been the only fish found to be infected with CCV, and variations in susceptibility to CCV have been recorded depending on fish strain. The age of the fish is extremely important for overt infection. Although experimental data suggest that older fish are susceptible to natural outbreaks of acute CCVD (11), the disease occurs almost exclusively in fish that are less than 1 year of age, and generally less than 4 months of age. Water temperature is the critical environmental factor. The mortality rate is high above 27°C, but readily decreases and ceases below 18°C.

Diagnosis of CCVD is based on virus isolation in cell culture. Confirmatory testing is by immunological identification by neutralisation, immunofluorescence, enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR). Rapid techniques by immunofluorescence tests or ELISA are suitable mainly for diagnosis in clinically infected fish. Because virus proteins or infectious virus is not produced,
control methods or antigen-based testing is of little use for carrier screening. Instead, detection of neutralising antibodies in a population of fish and, more recently, the use of PCR to detect latent CCV genomic DNA is of more use.

Control methods currently rely on maintaining relatively low stocking densities and avoiding stressful handling of young fish during the summer months. Also, control policies and hygiene practices have been used, where practical, in catfish husbandry. The incubation of eggs and rearing of fry and juveniles in facilities separated from carrier populations are critical to preventing the occurrence of CCVD in a CCV-free fish production site. Because virus is only detected during active outbreaks, defining CCV-free status has been done largely from historical data or identifying populations that are seronegative to the virus. Recent use of PCR and hybridisation probes to detect latent CCV genomic DNA suggests that CCV is present in many populations that have no history of the disease (2, 5, 9, 21). Vaccination, although experimentally promising (19, 20, 23, 24), is not in use at this time.

**DIAGNOSTIC PROCEDURES**

The diagnosis of channel catfish virus disease (CCVD) is generally based on the isolation of channel catfish virus (CCV) in cell culture followed by its immunological or nucleic-acid-based identification (conventional approach). Alternatively, the immunological demonstration of CCV antigen in infected fish tissues can be used. The conventional approach is most common because the virus produces rapid cytopathic effect (CPE) in cell culture and there are no commercial sources of CCV-specific antiserum, and custom-produced antisera to CCV is often of low titre or has cross-reaction with fish tissue.

Due to insufficient knowledge of the serology of fish virus infections, the detection of fish antibodies to viruses has not yet been recognised as a valuable diagnostic method for assessing the viral status of fish populations. However, the use of direct culture or detection of viral antigen is of little use in detecting carrier fish. Therefore, the identification of antibodies to CCV has more merit in screening carrier populations (8, 14). The antibody titres in carrier populations vary seasonally, with the lowest titres occurring in the late winter and early spring (3). 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:

- **Clinically affected fish:** Whole fry or small juveniles (body length ≤ 3 cm), viscera including kidney (3 cm ≤ body length ≤ 6 cm) or, for larger size fish, kidney, and spleen.

- **There is no definitive test for asymptomatic fish** (apparently healthy fish): Cell culture or antigen detection of latent virus is not possible. Occasional recrudescence of latent infection has been detected in adult catfish after spawning (16) or during winter months or after dexamethazone treatment (4), but these methods are not reliable enough for inspection purposes. Detection of latent virus DNA can be done using polymerase chain reaction (PCR) on kidney, fin or gill tissue (2, 5, 10). However, these tests were developed on experimentally infected fish and have not been applied to production systems. Therefore, their use or reliability in detecting CCV in populations or predicting the potential for vertical transmission has not been documented.

**Sampling procedures:** see Chapter I.1. Section B.
1. **STANDARD SCREENING METHOD FOR CCVD**

1.1. Isolation of CCV in cell culture

**Cell line to be used: CCO**

*a) Inoculation of cell monolayers*

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

ii) Allow virus to adsorb for 0.5–1 hour at 25–30°C. Then (without withdrawing the inoculum), add the cell culture medium buffered at pH 7.6 and supplemented with 10% fetal calf serum (FCS) (1 ml/well for 24-well drained 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 ×40–100 magnification for 10 days. The use of a phase-contrast microscope is recommended. CPE is extensive and rapidly progressing in cultures from overtly diseased individuals. CPE consists of cell fusion (syncytium) formation and contraction leaving cytoplasmic spindles irradiating from the syncytium to points on the flask surface where the cells were originally attached.

ii) Maintain the pH of the cell culture medium at between 7.3 and 7.6 during incubation. This can be achieved by the addition to the inoculated cell culture medium of bicarbonate buffer (for tightly closed cell culture flasks or in a CO₂ incubator), 2 M Tris buffer solution or by using 25 mM HEPES-buffered medium (HEPES = N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid).

iii) If CPE appears in those cell cultures inoculated with the dilutions of the tested homogenate supernatants, identification procedures must be undertaken immediately (see Section 1.2. below).

If a fish health surveillance/control programme is being implemented, provisions may 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 develops in the inoculated cultures (despite normal progression of CPE in the virus controls), the inoculated cultures should be subcultured for a further 7 days. Should the sample inoculated with the virus control fail to develop CPE, the process should be repeated with fresh susceptible cells and new batches of samples.

*c) Subcultivation procedures*

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

ii) Inoculate cell monolayers as described above in Section 1.1.a.

iii) Incubate and monitor as described in Section 1.1.b.

iv) Perform a second (and last) subcultivation step if the first one remains virus-negative.
1.2. Identification of CCV

a) Neutralisation test (Note: when developing antisera for CCV most researchers have found a weak neutralising antibody response in rabbits; cross-reaction with cellular components also often occurs)

i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge at 2000 g for 15 minutes at 4°C to remove cell debris.

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 neutralising antibody [NAb] solution must have a 50% plaque reduction titre of at least 2000.)

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

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

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

vi) Transfer aliquots of each of the above mixtures on to cell monolayers (inoculate two cell cultures per dilution) and allow adsorption to occur for 0.5–1 hour at 25°C; 24- or 12-well cell culture plates are suitable for this purpose, using a 50 µl inoculum.

vii) When adsorption is complete, add the 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 the cell culture medium and staining the 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 that 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, conduct an indirect fluorescent antibody test (IFAT) with the suspect sample, perform an enzyme-linked immunosorbent assay (ELISA) or use a CCV-specific nucleic-acid-based assay.

b) Indirect fluorescent antibody test

i) Prepare monolayers of cells in 2 cm² wells of cell culture plastic plates or on cover-slips in order to reach around 80% confluency, which is usually achieved within 4 hours of incubation at 30°C (seed six cell monolayers per virus isolate to be identified, plus two for positive and two for negative controls).

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 tenfold 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 5000–10,000 plaque-forming units (PFU)/ml in the cell culture medium.

iv) Incubate at 25°C for 18 hours.

v) Remove the cell culture medium, rinse once with 0.01 M phosphate buffered saline (PBS), pH 7.2, then three times briefly with cold acetone (stored at −20°C) for cover-slips or a mixture of acetone 30%/ethanol 70%, also at −20°C, for plastic wells.
vi) Let the fixative act for 15 minutes. 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 minutes and process immediately or freeze at –20°C.

viii) Prepare a solution of purified antibody or serum to CCV in 0.01 M PBS, pH 7.2, containing 0.05% Tween 80 (PBST), at the appropriate dilution (which has been established previously, there is no commercial source for CCV-specific antiserum).

ix) Rehydrate the dried cell monolayers by four rinsing steps with the PBST solution, and remove this buffer completely after the last rinsing.

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

xi) Rinse four times with PBST.

xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate (FITC)-conjugated 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 four times with PBST.

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

xv) Examine under incident UV light using a microscope with ×10 eye pieces and ×20–40 objective lens having a high numerical aperture. Positive and negative controls must be found to give the expected results prior to any other observation.

c) Enzyme-linked immunosorbent assay

i) Coat the wells of microplates designed for ELISAs with appropriate dilutions of purified immunoglobulins (Ig) or serum specific for CCV, in 0.01 M PBS, 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 four times with 0.01 M PBS 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 four times with PBST.

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

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

viii) Rinse four times with PBST.

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

x) Incubate for 1 hour at 37°C.

xi) Rinse four times with PBST.

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

xiii) Rinse four times with PBST.
xiv) Add the substrate and chromogen. Stop the course of the test when positive controls react, and monitor the results.

d) Polymerase chain reaction

There are several published PCR assays for CCV (2, 5, 10). As stated in Chapter I.1. Section C.3.5., PCR is very susceptible to false-positive and false-negative results. Therefore each assay and tissue extraction should include a negative control to rule out contamination. PCR reaction set-up should be done in a separate physical location from where the PCR products are evaluated. PCR is a useful method for identifying the virus after isolation in culture. The following is a modification of the method of Boyle & Blackwell (5) and uses a modified target as an internal control (12). The 5’–3’ sequence of the upper primer is TCA-TCC-GAA-TCC-GAC-AAC-TGA and that of the lower primer is CCA-AGA-TCG-CGG-AGA-AAC. To minimise the potential for contamination, all sample preparation and reaction set-up should be done with aerosol-preventing pipette tips.

- Sample preparation

i) Collect 1–0.5 ml of cell culture supernatant from affected wells into a 1.5 ml microcentrifuge tube.

ii) Centrifuge at maximum speed in microfuge (18,000–20,000 g) for 30 minutes.

iii) Discard supernatant and resuspend the pellet in 10 µl proteinase K buffer (50 mM KCl, 15 mM Tris-HCl pH 8.3 and 0.5% Nonidet P-40) containing 0.5 mg/ml proteinase K and incubate at 55°C for 1 hour.

iv) Heat inactivate the proteinase K at 95°C for 10 minutes. Centrifuge at maximum speed for 10 seconds. Use 3 µl in the PCR reaction.

Other standard DNA extraction methods may be used.

- PCR set-up

The mastermix should be made-up in a separate location from areas where diagnostic samples are prepared and PCR product are analysed.

i) Make-up the mastermix. Prepare at least one extra aliquot for each 10 reactions planned. Include a positive and a negative (water blank) control for every 10 samples.

<table>
<thead>
<tr>
<th>Per sample:</th>
<th>distilled water 33.6 µl</th>
<th>10× buffer 5.0 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Upper primer (50 pmole/µl) 0.4 µl</td>
<td>Lower primer (50 pmole/µl) 0.4 µl</td>
</tr>
<tr>
<td></td>
<td>dNTPs (2.5 mM each) 2.0 µl</td>
<td>25 mM MgSO₄ 5.0 µl</td>
</tr>
<tr>
<td></td>
<td>Internal control (0.025 pg/µl) 0.3 µl</td>
<td>Taq polymerase (5 U/µl) 0.3 µl</td>
</tr>
</tbody>
</table>

ii) Add 47 µl per reaction tube.

iii) Add 3 µl of sample or water (negative control).

iv) If using a thermocycler without a hot lid, add 50 µl of mineral oil.

v) Run the reaction. Use 30 cycles of 93°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds.

vi) Electrophorese 10 µl of the product on a 10% polyacrylamide gel with a 100 bp size ladder. Stain the gel with ethidium bromide. Observe and photograph under UV transillumination (17).
Chapter 2.1.6. - Channel catfish virus disease

- **Evaluate results**

The internal control produces a 149 bp product. CCV produces a 136 bp product. Expect a 149 bp band within the negative control and on negative samples. Expect a 136 bp product and a 149 bp product on CCV-positive samples. If CCV is present at a high level, the 149 bp band may be missing. If no bands are present, the PCR reaction did not work indicating a failure of one of the PCR components or an inhibitor in the sample. If the negative control shows a 136 bp product, then there is a contaminant in the PCR set-up and the assay must be redone. If an aberrant size band is produced or to confirm that the PCR product is from CCV, the PCR can be redone using no internal standard and the product can be directly sequenced. The sequence can then be evaluated using BLAST on the National Center for Biological Information internet site (http://www3.ncbi.nlm.nih.gov/BLAST/) to identify sequences with high homology. The PCR amplifies the region from 107827 to 107962 of the CCV genome (GenBank accession M75136) representing a portion of open reading frame 73 within ORF (9). Expect at least 95% identity to this sequence.

**Figure 1.** Sequence of CCV PCR product. Italic underlined portions indicate primer sequences.

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2. Diagnostic Methods for CCV

2.1. Virus isolation with subsequent identification

As in Section 1.1. and 1.2.

2.2. Indirect fluorescent antibody test

- **Test procedure**
  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 Section B.3.1. in Chapter I.1.) together with the other organs required for virus isolation in case this becomes necessary later.
  iv) Allow the imprint to air-dry for 20 minutes.
  v) Fix with acetone or ethanol/acetone and dry as indicated in Section 1.2.b. steps v–vii.
  vi) Rehydrate the above preparations (see Section 1.2.b. step ix) and block with 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C.
  vii) Rinse four times with PBST.
  viii) Treat the imprints with the solution of antibody to CCV and rinse as indicated in Section 1.2.b.
  ix) Block and rinse as previously in steps vi and vii.
  x) Reveal the reaction with suitable FITC-conjugated specific antibody, rinse and observe as indicated in Section 1.2.b. steps xii–xv.

If the test is negative, process the organ samples stored at 4°C for virus isolation in cell culture as described in Section 1.1.
2.3. Enzyme-linked immunosorbent assay

a) Microplate processing

As described in Section 1.2.c. of this chapter up to step iv (inclusive).

b) Sampling procedures

See the following sections in Chapter I.1.:
   B.1. for the selection of fish specimens
   B.2. for the selection of materials sampled.

c) Processing of organ samples

See the following sections in Chapter I.1.:
   B.3.1. for transportation
   B.3.2. for virus extraction and obtaining organ homogenates.

d) The enzyme-linked immunosorbent assay procedure

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 the homogenate with 2% Triton X-100 as described in Section 1.2.c. step v, and 2 mM of phenyl methyl sulfonide fluoride; mix gently.

   Complete the other steps of the procedure described in Section 1.2.c.

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

2.4. Polymerase chain reaction

As stated in Chapter I.1. Section C.3.5., PCR is very susceptible to false-positive and false-negative results. Therefore each assay and tissue extraction should include a negative control to rule out contamination. PCR reaction set-up should be done in a separate physical location from that where the PCR products are evaluated. Thus the use of nested PCR protocols for inspection purposes is not recommended. The PCR assay given in Section 1.2.d. can be modified for direct detection of CCV in overtly infected fish. This is done by centrifuging 1.5 ml of a 1/10 dilution of the homogenate at 8000 g for 5 minutes. The supernatant is then used the same way as the cell culture supernatant in Section 1.2.d. If this assay is used to detect carrier fish, the DNA must be purified from the tissue (using a commercially available kit such as the Puregene DNA isolation kit, Gentra Systems, Minneapolis Minnesota, USA). To increase the detection limit to that needed to detect a carrier state, use 0.5 µg of DNA, leave out the internal control DNA, and use 35–40 cycles. In performing PCR on tissue extracts, it is recommended that a parallel sample be run that has been spiked with dilute purified CCV DNA or cloned target fragment (such as the internal standard used above) as a positive control to rule out the presence of inhibitors giving false-negative results.

REFERENCES


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**NB:** There is an OIE Reference Laboratory for Channel catfish virus disease (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
VIRAL ENCEPHALOPATHY AND RETINOPATHY

SUMMARY

Viral encephalopathy and retinopathy (VER), or viral nervous necrosis (VNN) has been reported as a serious disease of larval and juvenile and sometimes older marine fish that occurs world-wide except for Africa (23). To date, the disease has been reported in at least 30 fish species, with the greatest impact being in sea bass (Lates calcarifer [10] and Dicentrarchus labrax [21]), groupers (Epinephelus akaara [22], E. fuscogutatus [3], E. malabaricus [6], E. moara [23], E. septemfasciatus [9], E. tawina [4], E. coioides [19] and Cromileptes altivelis [34]), jack (Pseudocaranx dentex [21]), parrotfish (Oplegnathus fasciatus [33]), puffer (Takifugu rubripes [25]), and flatfish (Verasper moser i [32], Hippoglossus hippoglossus [7/1], Paralichthys olivaceus [26], Scophthalmus maximus [7/1]).

Virus particles of about 25–30 nm in diameter have been visualised in affected fish, and the agents in striped jack, barramundi, and European sea bass have been characterised and placed in the genus Betanodavirus, family Nodaviridae (5, 21). Immunological studies have shown relationships between striped jack nervous necrosis virus (SJNNV, the type species of the genus Betanodavirus) and the other betanodaviruses. Genomic classification of betanodaviruses has shown close relationships, with major groupings being SJNNV-type, tiger puffer nervous necrosis virus (TPNNV)-type, barfin flounder nervous necrosis virus (BFNNV)-type and red-spotted grouper nervous necrosis virus (RGNNV)-type (27). Complete nucleotide sequences of RNA1 and RNA2 of SJNNV and GGNNV (a grouper betanodavirus) have been reported (15, 29).

All diseases are characterised by a variety of neurological abnormalities, such as erratic swimming behaviour (spiral, whirling or belly-up at rest) and vacuolation of the central nervous tissues. Usually there is also vacuolation of the nuclear layers of the retina. In general, younger fish have more severe lesions; older fish have less extensive lesions and these may show a predilection for the retina (23). Intracytoplasmic inclusions have been described in brain cells of European sea bass, barramundi, Japanese parrotfish, and brownspotted grouper. Neuronal necrosis has been described in most species.

Interesting differences with regard to the occurrence and severity of the diseases are shown in Table 1. There are considerable variations in the age at which disease is first noted and the period over which mortality occurs. In general, the earlier the signs of disease occur, the greater is the rate of mortality. Although disease occurrence at the juvenile stages in some species is very rare, mass mortalities often occur at juvenile to young stages in the other fish species, but usually do not reach 100%, indicating the age-dependence of susceptibility (23). Mortalities have been reported in production-size European sea bass (18) and grouper (9), but even in these cases mortalities were greatest in younger fish.

It has been demonstrated that vertical transmission of the causative agent occurs in Pseudocaranx dentex and this fact is reflected by the early occurrence of clinical disease. This finding led to the successful control of VNN of larval striped jack, where elimination of virus-carrying broodstock by reverse-transcription polymerase chain reaction and disinfection of fertilised eggs by ozone were applied (20, 24). Paradoxically, ovarian infection has also been reported in Dicentrarchus labrax in which disease is usually not seen until about 30 days post-batch. The mode of transmission/introduction of the viruses, other than in gametes and by cohabitation, 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 (8) and therefore readily translocated by commercial activities. Vaccination using a recombinant capsid protein is at the experimental stage (14, 30).

Table 1. Important features of VER/VNN 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>Lates calcarifer</td>
<td>9 days post-hatch</td>
<td>15–18 days post-hatch</td>
<td>≥24 days post-hatch</td>
<td>50–100%/month</td>
<td>100% in &lt;1 month</td>
</tr>
<tr>
<td>Dicentrarchus labrax</td>
<td>10 days post-hatch</td>
<td>25–40 days post-hatch</td>
<td>-</td>
<td>10%/month</td>
<td>-</td>
</tr>
<tr>
<td>Oplegnathus fasciatus</td>
<td>6–25 mm total length</td>
<td>-</td>
<td>&lt;40 mm total length</td>
<td>-</td>
<td>Up to 100%</td>
</tr>
<tr>
<td>Epinephelus akaara</td>
<td>14 days post-hatch (7–8 mm total length)</td>
<td>9–10 mm total length</td>
<td>&lt;40 mm total length</td>
<td>80%</td>
<td>Up to 100%</td>
</tr>
<tr>
<td>Epinephelus malabaricus</td>
<td>-</td>
<td>20–50 mm total length</td>
<td>-</td>
<td>50–80%</td>
<td>-</td>
</tr>
<tr>
<td>Pseudocaranx dentex</td>
<td>1 day post-hatch</td>
<td>1–4 days post-hatch</td>
<td>&lt;20 days post-hatch (8 mm total length)</td>
<td>100%</td>
<td>-</td>
</tr>
<tr>
<td>Scophthalmus maximus</td>
<td>&lt;21 days post-hatch</td>
<td>-</td>
<td>Body weight 50–100 mg</td>
<td>-</td>
<td>Up to 100%</td>
</tr>
</tbody>
</table>

DIAGNOSTIC PROCEDURES

Presumptive diagnosis of viral encephalopathy and retinopathy (VER) or viral nervous necrosis (VNN) can be made on the basis of the appearance of vacuoles in the brain, spinal cord and/or retina as seen by light microscopy. However, individual fish with the presence of only a few vacuoles in the nervous tissues pose a difficult diagnostic problem.

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 reported particles vary in size from 22 to 34 nm arranged intracytoplasmically in crystalline arrays, or as aggregates and single virions both intra- and extracellularly. The virus is nonenveloped and icosahedral in shape.

All betanodaviruses can be detected by the indirect fluorescent antibody test (IFAT) or immunohistochemistry with a rabbit anti-SJNNV serum (12, 16). Most can be detected by reverse-transcription polymerase chain reaction (RT-PCR) with a single primer set designed to amplify the T4 region (427 bases) of SJNNV coat protein gene (27, 28) (this primer set can be used for detection of all the genotypic variants of betanodaviruses with only one exception [31]). Although other immunological methods, such as the enzyme-linked immunosorbent assay (ELISA) or neutralisation test, are available for virus identification, they can only be used for some of betanodaviruses because of limited serological information.

The betanodaviruses can be cultured in a fish cell line, SSN-1, which is derived from striped snakehead (7, 16). A clonal cell line, E-11, derived from the SSN-1 cell line is useful for qualitative and quantitative analyses of all the betanodaviruses (17). It is notable that both SSN-1 and E-11 cells are infected by a spontaneously productive C-type retrovirus designated SnRV (13, 17).
Due to insufficient knowledge of the serological responses of fish to virus infections, the detection of fish antibodies to viruses has not thus far been accepted as a routine screening method for assessing the viral status of fish populations.

Fish material suitable for virological examination is:

- **Asymptomatic fish** (apparently healthy fish): whole larvae or small juveniles; brain, spinal cord, and eyes for larger size fish and/or ovarian fluid from broodfish at spawning time.

- **Clinically affected fish**: whole larvae or small juveniles; brain, spinal cord, and eyes for larger size fish.

**Sampling procedures**: see Chapter I.1. Section B.

1. **Standard Screening Method for VER/VNN**

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

   **Cell line to be used**: SSN-1 or E-11 (a cell clone of SSN-1)

   **a) Inoculation of cell monolayers**

   i) Fish samples are homogenised with nine volumes of Hanks’ balanced salt solution (HBSS), centrifuged and filtered (0.2 µm membrane filter).

   ii) Tenfold dilutions of the virus filtrate are inoculated in monolayers of cells cultured using Leibovitz L-15 medium or other medium supplemented with 5% fetal bovine serum (FBS). Inoculate at least 2 cm² of drained cell monolayer with 100 µl of each dilution.

   iii) Allow to adsorb for 1 hour at room temperature, add the medium supplemented with 5% FBS and incubated at 20–25°C.

   **Note**: Optimal growth temperatures are different among the four genotypic variants: 25–30°C for RGNNV-type, 20–25°C for SJNNV-type, 20°C for TPNNV-type, and 15–20°C for BFNNV-type (16, 17).

   **b) Monitoring incubation**

   i) Follow the course of infection in positive controls and other inoculated cell cultures by daily microscopic examination for 10 days.

   ii) If cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of the tested homogenate supernatants, betanodavirus identification procedures must be undertaken (see Section 1.2. below).

   **Note**: CPE in SSN-1 or E-11 cells is characterised by thin or rounded, refractile, granular cells with vacuoles, the monolayer then partially or completely disintegrates (7, 16).

   iii) If no CPE occurs after 10 days of incubation, subcultivation of the inoculated cell cultures must be performed.

   **c) Subcultivation procedures**

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

   ii) Inoculate cell monolayers as described in Section 1.1.a.

   iii) Incubate and monitor as described in Section 1.1.b.
1.2. Identification of betanodavirus isolated in cell culture

a) Indirect fluorescent antibody test
   i) Prepare monolayers of cells in 2 cm² wells of cell culture plastic plates or on cover-slips (or chamber slides) in order to reach around 70% confluency, which is usually achieved within 24 hours of incubation at 25°C.
   ii) Inoculate the virus suspensions to be identified by making tenfold dilution steps directly in the cell culture wells or flasks.
   iii) Incubate at 20°C or 25°C for 48–72 hours (See Section 1.1.a. Note).
   iv) Remove the culture medium, rinse once with PBS, then fix with methanol for 10 minutes.
   v) Allow the cell monolayers to air-dry.
   vi) Treat the cell monolayers with a rabbit anti-betanodavirus serum for 30 minutes at 37°C in a humid chamber, and rinse four times with PBS–Tween 80 (PBST).
   vii) Treat the cell monolayers for 30 minutes at 37°C with commercially available fluorescein isothiocyanate-conjugated anti-rabbit Ig antibody, and rinse with PBST.
   viii) Examine the treated cell monolayers on plates immediately, or mount the cover-slips using glycerol saline, pH 8.5, prior to microscopic observation.

b) Reverse-transcription polymerase chain reaction
   i) Total RNA is extracted from virus-inoculated cells by a commercially available RNA extraction kit according to the manufacturer’s instructions.
   ii) There are several published primers for RT-PCR amplification of betanodaviruses. Primers, R3 (5’-CGA-GTC-AAC-ACG-GGT-GAA-GA-3’) and F2 (5’-CGT-GTC-AGT-CAT-GTG-TCG-CT-3’), designed to amplify the T4 region (427 bases) of SJNNV coat protein gene, are available for all genotypic variants (27).

2. Diagnostic Methods for Clinically Diseased Fish

2.1. Direct detection in fish tissues

a) Indirect fluorescent antibody test
   i) Fish samples fixed in 10% buffered formalin are dehydrated and embedded in paraffin wax. Deparaffinise sections and rehydrate to PBS.
   ii) The sections are treated with 0.1% trypsin in PBS at 37°C for 30 minutes.
   iii) After washing with cold PBS, proceed as described in Section 1.2.a. steps vi to viii.
   iv) Specific fluorescence is observed in the cytoplasm of the affected cells in brain, spinal cord, or retina.

b) Immunohistochemistry (avidin-biotin-alkaline phosphatase technique) (12)
   i) Prepare paraffin sections as described above in Section 2.1.a.
   ii) Add a blocking solution, for example 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 20 minutes.
   iii) Incubate with the anti-betanodavirus rabbit serum for 30 minutes, and wash in TBS.
   iv) Add biotinylated goat anti-rabbit Ig for 30 minutes, and wash in TBS.
   v) Add streptavidin alkaline phosphatase complex, incubate for 30 minutes, and wash in TBS.
   vi) Add fuchsin chromogen reagent for 5 minutes, and wash in tap water.
vii) Counterstain with haematoxylin.

viii) A positive result is indicated by red colour.

c) Reverse-transcription polymerase chain reaction

i) The fish sample is homogenised with distilled water treated with 0.1% diethyl pyrocarbonate.

ii) Centrifuge at 10,000 g for 10 minutes.

iii) Using the supernatant, continue as described in Section 1.2.b.

2.2. Virus isolation in cell culture

See Section 1.1.

REFERENCES


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NB: There are OIE Reference Laboratories for Viral encephalopathy and retinopathy (see Table at the end of this Aquatic Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
INFECTIOUS PANCREATIC NECROSIS

SUMMARY

Infectious pancreatic necrosis (IPN) is a highly contagious viral disease of young fish of salmonid species held under intensive rearing conditions (14, 32, 33). The disease most characteristically occurs in rainbow trout (Oncorhynchus mykiss), brook trout (Salvelinus fontinalis), brown trout (Salmo trutta), Atlantic salmon (Salmo salar), and several Pacific salmon species (Oncorhynchus spp.). Susceptibility generally decreases with age, with resistance to clinical disease in salmonid fish usually being reached at about 1500 degree-days (value obtained by multiplying the age in days by the average temperature in degrees centigrade during the lifespan) (11), except for Atlantic salmon smolts, which can be affected after transfer from fresh water to seawater (29). The first sign of an outbreak in salmonid fry is frequently a sudden and usually progressive increase in daily mortality, particularly in the faster growing individuals. Clinical signs include darkening pigmentation, a pronounced distended abdomen and a corkscrew/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 (17) and quantity (24), host and environment (10). For further details see reviews by Hill (14), Reno (27) and Wolf (32).

The disease is transmitted both horizontally via the water route and vertically via the egg (3, 4, 12). Surface disinfection of eggs is not entirely effective in preventing vertical transmission (6).

The disease has a wide geographical distribution, occurring in most major salmonid-farming countries of North and South America, Europe and Asia. Oceania is free of the disease.

IPN virus (IPNV), or viruses showing serological relatedness to IPNV, have been reported to cause diseases in some farmed marine fish species, such as yellowtail (Seriola quinqueradiata) (21), turbot (Scophthalmus maximus) (7, 20, 22), dab (Limanda limanda) (25), halibut (Hippoglossus hippoglossus) (20, 28) and Atlantic cod (Gadus morhua). Subclinical covert infections have been detected in a wide range of estuarine and freshwater fish species, such as lough (Misgurnus anguillicaudatus) (8), pike (Esox lucius) (2) and numerous other species in the families Anguillidae, Atherinidae, Bothidae, Carangidae, Cottidae, Clupeidae, Cobitidae, Coregonidae, Cyprinidae, Esocidae, Moronidae, Paralichthyidae, Percidae, Poeciliidae, Sciaenidae, Soleidae and Thymallidae (27).

The causative agent, IPNV, is a bi-segmented double-stranded RNA virus belonging to the family Birnaviridae (see review by Dobos & Roberts [10]). Isolates display wide antigenic diversity (11, 16, 19, 23) and fall into two serogroups that do not cross-react in serum neutralisation tests (3, 25, 31), with the majority of strains belonging to serogroup A, which comprises at least nine serotypes (16). Isolates also show marked differences in degrees of virulence (14, 15, 17).

Control methods currently rely on the implementation of control policies and of hygiene practices in salmonid husbandry, through the avoidance of the introduction of fertilized eggs originating from IPNV-carrier broodstock, and the use of a protected water supply (e.g. spring or borehole pond) where the ingress of fish, particularly possible virus carriers, is prevented. In outbreaks, a reduction in the population density (‘thinning out’) may help to reduce the overall mortality. No treatment or entirely effective vaccine is available at present.
DIAGNOSTIC PROCEDURES

The screening procedure for infectious pancreatic necrosis (IPN) is based on IPN virus (IPNV) isolation tests in cell culture (1) followed by immunological identification, either by serum neutralisation (16) or enzyme-linked immunosorbent assay (ELISA) (9), of virus isolated. Diagnosis of clinical cases is normally based on histology (18) and/or immunological demonstration of IPNV antigen in infected tissues (13), confirmed by isolation and immunological identification of IPNV in tissue culture, as for screening.

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

Fish material suitable for virological examination is:

- **Asymptomatic fish** (apparently healthy fish): liver, kidney and spleen (any size fish) and/or ovarian fluid from broodfish at spawning time.

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

**Sampling procedures**: see Chapter I.1. Section B.

1. **STANDARD SCREENING METHOD FOR IPN**

1.1. Isolation of IPNV in cell culture

**Cell line to be used: BF-2 or CHSE-214 or RTG-2**

*a) Inoculation of cell monolayers*

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

ii) Allow to adsorb for 0.5–1 hour at 10–15°C and, without withdrawing the inoculate, add the cell culture medium buffered at pH 7.6 and supplemented with 2% fetal calf serum (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 ×40–100 magnification for 7 days. The use of a phase-contrast microscope is recommended.

ii) Maintain the pH of the cell culture medium at between 7.3 and 7.6 during incubation. This can be achieved by the 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 medium (HEPES = N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid).

iii) If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of the homogenate, IPNV identification procedures must be undertaken immediately (see Section 1.2. 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) Subject cell culture monolayers 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 cell monolayers as described above in Section 1.1.a.

iii) Incubate at 15°C and monitor as described in above Section 1.1.b.

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

v) If CPE occurs, the IPNV identification procedures must be undertaken immediately.

1.2. Identification of IPNV isolated in cell culture

a) Neutralisation test

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

ii) Mix aliquots of each dilution with equal volumes of a neutralising antibody (NAb) solution against the indigenous serotypes of IPNV known in the region. If IPNV is not known to be indigenous to the region, mix aliquots of each dilution with equal volumes of a pooled NAb solution against all IPNV serotypes (16). Similarly, treat aliquots of each virus dilution with cell culture medium. In parallel, a neutralisation test must be performed against homologous IPNV (positive neutralisation test).

(The NAb solution must have a 50% plaque reduction titre of at least 2000.)

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

• a homologous virus strain (positive neutralisation test)

• a heterologous virus strain (negative neutralisation test).

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

v) Transfer aliquots of each of the above mixtures on to cell monolayers (inoculate two cell cultures per dilution).

vi) Incubate at 15°C.

vii) 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 the cell culture medium and staining the cell monolayers with a solution of 1% crystal violet in 20% ethanol.

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

ix) If CPE develops in the presence of antiserum against the indigenous serotypes of IPNV, the neutralisation test must be repeated using pooled antibody against the non-indigenous serotypes of IPNV.

b) Indirect fluorescent antibody test

i) Prepare monolayers of cells in 2 cm$^2$ wells of cell culture plastic plates or on cover-slips in order to reach around 80% confluency, which is usually achieved within 24 hours of incubation at 22°C (seed six cell monolayers per virus isolate to be identified, plus two for positive and two for negative controls). The FCS content of the cell culture medium can be reduced to 2–4%.

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 tenfold dilution steps directly in the cell culture wells or flasks.
iii) Dilute the control virus suspension of IPNV in a similar way, in order to obtain a virus titre of about 5000–10,000 plaque-forming units (PFU)/ml in the cell culture medium.

iv) Incubate at 15°C for 24 hours.

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

vi) Let the fixative act for 15 minutes. 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 minutes and process immediately or freeze at −20°C.

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

ix) Rehydrate the dried cell monolayers by four rinsing steps with the PBST solution, and remove this buffer completely after the last rinsing.

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

xi) Rinse four times with PBST.

xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate (FITC)-conjugated antibody to the immunoglobulin (Ig) 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 four times with PBST.

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

xv) Examine under incident UV light using a microscope with ×10 eye pieces and ×20–40 objective lens having high numerical aperture. Positive and negative controls must be found to give the expected results prior to any other observation.

c) Enzyme-linked immunosorbent assay

i) Coat the wells of microplates designed for enzyme-linked immunosorbent assays (ELISAs) with appropriate dilutions of purified Ig specific for IPNV, in 0.02 M carbonate buffer, 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 (MAbs) specific for certain domains of the capsid protein are suitable.

ii) Incubate overnight at 4°C.

iii) Rinse twice with 0.01 M PBS.

iv) Block with bovine serum albumin (BSA) (1.5% in carbonate buffer) or other blocking solution for 1 hour at 37°C (300 µl/well).

v) Rinse four times with PBS 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 two- or four-step dilutions of the suspension of virus to be identified and of the non-infected cell culture harvest (negative control), and the IPNV control virus (positive control); allow to react with the coated antibody to IPNV for 30 minutes at 37°C.
Chapter 2.1.8. - Infectious pancreatic necrosis

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

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

x) Incubate for 30 minutes at 37°C.

xi) Rinse and wash with PBST as in step viii above.

xii) Add 200 µl of HRPO-conjugated streptavidin or ExtrAvidin (Sigma) to those wells that have received the biotin-conjugated antibody and repeat steps x and xi.

xiii) Add 200 µl of the substrate and chromogen. Stop the course of the test when positive controls react, and read the results.

xiv) Alternatively, add substrate and chromogen to those wells containing the peroxidase-conjugated antibody and proceed as above.

(The procedure described above is just one example of an ELISA. Other procedures that use smaller volumes [50–100 µl] of reagents and/or that use alkaline phosphatase as the enzyme are also acceptable.)

2. DIAGNOSTIC METHODS FOR CLINICALLY DISEASED FISH

2.1. Direct detection in fish tissues

a) 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 Chapter I.1. Section B.3.1.) together with the other organs required for virus isolation in case this becomes necessary later.

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

v) Fix with acetone or ethanol/acetone and dry as indicated in Section 1.2.b. steps v–vii.

vi) Rehydrate the above preparations (see Section 1.2.b. step ix) and block with 5% skim milk or 1% BSA, in PBST for 30 minutes at 37°C.

vii) Rinse four times with PBST.

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

ix) Block and rinse as described previously in steps vi and vii.

x) Reveal the reaction with suitable FITC-conjugated specific antibody, rinse and observe as indicated in Section 1.2.b. steps xii–xv.

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

b) Enzyme-linked immunosorbent assay

i) Microplate processing

As in Section 1.2.c. of this chapter up to step iv (inclusive).
ii) Sampling procedures

See the following sections in Chapter I.1.:
B.1. for the selection of fish specimens
B.2. for the selection of materials sampled.

iii) Processing of organ samples

See the following sections in Chapter I.1.:
B.3.1. for transportation
B.3.2. for virus extraction and obtaining organ homogenates.

iv) Enzyme-linked immunosorbent assay procedure

- Set aside an aliquot of 1/4 of each homogenate in case further virus isolation in cell culture is required.
- Add a volume of 100 mM phenyl methyl sulphonide fluoride (PMSF) to the homogenate to a final concentration of 2 mM PMSF, and mix gently.
- Complete the other steps of the procedure described in Section 1.2.c.

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

c) Co-agglutination test

IPNV may be detected by a rapid co-agglutination test method (30). The test comprises two test solutions – an anti-IPNV solution, consisting of Staphylococcus aureus cells (Phadebact coating kit, Boule Diagnostics AB, Sweden) coated with rabbit-anti-IPNV antibody, according to the manufacturers instructions, and a negative control solution of S. aureus cells coated with normal rabbit serum – and cardboard slides, a stomacher plastic bags and PBS.

The procedure for the test is as follows:

i) Use 0.5–1 g tissue material, preferably kidney, but if small fry are to be examined, all of the visceral organs or whole fry except head and tail may be used. Homogenise the tissue sample with 0.5–1 ml of PBS.

ii) Centrifuge the homogenate at 2000 \( g \) for 10 minutes, remove the supernatant and dilute in an equal volume of PBS.

iii) Add one drop of the anti-IPNV solution and one drop of the negative control solution separately to a cardboard slide. Add one drop of the diluted homogenate (see step ii) separately to the drop containing anti-IPNV and to the drop containing negative control solution. Use a bacterial loop or syringe point to mix the contents of the drops.

iv) A positive reaction is shown as agglutination in the sample where anti-IPNV antibody is present, but not in the negative control solution.

d) Immunohistochemistry

i) **Fixation:** The tissues for examination have to be fixed in aldehyde fixatives (10% formaldehyde, 3% paraformaldehyde, 2.5% glutaraldehyde) for 48 hours or in absolute alcohol or 96% alcohol. Tissues for freeze sections are frozen in Freon gas or in liquid nitrogen as fast as possible. Such tissues must be kept at –70°C prior to and after sectioning.

ii) **Pretreatment of tissue samples:** After fixation, the sample is processed through a histokinette according to normal histological procedures and embedded in liquid paraffin. Freeze sections are thawed, dried by a fan without heat and fixed in acetone, acetone/methanol, ethanol or other similar solvents.
iii) Histolological sections are put on slides coated with poly-L-lysine. (Poly-L-lysine glass is prepared in the following manner: 0.5% poly-L-lysine is dissolved in distilled water to reach a user concentration of 0.05%. The slide is left in poly-L-lysine solution for 30 minutes, and then rinsed for 5 minutes in running tap water. The slides are air dried over night. Note: Poly-L-lysine-coated slides are also commercially available.)

iv) The sections are then deparaffinised and hydrated in the following way:

- Heat the chamber for 25 minutes at 56–58°C. The temperature must not exceed 60°C. Sections are then placed in the following xylene/ethanol baths in a fume-hood:
  - 5 minutes in xylene,
  - 5 minutes in xylene,
  - 5 minutes in absolute alcohol,
  - 5 minutes in absolute alcohol,
  - 5 minutes in 96% alcohol,
  - 5 minutes in 70% alcohol,
  - 5 minutes in running tap water.

v) Blocking of endogenous peroxidase activity can be done by two different methods:

- H₂O₂/methanol (30% H₂O₂ diluted in 3% methanol) in staining jars. The sections are kept in this solution for 20 minutes and then washed in PBS.
- Phenylhydrazine diluted in PBS to a final concentration 0.05%. The PBS must be preheated to 37°C before phenylhydrazine is added. Mix well. The sections are covered with this solution and incubated, in a humid chamber, at 37°C for 40 minutes and then washed in PBS.

vi) Pretreatment of sections to unmask antigens: As antigens may be masked by methylene bridges from the formalin fixation, it is necessary to break the cross-bindings. This can be done by pretreatment in a microwave oven, or by trypsin treatment or by boiling under pressure/autoclaving.

vii) Immunohistochemical procedure

After deparaffinisation and unmasking procedures are completed, the sections are overlaid with 5% (w/v) BSA for 20 minutes. The solution is then blotted off the slides (without washing) and the specific polyclonal or MAbs against IPNV are incubated at concentrations/dilutions that will give an optimal signal to noise ratio. After washing for 5 minutes in Tris-buffered saline (TBS, pH 7.4), the secondary antibody, biotinylated anti-rabbit, diluted from 1/300 to 1/500, or anti-mouse immunoglobulin, is incubated for 30 minutes. After washing in TBS, streptavidin alkaline phosphatase (Boehringer Mannheim GmbH 1089-161) diluted 1/1000 (30 minutes incubation), avidin/biotin/alkaline/phosphatase (Dakopatts, D 365) or avidin/biotin/peroxidase (ABC-PO, Vector PK-4001) (the two latter complexes diluted according to the manufacturer’s instructions) should be incubated for 30–45 minutes at room temperature. After washing, the specimens are incubated with either Fast Red in the case of alkaline phosphatase or with AEC (3-amino-9-ethlycarbazole, 0.27 g/litre, Sigma A-5754) in the case of peroxidase. Fast Red TR salt (Sigma F 1500) (1 g/litre) should be dissolved in TBS, and naphthol AS-MX phosphate (0.2 g/litre), N,N-dimethylformamide (20 ml/litre) (Sigma D-4254) and 1 mM levamisole are then added. Levamisole should be added as an inhibitor of endogenous alkaline phosphatase. The sections are incubated for 20 minutes. AEC is dissolved in N,N-dimethylformamide (67 ml/litre) in 0.1 M acetate buffer, pH 5.2, and 0.03% H₂O₂ is added. The sections should be incubated for 15 minutes. The sections are then washed in running tap water for 10 minutes and counterstained with Mayer’s haematoxylin for 2 minutes. Finally, the sections are washed in tap water for another 2 minutes and cover-slips are applied with an aqueous medium (Aquamount, BDH-Laboratory Supplies, UK, 36086). All incubations, unless otherwise stated, are performed at room temperature in a humidified chamber.
Performance controls should include application of a non-immune serum (normal rabbit serum) at the same dilution as the immune serum or of heterologous MAbs (of the same isotype) at almost identical protein concentrations. Tissue sections from non-infected fish (same species as subject to analysis) should be incubated with immune and non-immune serum-specific or heterologous MAbs. Estimation of end-point dilution value (the highest dilution giving a positive reaction that was discernible from background) must be performed for antibody solutions using a 60-minute incubation at 37°C to validate the method.

2.2. Virus isolation in cell culture

See Section 1.1.

REFERENCES


* * *

**NB:** There is an OIE Reference Laboratory for Infectious pancreatic necrosis (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: [www.oie.int](http://www.oie.int)).
CHAPTER 2.1.9.
INFECTIOUS SALMON ANAEMIA

1. CASE DEFINITION

Infectious salmon anaemia (ISA) is a disease of farmed Atlantic salmon (Salmo salar) (47) caused by the orthomyxovirus infectious salmon anaemia virus (ISAV) (13, 24, 31). ISA primarily affects fish held in seawater or fish exposed to seawater. However, indications of disease outbreaks in fish held in freshwater have also been reported (37). The disease may appear as a systemic and lethal condition characterised by severe anaemia and haemorrhages in several organs. The most prominent signs observed are pale gills, exophthalmus, distended abdomen, and petechia in the eye chamber; skin haemorrhages in the abdomen and scale oedema may occur. The major findings on post-mortem examination are circulatory disturbances in several organs caused by endothelial injury in peripheral blood vessels. These clinical manifestations of ISA are found in four organs: the liver, kidney, gut or gill. The liver manifestation is characterised by dark livers caused by haemorrhagic liver necrosis. The kidney manifestation is characterised by moderately swollen kidneys with interstitial haemorrhaging and tubular necrosis. The gut manifestation is characterised by dark red guts caused by haemorrhaging within the intestinal wall but not into the gut lumen. In addition to being pale, the gill manifestation includes, in some cases, accumulation of blood, especially in the central venous sinus of the gill filaments. Lesions in the liver and gut are easily visible while lesions in the gills and kidney are less obvious. In some ISA outbreaks, one of the organ manifestations can dominate, while in other outbreaks all manifestations can be found. In individual fish all manifestations may be found to some extent. Outbreaks dominated by either the liver or kidney manifestation appear to be most common. Haematocrit <10 in end stages may be observed. Diseased Atlantic salmon presenting with anaemia and circulatory disturbances should be examined for ISAV infection as detailed below, if there are no other, obvious cause to explain their condition.

Mortality during an outbreak of ISA may vary significantly. Daily mortality in affected net pens may initially range from 0.5 to 1%, but may increase with time. Cumulative mortality ranges from moderate to high and may exceed 90% in severe cases. The disease usually starts in one net pen and it may take many months before the disease develops in neighbouring net pens. Although natural outbreaks of ISA have been recorded in farmed Atlantic salmon only, subclinically infected feral Atlantic salmon, brown trout and sea trout (S. trutta) have been identified (41). ISAV has also been detected in two marine species, pollock (Pollachius virens) and cod (Gadus morhua) (28). Corroborative diagnostic criteria are summarised in Section 5 of this chapter.

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

a) Agent factors

- Aetiological agent, agent strains

ISAV is an enveloped virus, 100–130 nm in diameter, with a genome consisting of eight single-stranded RNA segments having negative polarity, and with haemagglutinating, receptor-destroying and fusion activity (6, 9, 11, 13, 31).

The morphological, physiochemical and genetic properties of ISAV are consistent with those of the Orthomyxoviridae (42), and ISAV has recently been classified as the type species of the new genus Isavirus within this virus family. The nucleotide sequences of all eight genome

segments have been described (4). Four major structural proteins have been identified, including a 68 kDa nucleoprotein, a 22 kDa matrix protein, a 42 kDa haemagglutinin-esterase protein and a 50 kDa surface glycoprotein with putative fusion activity, coded by genome segments 3, 8, 6 and 5, respectively (11).

Sequence analysis of various gene segments has revealed differences between isolates both within and between defined geographical areas (2, 7, 20, 25). Analysis of the haemagglutinin-esterase gene has demonstrated the presence of two major groups of ISAV isolates, one European and one North American group. The European group may further be divided into three major groups as suggested by Nylund et al. (35). A small, (hypervariable) highly polymorphic region (HPR) of the haemagglutinin gene has been identified (25), and all the European ISAV isolates can be genotyped according to variations in this region. However, there is no direct correlation between phylogenetic groups and HPR groups.

- **Survival outside the host**

ISAV RNA has been detected by reverse-transcription polymerase chain reaction (RT-PCR) in seawater sampled at farming sites with ISAV-positive Atlantic salmon (26). It is difficult to estimate exactly how long the virus may remain infectious in the natural environment because of a number of factors, such as the presence of particles or substances that may bind or inactivate the virus, UV irradiation and temperature. Exposing cell-culture-propagated ISAV to 15°C for 10 days or to 4°C for 14 days had no effect on virus infectivity (13).

- **Stability of the agent**

ISAV is sensitive to UV irradiation (UVC): a 3-log reduction in infectivity in sterile fresh water and seawater was obtained with a UVC dose of approximately 35 J m⁻² and 50 J m⁻², respectively, while the corresponding value for ISAV in wastewater from a fish processing plant was approximately 72 J m⁻² (40). Recent studies have shown that ISAV can be inactivated by ozonated seawater (unpublished results). Isolated ISAV may survive for weeks at low temperatures, but virus infectivity is lost within 30 minutes of exposure at 56°C (13, 48). Incubation of tissue homogenate from ISA-diseased fish at pH 4 or pH 12 for 24 hours inactivated ISA infectivity. Incubation in the presence of chlorine (100 mg/ml) for 15 minutes also inactivated virus infectivity (48).

- **Life cycle**

The main infection route is most likely through the gills (29, 49) but infection via the intestine cannot be excluded. Endothelial cells seem to be the primary target cells for ISAV by electron microscopy (16, 23). This has recently been confirmed by immunohistochemical examination of several organs (unpublished results). Virus replication has also been demonstrated in leukocyte cells (5, 16, 46), and sinusoidal macrophages in kidney tissue stain positive for ISAV using immunohistochemistry. As endothelial cells are the target cells, virus replication may occur in several organs.

The haemagglutinin-esterase molecule of ISAV, like the haemagglutinin of other orthomyxoviruses, is essential for binding the virus to sialic acid residues on the cell surface. In the case of ISAV, the virus binds to glycoproteins containing 4-O-acetylated sialic acids, which also serve as substrate for the receptor-destroying enzyme (15). Further uptake and replication seem to follow the pathway described for influenza A viruses, indicated by demonstration of low-pH-dependent fusion (9), inhibition of replication by Actinomycin D and α-amanitin (13, 44), early accumulation of nucleoprotein followed by matrix protein in the nucleus (1, 11) and budding from the cell surface (6, 16).

The route of shedding of ISAV from infected fish may be through natural secretions (49).

- **b) Host factors**

- **Susceptible host species**

Natural outbreaks of ISA have been recorded in farmed Atlantic salmon only, while subclinically infected feral Atlantic salmon, brown trout and sea trout (S. trutta) have been
identified by RT-PCR (41). ISAV has also been detected in two marine species, pollock (*Pollachius virens*) and cod (*Gadus morhua*), but only in fish collected in the vicinity of cages with Atlantic salmon exhibiting ISA (28).

Following experimental infection, replication of ISAV has been demonstrated in several fish species, including brown trout, sea trout, rainbow trout (*Oncorhyncus mykiss*), Arctic char (*Salvelinus alpinus*) and herring (*Clupea harengus*) (33, 34, 36, 38).

Attempts have been made to induce infection or disease in *P. virens*, but with negative results (45).

- **Susceptible stages of the host**
  In Atlantic salmon, disease outbreaks are mainly reported in seawater cages; only a few cases have been reported in the freshwater stage, including one case in yolk sac fry (37). Furthermore, ISA has been experimentally induced in both Atlantic salmon fry and parr kept in freshwater. Genetics may also play an important role in the susceptibility of Atlantic salmon to ISA, as a functional association between disease resistance and major histocompatibility (MHC) class I and II polymorphism has been demonstrated (14).

- **Target organs and infected tissue**
  Endothelial cells in many organs (heart, liver, kidney, spleen and others).

- **Persistent infection in lifelong carriers**
  Persistent infection in lifelong carriers has not been documented in Atlantic salmon, but at the farm level, infection may persist in the population by continuous infection of new individuals that do not cause any recognisable disease problems. Experimental infection of rainbow trout and brown trout indicates that persistent infection in these species is possible (33, 36).

- **Vectors**
  Passive transfer of ISA by salmon lice (*Lepeophtheirus salmonis*) has been demonstrated under experimental conditions (39).

c) **Disease pattern**

- **Transmission mechanisms**
  The disease is spread horizontally by water-borne transmission as shown by experimental infection studies. There is no strong evidence for vertical transmission through infected gonadal products. It has been suggested that the spread of disease over long distances is caused by transportation of smolt, either infected prior to shipping or by well boats contaminated with ISAV. Contamination of well boats may be due either to previous transport of infected fish or through intake water from areas with farms harbouring diseased fish.

  Epidemiological studies (17, 50) have shown that the risk of ISA transmission is closely linked to husbandry practices in aquaculture and horizontal transmission. Geographical proximity (<5 km) to farms with ISA outbreaks or slaughterhouses/processing plants releasing contaminated water, numerous smolt deliveries and the use of well boats, and sharing staff and equipment are all considered significant risk factors.

- **Prevalence**
  In a net pen containing diseased fish, the prevalence can vary extensively, while in adjacent net pens ISAV may be difficult to detect, even by the most sensitive methods. Therefore, for diagnostic investigations it is important to sample from net pens containing diseased fish.
Chapter 2.1.9. - Infectious salmon anaemia

- Geographical distribution

Initially reported in Norway in the mid-1980s, ISA has since then been reported in Canada (New Brunswick in 1996 and Nova Scotia in 2000), the United Kingdom (Scotland in 1998 and later in the Shetland Islands), the Faroe Islands (2000) and the USA (Maine in 2001) (3, 27, 43). The causal virus has been isolated from Coho salmon from Chile (18) and from rainbow trout in Ireland in 2002.

- Mortality and morbidity

Morbidity and mortality may vary greatly within and between different net pens in a seawater fish farm, and between different fish farms. Morbidity and mortality within a net pen may start at very low levels. Typically, daily mortality ranges from 0.5 to 1% in affected cages. Without intervention, mortality increases and seems to peak in early summer and winter. The range of cumulative mortality during an outbreak is from insignificant to moderate, but in severe cases, cumulative mortality exceeding 90% may be experienced. Initially, an outbreak of ISA may be limited to one or two net pens over a long period and the spread to other net pens may take months. In outbreaks where affected smolts have been infected in well boats during transport, a simultaneous outbreak may occur.

- Economic and/or production impact of the disease

ISA is regarded as an important disease in the countries where it has been reported. Most countries have therefore initiated general legislative measures and/or control programmes to combat the disease. The economic and production impact on the farming industry lies mainly in losses caused by the disease itself, and to the implementation of governmental measures such as contingency plans and control programmes.

d) Control and prevention

- Vaccination

Vaccination against ISA has been carried out in North America during the past 5 years, but the currently available vaccines do not seem to offer complete protection. The vaccines, which are inactivated, whole virus vaccines, do not give virus clearance in immunised fish, which may thus become virus carriers (19).

- General husbandry practices

The incidence of ISA may be greatly reduced by implementation of legislative measures or husbandry practices regarding the movement of fish, mandatory health control, transport and slaughterhouse regulations. Specific measures including restrictions on affected, suspected and neighbouring farms, enforced sanitary slaughtering, generation segregation ('all in/all out') as well as disinfection of offal and wastewater from fish slaughterhouses and fish processing plants may also contribute to reducing the incidence of the disease.

3. Diagnostic methods

The diagnosis of ISA was initially based on clinical and pathological findings only (10). Following the isolation of the causative agent, a number of direct methods for detection of virus and confirmation of the diagnosis have been established. These include isolation of the virus in cell culture followed by immunological identification (6, 12), immunological demonstration of ISAV antigen in tissues (12) and PCR techniques (8, 31). Differential diagnoses are: other anaemic and haemorrhagic conditions, and winter ulcer and septicaemias caused by infections with Moritella viscosa.

a) Field diagnostic methods

- Clinical signs

Generally, ISA-infected fish appear lethargic and may keep close to the wall of the net pen. The most prominent external signs are pale gills (except in cases with blood stasis in the gills),
exophthalmus, distended abdomen, petechia in the eye chamber, sometimes skin
haemorrhages especially of the abdomen, and scale oedema.

b) Clinical methods

Fish infected with ISAV may show a range of pathological changes, from none to severe,
depending on factors such as infective dose, temperature, age, immune status, virus strain and
pathogenicity, seasonal variation, etc. None of the described lesions is pathognomonic to ISA.
Circulatory disturbances are always present. The following findings have been described to be
consistent with ISA:

- Gross pathology
  - Yellowish or blood-tinged fluid in peritoneal and pericardial cavities.
  - Oedema of the swim bladder.
  - Small haemorrhages of the visceral and parietal peritoneum.
  - Focal or diffusely dark red liver. A thin fibrin layer may be present on the surface.
  - Swollen, dark red spleen with rounded margins.
  - Dark redness of the intestinal wall mucosa in the blind sacs, mid- and hind-gut, without
    blood in the gut lumen of fresh specimens.
  - Swollen, dark red kidney with blood and liquid effusing from cut surfaces.
  - Pinpoint haemorrhages of the skeletal muscle.

- Haematological findings:
  - Haematocrit <10 in end stages (25–30 often seen in less severe cases).
  - Blood smears with degenerate and vacuolised erythrocytes and the presence of
    erythroblasts with irregular nuclear shape. A reduction in the proportion of leukocytes
    relative to erythrocytes, with the largest reduction being among lymphocytes and
    thrombocytes.

A haematocrit value below 10 is not a unique finding for ISA. Fish with disease conditions
such as ulcerations and erythrocytic inclusion body syndrome, may regularly demonstrate
haematocrit values this low.

- Microscopic pathology
  The following histological findings are typical for the disease and include:
  - Numerous erythrocytes in the central venous sinus and lamellar capillaries where
    erythrocyte thrombi also form in the gills.
  - Multifocal to confluent haemorrhages and/or hepatocyte necrosis at some distance from
    larger vessels in the liver. Focal accumulations of erythrocytes in dilated hepatic sinusoids.
  - Accumulation of erythrocytes in blood vessels of the intestinal lamina propria and
eventually haemorrhage into the lamina propria.
  - Spleen stroma is distended by erythrocyte accumulation.
  - Slight multifocal to extensive diffuse interstitial haemorrhage with tubular necrosis in the
    haemorrhagic areas, erythrocyte accumulation in the glomeruli in the kidney.
  - Erythrophagocytosis in the spleen and secondary haemorrhages in liver and kidney.

- Electron microscopy/cytopathology
  Virus has been observed in endothelial cells throughout the body by electron microscopy of
tissue preparations, but this method has not been used for diagnostic purposes.
c) **Agent detection and identification methods**

- **Direct detection methods**

  i) **Microscopic methods**

    • **Smears**

      a) **Indirect fluorescent antibody test**

      An indirect fluorescent antibody test (IFAT) using anti-ISAV haemagglutinin-esterase (HE) monoclonal antibody (MAb) (mix of 3H6F8 and 10C9F5) on frozen tissue sections of kidney, heart and liver or on kidney imprints has given positive reactions in both experimentally and naturally infected Atlantic salmon. Suspected cases (see Section 5) may be confirmed with a positive IFAT.

      i) **Preparations of tissue imprints**

      A small piece of the mid-kidney is briefly blotted against absorbant paper to remove excess fluid, and several imprints in a thumbnail-sized area are fixed on poly-L-lysine-coated microscope slides. The imprints are air-dried, fixed in chilled 100% acetone for 10 minutes and stored either at 4°C for a few days or at –80°C until use.

      ii) **Preparations of cryosections**

      Tissue samples from kidney, liver and heart are collected from moribund fish, frozen in isopentane, chilled in liquid nitrogen, and stored at –80°C. Sections are cut on a cryostat, placed on poly-L-lysine-coated slides, fixed in chilled 100% acetone for 10 minutes and stored at –80°C until use.

      iii) **Staining procedure**

      After blocking with 5% nonfat dry milk in phosphate buffered saline (PBS) for 30 minutes, the preparations are incubated for 1 hour with diluted (e.g. 1/100) anti-ISAV MAb supernatant, followed by three washes. For the detection of bound antibodies, the preparations are incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ig for 1 hour. PBS with 0.1% Tween 20 is used for washing. All incubations are performed at room temperature.

    • **Fixed sections**

      b) **Immunohistochemistry (IHC)**

      Polyclonal antibody against ISAV nucleoprotein is used on paraffin sections from formalin-fixed tissue. This IHC staining has given positive reactions in both experimentally and naturally infected Atlantic salmon. Preferred organs are mid-kidney and heart (transitional area including all three chambers and valves). Suspected cases due to pathological signs are verified with a positive IHC. Histological sections are prepared according to standard methods.

      i) **Preparation of tissue sections**

      The tissues are fixed in neutral phosphate buffered 10% formalin for at least 1 day, dehydrated in graded ethanol, cleared in xylene and embedded in paraffin, according to standard protocols. Approximately 5 µm thick sections (for IHC sampled on poly-L-lysine coated slides) are heated at 56–58°C (maximum 60°C) for 20 minutes, dewaxed in xylene, rehydrated through
graded ethanol, and stained with haemalun and eosin for pathomorphology and IHC as described below.

ii) Staining procedure for IHC

Control sections with and without ISAV in pathologically altered tissues are included in every IHC staining procedure. All incubations are done at room temperature on a rocking table, unless otherwise stated.

a) Place rehydrated sections in 200 ml 0.1 M citrate buffer (10 mM citric acid, pH adjusted to 6.0 with NaOH) in a micro-oven. Boil for 2 × 6 minutes and after each heating leave sections for 5 and 15 minutes, respectively, in the warm solution. Rinse in TRIS-buffered saline (TBS; Tris/HCl 50 mM, NaCl 150 mM, pH 7.6).

b) Incubate for 20 minutes with blocking solution (2% [v/v] normal goat serum in TBS with 5% [w/v] non-fat dry milk).

c) Pour off blocking solution without washing.

d) Incubate at 4°C over night with primary antibody (rabbit antibody against ISAV nucleoprotein) at the recommendd dilution in TBS with 1% (w/v) non-fat dry milk. If necessary absorb by incubating, over night at 4°C, 6 ml of antiserum of appropriate dilution in phosphate buffered saline (PBS) on acetone-fixed cells (ASK or SHK-1 infected with ISAV) in a 175 cm² tissue culture flask.

e) Rinse twice for 5 minutes in TBS and 5 minutes in TBS with 0.1% (v/v) Tween 20.

f) Incubate for 60 minutes with biotinylated anti rabbit IgG (Vector AK 5001) diluted 1/200 in TBS with 2.5% BSA.

g) Rinse twice for 5 minutes in TBS and 5 minutes in TBS with Tween.

h) Incubate for 45 minutes with ABC/AP complex from kit (Vectastain PK 4000) diluted in TBS.

i) Rinse twice for 5 minutes in TBS and 5 minutes in TBS with Tween.

j) Visualise by incubating for 20 minutes with Fast Red TR (2.0 mg naphthol AS-MX phosphate, 0.2 ml N,N-dimethylformamide, 0.01 ml 1.0 M levamisole and 10 mg Fast red TR in 9.8 ml TBS, pH 8.2).

k) Rinse in water.

l) Counter-stain in Harris haematoxylin for approximately 2 minutes.

m) Rinse first in water and then in TBS with Tween.

n) Mount with Aquamount or similar.

ii) Interpretation

Negative control sections should not have any significant colour reactions. Positive control sections should have clearly visible red-coloured cytoplasmic and intranuclear staining of endothelial cells in blood vessels or heart endocardium. A test sample section should only be regarded as positive if clear, intranuclear red staining of endothelial cells is found. The intranuclear localisation is particular to the orthomyxovirus nucleoprotein during a stage of the virus replication. Concurrent cytoplasmic staining is often dominant. Cytoplasmic and other staining patterns without intranuclear localisation must be considered as nonspecific or inconclusive.

The strongest positive staining reactions are usually obtained in endothelial cells of heart and kidney. Endothelial staining reactions within very extensive
haemorrhagic lesions can be slight or absent, possibly due to lysis of infected endothelial cells.

**ii) Agent isolation and identification**

- **Cell culture**

Infected material suitable for virological examination is: spleen, heart and kidney.

SHK-1 (6), ASK (8) or other susceptible cell lines, such as TO (51) and CHSE-214 (21), may be used, but strain variability and the ability to replicate in different cell lines should be taken into consideration. The SHK-1 cells (30), and probably also ASK cells, seem to support growth of the hitherto known virus isolates. A more distinct cytopathic effect (CPE) may appear in ASK cells.

The SHK-1 and ASK cells are grown at 20°C in Leibovitz’s L-15 cell culture medium supplemented with fetal bovine serum (5% or 10%), L-glutamine (4 mM), gentamicin (50 µg/ml) and 2-mercaptoethanol (40 µM) (may be omitted).

For virus isolation, cells grown in 25 cm² tissue culture flasks or multi-well cell culture plates, which may be sealed with parafilm to stabilise the pH of the medium, may be used. Cells grown in 24-well plates may not grow very well into monolayers, but this trait may vary between laboratories and according to the type of cell culture plates used. Serially diluted ISAV-positive controls should be inoculated in parallel with the tissue samples as a test for cell susceptibility to ISAV.

a) **Inoculation of cell monolayers**

Following the virus extraction procedure described in Section B.3.2 of chapter I.1, inoculate actively growing monolayers (1–3-day-old cultures) with a small volume of tissue homogenate supernatant (e.g. 1–1.5 ml per flask or well) after removal of the growth medium. Dilute tissue supernatant with L-15 medium without serum to final dilutions of tissue material of 1/50 and/or higher. Allow 3–4 hours’ incubation at 15°C followed by removal of the inoculate and addition of fresh, fully supplemented growth medium. Using this procedure, cytotoxicity is seldom observed even at the lowest dilution of supernatant. Alternatively, a 1/1000 dilution and direct inoculation without medium replacement can be used.

When fish samples come from production sites where infectious pancreatic necrosis virus (IPNV) is regarded as ubiquitous, the tissue homogenate supernatant should be incubated (for a minimum of 1 hour at 15°C) with a pool of antisera to the indigenous serotypes of IPNV prior to inoculation.

b) **Monitoring incubation**

Inoculated cell cultures (kept at 15°C) are examined at regular intervals (at least every 7 days) for the occurrence of a CPE. Typical CPE due to ISAV appears as vacuolated cells that subsequently round up and loosen from the growth surface. If a CPE consistent with that described for ISAV or IPNV appears, an aliquot of the medium for virus identification as described below must be collected. In the case of an IPNV infection, re-inoculate cells with tissue homogenate supernatant that has been incubated with a lower dilution of IPNV antisera. If no CPE has developed after 14 days, subculture to fresh cell cultures.

c) **Subcultivation procedure**

Aliquots of medium (supernatant) from the primary cultures are collected 14 days (or earlier when obvious CPE appears) after inoculation. Supernatants from wells inoculated with different dilutions of identical samples may be pooled.
Supernatants are inoculated into fresh cell cultures as described for the primary inoculation: remove growth medium, inoculate monolayers with a small volume of diluted supernatants (1/5 and higher dilutions) for 3–4 hours before addition of fresh medium. Alternatively, add supernatants (final dilutions 1/10 and higher) directly to cell cultures with growth medium.

Inoculated cell cultures are incubated for at least 14 days and examined at regular intervals as described for the primary inoculation. At the end of the incubation period, or earlier if obvious CPE appears, medium is collected for virus identification as described below. Cell cultures with no CPE should always be examined for the presence of ISAV by IFAT, haemadsorption or by PCR because virus replication may occur without development of apparent CPE.

- **Antibody-based antigen detection methods**
  
  a) **Virus identification by IF-AT**

  i) Prepare monolayers of cells in appropriate tissue culture plates (e.g. 96-well or 24-well plates), in slide flasks or on plastic cover-slips dependent on the type of microscope available (inverted microscope equipped with UV light is necessary for monolayers grown on tissue culture plates). SHK-1 cells grow rather poorly on glass cover-slips. The necessary monolayers for negative and positive controls must be included.

  ii) Inoculate the monolayers with the virus suspensions to be identified in tenfold dilutions, two monolayers for each dilution. Add positive virus control in dilutions known to give good staining reaction.

  iii) Incubate inoculated cell cultures at 15°C for 7 days or, if CPE appears, for a shorter time.

  iv) Remove cell culture medium and rinse once with 80% acetone. Add 80% acetone and let the fixative act for 20 minutes at room temperature. Remove the fixative and air dry for 1 hour. The fixed cell cultures may be stored dry for less than a week at 4°C or at −20°C for longer storage.

  v) Prepare a solution of antibody against ISAV (anti ISAV MAb 3H6F8 or MAb mix 3H6F8/10C9F5) at an appropriate dilution in PBS containing 0.5% skimmed dry milk. Treat the cell monolayers with the antibody solution for 1 hour at room temperature or 30 minutes at 37°C.

  vi) Rinse twice with PBS/0.05% Tween 20. Keep the monolayers in the dark for 1–2 minutes before removal of the last washing solution.

  vii) Treat the cell monolayers with FITC-conjugated goat anti-mouse immunoglobulin (or if antibody raised in rabbits is used as the primary antibody, use FITC-conjugated antibody against rabbit immunoglobulin) according to the instructions of the supplier. Dilute the conjugate with PBS to appropriate working dilution and incubate for 1 hour at room temperature or 30 minutes at 37°C in the dark.

  viii) Rinse once with PBS/0.05% Tween 20 as described above.

  ix) To stain the nuclei (red colour) add propidiumidol (100 µg/ml in aqua dest.) and incubate for 1–2 minutes at room temperature in the dark followed by rinsing once with PBS/0.05% Tween 20.

  x) If the plates cannot be examined immediately, add a solution of 1,4-diazabicyclooctane (DABCO 2.5% in PBS, pH 8.2) or similar reagent as an anti-fade solution.
• Molecular techniques

a) Reverse-transcription polymerase chain reaction (RT-PCR)

Samples of fish tissue (kidney and heart) to be examined for ISAV by RT-PCR must be transported in a way that prevents degradation of RNA. The samples must be transported on ice, or in a solution or medium designed to retain RNA.

Total RNA is extracted from small pieces of tissues or from tissue homogenates, or from infected cell layers. Several methods for RNA extraction are available (see ref. 30 for details). Methods based on silica-membrane columns have the advantage that they do not involve the use of hazardous chemicals compared with the classical phenol–chloroform method. The concentration and purity of the RNA should be estimated by measuring the optical density at 260 nm and at 280 (OD$_{260}$ and OD$_{280}$). The resulting ratio OD$_{260}$/OD$_{280}$ should be between 1.7 and 2.1 depending on the diluent. Special kits are now available for isolation of RNA from body fluids (serum/plasma) or from cell culture supernatants containing little cellular RNA. In these kits, extra carrier RNA is included to improve the extraction of viral RNA, but the disadvantage is that the amount of RNA extracted from the sample cannot be estimated.

Since the first RT-PCR for ISAV was reported in 1997 (31), several attempts have been made to optimise the method (see ref. 29 for a review). A two-step RT-PCR can be performed whereby the RT and PCR steps are run in separate tubes. The introduction of one-step procedures, where the two reactions are run in a single tube, has been successful regarding sensitivity of the test. However, in this case, no cDNA is left for use in additional amplifications, which may be a disadvantage if several primer sets should be included in the examination.

Several primer sets for ISAV RT-PCR have been reported and some are presented in the table below. The primer sets derived from genomic segment 8 have been used by several laboratories and found suitable for detection of ISAV during disease outbreaks and in carrier fish (8, 29). The ILA1/ILA2 primer set was also used for detection of ISA in Canada and Scotland (27). The primer set derived from segment 6 is suitable for detection of virus strains that may show some variation in the amplified region of segment 8. The segment-6 primers may be useful for verification of PCR results based on segment-8 primers as an alternative to sequencing the PCR product.

<table>
<thead>
<tr>
<th>Primer sequence (5'–3')</th>
<th>Designated Genomic segment</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGC-TAT-CTA-CCA-TGA-ACG-AAT-C</td>
<td>ILA1</td>
<td>8</td>
<td>155</td>
</tr>
<tr>
<td>GCC-AAG-TGT-AAG-TAG-CAC-TCC</td>
<td>ILA2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAA-GAG-TCA-GGA-TGC-CAA-GAC-G</td>
<td>FA-3</td>
<td>8</td>
<td>211</td>
</tr>
<tr>
<td>GAA-GTC-GAT-GAA-CTG-CAG-CGA</td>
<td>RA-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGA-ATC-TAC-AAG-GTC-TGC-ATT-G</td>
<td>Seg6U</td>
<td>6</td>
<td>130</td>
</tr>
<tr>
<td>CTT-CAA-AGG-TGT-CTG-ACA-CGT-A</td>
<td>Seg6L</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The use of real-time RT-PCR may increase the specificity and probably also the sensitivity of the test (32), especially when including a sequence-specific probe. This method is more rapid compared with conventional one-tube RT-PCR, the risk of
contamination may be reduced and it is possible to estimate the relative amount of viral RNA in the sample.

- **Agent purification**

  ISAV propagated in cell culture can be purified by sucrose gradient centrifugation according to Falk *et al.* (13) or by affinity purification using immunomagnetic beads coated with anti-ISAV Mab as described by Falk *et al.* (11).

- **Indirect detection methods**

- **Serological methods**

  Both Atlantic salmon and rainbow trout develop a humoral immune response to the ISAV infection (22). Enzyme-linked immunosorbent assays (ELISAs) with either purified virus or lysates from ISAV-infected cell cultures have been established for detection of ISAV-specific antibodies. ELISA titres can be very high and appear to be quite specific for the nucleoprotein in Western blots (K. Falk, pers. comm.). The test is not standardised for surveillance or diagnostic use, but may be used as a supplement to direct virus detection and pathology in obscure cases. Furthermore, the level and distribution of seroconversion in an ISAV-infected population may give some information about the spread of infection.

4. **Suitability of tests against purpose of use**

<table>
<thead>
<tr>
<th>Method</th>
<th>Examination for virus infection in fish without clinical signs</th>
<th>Examination of diseased fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathology (macroscopic and histology)</td>
<td>Not suitable</td>
<td>Suitable (presumptive)</td>
</tr>
<tr>
<td>IFAT on kidney imprints</td>
<td>Not suitable</td>
<td>Suitable (confirmatory)</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>Not suitable</td>
<td>Suitable (confirmatory)</td>
</tr>
<tr>
<td>RT-PCR (with sequencing for confirmation/characterisation)</td>
<td>Suitable (not confirmatory for infectious virus)</td>
<td>Suitable (confirmatory together with other tests positive for ISA)</td>
</tr>
<tr>
<td>Cell culture</td>
<td>Suitable</td>
<td>Suitable (confirmatory)</td>
</tr>
<tr>
<td>Serology</td>
<td>Suitable (not confirmatory for infectious virus)</td>
<td>Suitable (not confirmatory)</td>
</tr>
</tbody>
</table>

5. **Corroborative diagnostic criteria**

Reasonable grounds to suspect fish of being infected with ISAV are outlined in Section 5.a below. The Competent Authority shall ensure that, following the suspicion of fish on a farm being infected with ISAV, an official investigation to confirm or rule out the presence of the disease will be carried out as quickly as possible, applying inspection and clinical examination, as well as collection and selection of samples and using the methods for laboratory examination as described in Section 3.

a) **Definition of suspect case**

  ISA shall be suspected if at least one of the following criteria is met:

i) Clinical and/or pathological changes consistent with ISA (Section 3.a and b), with or without clinical signs of disease;
ii) Isolation and identification of ISAV in cell culture from a single sample from any fish on the farm as described in Section 3.c.ii;

iii) Evidence for the presence of ISAV from two independent laboratory tests such as RT-PCR (Section 3.c.ii) and IFAT on tissue imprints (Section 3.c.ii);

iv) Transfer of live fish from a farm where ISA may be suspected to be present to farms without suspicions of ISA;

v) Any other epidemiological links to ISA-suspected or confirmed farms;

vi) Detection of antibodies to ISAV.

b) Definition of confirmed case

The following criteria in i) or ii) or iii) should be met for confirmation of ISA:

i) Mortality, clinical signs and pathological changes consistent with ISA (Section 3.a and b), and detection of ISAV by one or more of the following methods:
   a) isolation and identification of ISAV in cell culture from at least one sample from any fish on the farm as described in Section 3.c.ii;
   b) detection of ISAV by RT-PCR by the methods described in Section 3.c.ii;
   c) detection of ISAV in tissue preparations by means of specific antibodies against ISAV (e.g. IFAT on tissue imprints or fixed sections as described in Section 3.c.i).

ii) Isolation and identification of ISAV in cell culture from at least two independent samples from any fish on the farm tested on separate occasions as described in Section 3.c.ii;

iii) Isolation and identification of ISAV in cell culture from at least one sample from any fish on the farm with corroborating evidence of ISAV in tissue preparations using either RT-PCR (Section 3.c.ii) or IFAT (Section 3.c.ii).

6. DIAGNOSTIC/Detection METHODS TO DECLARE FREEDOM

Regular health inspections combined with investigation for ISA when increased mortality is associated with one of the given clinical signs may be satisfactory in regions where ISA never has been reported. In regions where ISA has been reported, testing for ISAV, preferentially by RT-PCR, at certain intervals should be carried out.

REFERENCES


* * *

**NB:** There are OIE Reference Laboratories for Infectious salmon anaemia (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: [www.oie.int](http://www.oie.int)).
1. **Case Definition**

Epizootic ulcerative syndrome (EUS) is considered to be an infection with an oomycete known as *Aphanomyces invadans* or *A. piscicida*. It is an epizootic condition of wild and farmed freshwater and estuarine fish.

2. **Information for the Design of Surveillance Programmes**

   a) **Agent factors**

   EUS is a seasonal epizootic condition of great importance in wild and farmed freshwater and estuarine fish; it has a complex infectious aetiology and is clinically characterised by the presence of invasive *Aphanomyces* infection and necrotising ulcerative lesions typically leading to a granulomatous response. EUS is also known as red spot disease (RSD), mycotic granulomatosis (MG) and ulcerative mycosis (UM). Recently, scientists proposed that EUS should be re-named as epizootic granulomatous aphanomycosis or EGA (1). At present, RSD, MG, UM and EGA are synonyms for EUS. The oomycete that causes EUS is known as *Aphanomyces invadans* or *A. piscicida*. Parasites and rhabdoviruses have also been associated with particular outbreaks, and secondary Gram-negative bacteria invariably infect EUS lesions.

   The genera *Aphanomyces* is a member of a group of organisms commonly known as the water moulds. Although long regarded to be a fungus because of its characteristic filamentous growth, this group, the Oomycetida, is not a member of the Eumycota but is classified with diatoms and brown algae in a group called the Stramenopiles or Chromista.

   b) **Host factors**

   EUS was first reported in farmed freshwater ayu (*Plecoglossus altivelis*) in Oita Prefecture, Kyushu Island, Japan in 1971 (7). It was later reported in estuarine fish, particularly grey mullet (*Mugil cephalus*) in eastern Australia in 1972 (17). The outbreak has extended its range through Papua New Guinea into South-East and South Asia, and recently into West Asia, where it has now reached Pakistan (12, 18). Outbreaks of ulcerative disease in menhaden (*Brevoortia tyrannus*) in the United States of America (USA) have been shown to be very similar to EUS in Asia (2).

   EUS causes disease and mortality in farmed and wild fishes. Region-wide, over 50 species of fish have been confirmed by histological diagnosis to be naturally affected by EUS (12): yellowfin seabream (*Acanthopagrus australis*); climbing perch (*Anabas testudineus*); swamp eel (*Flutia alba*); Catfishes, bagrid (Bagridae); silver perch (*Bidyanus bidyanus*); Atlantic menhaden (*Brevoortia tyrannus*); catla (*Catla catla*); striped snakehead (*Channa striatus*); mrigal (*Cirrhinus mrigala*); walking catfish (*Clarius batrachus*); flying barb (*Eionurus sp.*); bar-eyed goby (*Glossogobius giuris*); marble goby (*Oxyeleotris marmoratus*); goby (*Glossogobius sp.*); rohu (*Labeo rohita*); baramundi or seabass (*Lates calcarifer*); grey mullet or striped mullet (*Mugil cephalus*); mullets (*Mugilidae [Mugil spp.; Liza spp.*]); ayu (*Plecoglossus altivelis*); barb, pool (*Puntius sophore*); sand whiting (*Sillago ciliata*); catfishes, wells (*Siluridae*); snakeskin gourami (*Trichogaster pectoralis*); dwarf gourami (*Colisa lalia*); giant gourami (*Osphonemus goramy*); three-spot gourami (*Trichogaster trichopterus*); silver barb (*Puntius gonionotus*); spotted scat (*Scatophagus argus*); dusky flathead (*Platyccephalus fasciatus*); spiny turbot (*Psettodes sp.*); keti-Bangladeshi (*Rohtee sp.*); therapon (*Terapon sp.*); and common archer fish (*Toxotes chatareus*).
Some important culture species, including tilapia, milk fish and Chinese carp, have been shown to be resistant. Experimental infections demonstrated that goldfish are susceptible (10, 11), but common carp is resistant (19).

Suspect cases of natural infection with \textit{A. invadans} in species other than those listed above should be referred immediately to the appropriate OIE Reference Laboratory, whether or not clinical signs are associated with the findings.

EUS is transmitted horizontally. The \textit{Aphanomyces} zoospores can be horizontally transmitted from one fish to another through the water supply. It is believed that only the zoospores are capable of attaching to the damaged skin of fish and germinating into hyphae. If the zoospores cannot find the susceptible species or encounter unfavourable conditions, they can form secondary zoospores. The secondary zoospores can encyst in the water or pond environment waiting for conditions that favour the activation of the spores. How the \textit{Aphanomyces} pathogen or its spore survives after the outbreak is still unclear as outbreaks usually occur about the same time every year in endemic areas.

c) Disease pattern

EUS occurs mostly during periods of low temperatures or 18–22°C and after periods of heavy rainfall (3). These conditions favour sporulation of \textit{Aphanomyces invadans} (16), and low temperatures have been shown to delay the inflammatory response of fish to oomycete infection (4, 6). In some countries, outbreaks occur in wild fish first and then spread into fish ponds.

d) Control and prevention

Control of EUS in natural waters is probably impossible. In outbreaks occurring in small, closed water-bodies, liming water and improving water quality, together with removal of infected fish, is often effective in reducing mortalities and controlling the disease.

3. Diagnostic methods

Diagnosis of EUS is based on clinical signs and confirmed by histopathology. Diagnosis of EUS in clinically affected fish may be achieved by histopathology or by oomycete isolation. Positive diagnosis of EUS is made by demonstrating the presence of mycotic granulomas in histological section or isolation of \textit{Aphanomyces invadans} from internal tissues.

Fish material suitable for histopathological examination or oomycete isolation:

- **Clinically affected fish:** Kidneys, livers, muscular tissue (any size fish).

a) Field diagnostic methods

- EUS outbreaks have been associated with mass mortality of various species of freshwater fish in the wild (including rice-fields, estuaries, lakes and rivers) and in farms during periods of low temperatures and after periods of heavy rainfall. The early signs of the disease include loss of appetite and fish become darker. Infected fish may float below the surface of the water, and become hyperactive with a very jerky pattern of movement. Fish usually develop red spots or small to large ulcerative lesions on the body.

b) Clinical methods

- Red spots may be observed on the body surface, head, operculum or caudal peduncle. Large red or grey shallow ulcers, often with a brown necrosis, are observed in the later stages. Large superficial lesions occur on the flank or dorsum. Most species other than striped snakeheads and mullet will die at this stage. In highly susceptible species, such as snakehead, the lesions are more extensive and can lead to complete erosion of the posterior part of the body, or to
necrosis of both soft and hard tissues of the cranium, so that the brain is exposed in the living animal.

- Non-septate hyphae of *Aphanomyces invadans* (12–25 µm in diameter) can be observed in muscle squash preparations of the infected area around the lesion. Lesion scrapes generally show secondary fungal, bacterial and/or parasitic infections. Histopathological examination must demonstrate the presence of mycotic granulomas or isolation of *Aphanomyces invadans* from internal tissues.

c) Agent detection and identification methods

- **Histopathology**
  
  **Sampling procedure**
  
  i) Sample only live or moribund specimens of fish with clinical lesions.

  ii) Take samples of skin/muscle (<1 cm³), including the leading edge of the lesion and the surrounding tissue.

  iii) Fix the tissues immediately in 10% formalin. The amount of formalin should be 10 times the volume of the tissue to be fixed.

- **Histological procedure**
  
  Processing of the fixed tissue involves dehydration through ascending alcohol grades, clearing in a wax-miscible agent and impregnation with wax (5). The blocks of fish tissue are cut at about 5 µm and mounted on a glass slide. Before staining, the section must be completely de-waxed and stained in haematoxylin and eosin (H&E) (5). H&E and general fungus stains (e.g. Grocott’s) will demonstrate typical granulomas and invasive hyphae.

- **Confirmatory diagnosis**

  Early EUS lesions are erythematous dermatitis with no obvious oomycete involvement. *Aphanomyces invadans* hyphae are observed growing in skeletal muscle as the lesion progresses from a mild chronic active dermatitis and dermatitis to a severe locally extensive necrotising granulomatous dermatitis with severe floccular degeneration of the muscle. The oomycete elicits a strong inflammatory response and granulomas are formed around the penetrating hyphae.

- **Isolation of *Aphanomyces invadans* from internal tissues**

  The following are two methods of isolation of *A. invadans* or *A. piscicida* adapted from refs 8 and 20.

  - **Method 1:** Moderate, pale, raised, dermal lesions are most suitable for oomycete isolation attempts. Remove the scales around the periphery of the lesion and sear the underlying skin with a red-hot spatula so as to sterilise the surface. Using a sterile scalpel blade and sterile fine-pointed forceps, cut through stratum compactum underlying the seared area and, by cutting horizontally and reflecting superficial tissues, expose the underlying muscle. Ensure the instruments do not make contact with the contaminated external surface and thereby contaminate the underlying muscle. Using aseptic techniques, carefully excise pieces of affected muscle, approximately 2 mm³, and place on a Petri dish containing glucose/peptone (GP) agar with penicillin G (100 units/ml) and oxolinic acid (100 µg/ml). Seal plates, incubate at room temperature or at 25°C and examine daily. Repeatedly transfer emerging hyphal tips on to fresh plates of GP agar until cultures are free of contamination.

  - **Method 2:** Lesions located on the flank or tail of fish <20 cm in length can be sampled by cutting the fish in two using a sterile scalpel, and slicing a cross-section through the fish at the edge of the lesion. Flame the scalpel until red-hot and use this to sterilise the exposed
surface of the muscle. Use a small-bladed sterile scalpel to cut out a circular block of muscle (2–4 mm³) from beneath the lesion and place it in a Petri dish of GP medium (see Table 1) with 100 units/ml penicillin-K and 10 µg/ml oxolinic acid. Instruments should not contact the contaminated external surface of the fish. Incubate inoculated media at approximately 25°C and examine under a microscope (preferably an inverted microscope) within 12 hours. Repeatedly transfer emerging hyphal tips to plates of GP medium with 12 g/litre technical agar, 100 units/ml penicillin-K and 10 µg/ml streptomycin sulphate until axenic cultures are obtained. They may then be maintained at 10°C on GP agar and subcultured at intervals of no greater than 7 days. The oomycete isolate can also be maintained at 25°C on GY agar (1% glucose, 0.25% yeast extract and 1.5% agar) and transferred to fresh GY agar once every month (9).

• Identification of *Aphanomyces invadans*

*Aphanomyces invadans* does not produce any sexual structures and should thus not be diagnosed by morphological criteria alone. However the oomycete can be identified to the genus level by inducing sporogenesis and demonstrating typical asexual characteristics of *Aphanomyces* as described in ref. 12. *Aphanomyces invadans* is characteristically slow growing in culture and fails to grow at 37°C on GPY agar (Table 1). Detailed temperature–growth profiles are given in ref. 15. Two procedures that can be used to confirm *A. invadans* are bioassay and polymerase chain reaction (PCR) amplification of the rDNA of *A. invadans*.

• Inducing sporulation in *Aphanomyces invadans* cultures

The induction of asexual reproductive structures is necessary for identifying oomycete cultures as members of the genus *Aphanomyces*. To induce sporulation, place an agar plug (3–4 mm in diameter) of actively growing mycelium in a Petri dish containing GPY broth and incubate for 4 days at approximately 20°C. Wash the nutrient agar out of the resulting mat by sequential transfer through five Petri dishes containing autoclaved pond water, and leave overnight at 20°C in autoclaved pond water. After about 12 hours, the formation of acalyoid clusters of primary cysts and the release of motile secondary zoospores should be apparent under the microscope. Media for inducing sporulation are shown in Table 1.

• Bioassay

Fish can be experimentally infected by injecting intramuscularly a 0.1 ml suspension of 100+ motile zoospores in EUS-susceptible fish (preferably *Channa striata* or other susceptible species) at 20°C, and demonstrating histological growth of aseptate hyphae, 12–25 µm in diameter, in the muscle of fish sampled after 7 days, and of typical mycotic granulomas in the muscle of fish sampled after 14 days.

• Polymerase chain reaction amplification of the rDNA of *A. invadans*

Details regarding DNA preparation and PCR primers for amplification can be found in ref. 13.

• DNA preparation

Genomic DNA is extracted using the following procedure. About 50 mg of mycelium grown in glucose/peptone/yeast (GPY) medium is homogenised in 11 ml lysis buffer (50 mM Tris/HCl, pH 8.0, 20 mM ethylenediamine tetraacetic acid [EDTA], 2% sodium dodecyl sulphate (SDS)). Proteinase K is added to a final concentration of 1 mg/ml and the mixture is incubated overnight at 37°C with shaking. The sample is chilled on ice for 10 minutes; 5 ml saturated NaCl is added to the tube, which is mixed and then chilled for another 5 minutes. Precipitated protein is pelleted by centrifugation at 2000 g for 15 minutes at 4°C. The supernatant is transferred to a fresh tube and centrifuged again to ensure removal of the precipitate. RNase A is added to a final concentration of 20 µg/ml and the tube is incubated for 30 minutes at 37°C. Two volumes of 100% ethanol are added to the sample, which is then mixed and stored at
Chapter 2.1.10. - Epizootic ulcerative syndrome

20°C overnight. The sample is centrifuged at 2000 g for 15 minutes at 4°C. The resulting DNA pellet is washed with 10 ml ice-cold 75% ethanol and centrifuged again for 5 minutes. The pellet is vacuum-dried and re-suspended in 200 µl dH2O.

- **Diagnostic PCR technique**

Two sets of primers are designed from the ITS1 rDNA sequence for the specific amplification of *A. invadans* DNA. The first set, FP1 5’-AAG-GCT-TGT-GCT-GAG-CTC-ACA-CTC-3’ and FP2 5’-GAT-GGC-TAA-GGT-TTC-AGT-ATG-TAG-3’, are located at nucleotide positions 50–73 and 124–147 of the ITS1 and ITS2 amplification product. Reaction conditions are optimised, and in the final PCR technique the following reagents are added to a 50 µl reaction tube: 5–50 ng genomic DNA, 1 × Taq buffer, 200 µM NTPs, 25 pM each of the primers FP1 and FP2, 2.5 mM MgCl₂ and 1.25 units of Taq polymerase. Amplifications are performed with an initial denaturation of 3 minutes at 95°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 68°C and 30 seconds at 72°C, with a final extension of 5 minutes at 72°C. Amplification products are separated on 1.5% agarose gels, stained with ethidium bromide and visualised under UV illumination. The target amplicon is 98 bps.

All *A. invadans* isolates found so far belong to a single genotype, which facilitates identification. Alternatively, sequencing of the internal spacer region can be performed and the result compared with the sequence deposited in public gene data banks. *Aphanomyces invadans* is different to *A. astaci*, the aetiological agent of crayfish plague, as *A. invadans* does not have a sexual reproductive stage. Both pathogenic fungi can also be differentiated using molecular tools (14).

**Table 1. Media for isolation, growth and sporulation of Aphanomyces invadans cultures**

<table>
<thead>
<tr>
<th>GP (glucose/peptone) medium</th>
<th>GPY (glucose/peptone/yeast) broth</th>
<th>GPY agar</th>
<th>Autoclaved pond water</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 g/litre glucose</td>
<td>GP broth + 0.5 g/litre yeast extract</td>
<td>GPY broth + 12 g/litre technical agar</td>
<td>Sample pond/lake water known to support oomycete growth. Filter through Whatman 541 filter paper. Combine one part pond water with two parts distilled water and autoclave. pH to 6–7.</td>
</tr>
<tr>
<td>1 g/litre peptone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.128 g/litre MgSO₄.7H₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.014 g/litre KH₂PO₄</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.029 g/litre CaCl₂.2H₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.4 mg/litre FeCl₃.6H₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.8 mg/litre MnCl₂.4H₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.9 mg/litre CuSO₄.5H₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4 mg/litre ZnSO₄.7H₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4. **RATING OF TESTS AGAINST PURPOSE OF USE**

The methods currently available for surveillance, detection, and diagnosis of EUS are listed in Table 2. The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic sensitivity and specificity; C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and D = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation (see Chapter 1.1.2), their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
Table 2. EUS surveillance, detection and diagnostic methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance to declare freedom from infection</th>
<th>Presumptive diagnosis of infection or disease</th>
<th>Confirmatory diagnosis of infection or disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross signs</td>
<td>D</td>
<td>B</td>
<td>D</td>
</tr>
<tr>
<td>Direct observation of the oomycete hyphae in muscle or internal organs under microscope</td>
<td>C</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Histopathology of tissues and organs</td>
<td>B</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Isolation of <em>A. invadans</em> and confirmatory identification</td>
<td>C</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Antibody based assays to detect <em>A. invadans</em> antigen (IFAT, ELISA)</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Transmission EM of tissues</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>PCR of tissue extracts</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>PCR of pure isolate of <em>A. invadans</em></td>
<td>D</td>
<td>D</td>
<td>A</td>
</tr>
</tbody>
</table>

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

5. CORROBORATIVE DIAGNOSTIC CRITERIA

a) Definition of suspect case

A suspect case of EUS disease is defined as the presence of typical clinical signs of the disease in a population of susceptible fish OR presentation of typical histopathology in tissue sections OR isolation of the slow growing *Aphanomyces* without identification of the causative agent OR a single positive result from one of the diagnostic assays described above.

b) Definition of confirmed case

A confirmed case of EUS is defined as a suspect case that has produced typical mycotic granulomas in affected tissues or organs with subsequent identification of the causative agent by one of the assays described above OR a second positive result from a separate and different diagnostic assay described above.

6. DIAGNOSTIC/DETECTION METHODS TO DECLARE FREEDOM

The method for surveillance of susceptible fish populations for declaration of freedom from EUS is examination of the gross clinical signs and sampling of the diseased fish only for isolation of *A. invadans* or for histopathology examination to demonstrate absence of the *A. invadans*.

REFERENCES


* *

**NB:** There is an OIE Reference Laboratory for Epizootic ulcerative syndrome (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.1.11.

BACTERIAL KIDNEY DISEASE

(Renibacterium salmoninarum)

SUMMARY

Bacterial kidney disease (BKD) caused by Renibacterium salmoninarum occurs in most parts of the world where salmonid fish are cultured or occur in the wild (25, 26). Salmonids vary in their susceptibility to BKD, and Pacific salmon species of the genus Oncorhynchus are generally considered to be the most susceptible (21, 26, 49, 52). BKD can cause serious mortality in juvenile salmonids in both fresh water and seawater, and also in pre-spawning adults. The disease has been described throughout North America and in many countries in Europe, as well as Japan, Chile, and Iceland (2, 21, 25, 27). Most recorded outbreaks of BKD have occurred in fish culture facilities, and the spread of BKD has followed the expansion of salmonid culture (25). Clinical BKD has also been reported in feral fish (5, 36, 39, 45), including naturally spawning populations that have never been supplemented with hatchery fish (22, 51). Whereas the chronic nature of the disease has hindered accurate estimates of fish losses, particularly in feral fish populations, BKD remains one of the most important bacterial diseases affecting cultured salmonids. Losses as high as 80% in stocks of Pacific salmon and 40% in stocks of Atlantic salmon (Salmo salar) have been reported (25).

Renibacterium salmoninarum is a small (0.3–0.1 μm by 1.0–1.5 μm), nonmotile, nonspore-forming, non-acid-fast, Gram-positive diplobacillus (27). It typically causes a slowly progressing systemic infection, with overt disease rarely evident until fish are 6–12 months old (21). Fish with severe R. salmoninarum infections may show no obvious external signs, or may exhibit one or more of the following: lethargy; skin darkening; abdominal distension due to ascites; pale gills associated with anaemia; exophthalmus; haemorrhages around the vent; and cystic cavities in the skeletal muscle. Internal examination usually reveals the presence of focal to multifocal grayish-white nodular lesions in the kidney, and sometimes in the spleen and liver. In addition, there may be turbid fluid in the abdominal cavity, haemorrhages on the abdominal wall and in the viscera, and a diffuse white membranous layer (pseudomembrane) on one or more of the internal organs. In tissue sections of BKD lesions, R. salmoninarum is frequently observed within phagocytic cells, particularly macrophages. The bacterium appears to survive and perhaps replicate within these cells (4, 10, 31, 57).

The kidney disease bacterium can be transmitted both horizontally from infected fish sharing the water supply (6, 38), and vertically in association with eggs from infected parents (23, 43). As with other infectious diseases of salmonids that are difficult or impossible to treat, avoidance is recommended for the control of BKD in cultured salmonid stocks (1, 21). Because R. salmoninarum is often enzootic in wild salmonid populations (9, 22, 34), measures to control losses from BKD may be defeated by constant exposure of hatchery fish to waterborne bacteria shed into the water supply by wild fish residing upstream from the hatchery (33, 38). Salmonids reared in seawater present special problems because it is difficult to ensure adequate separation of groups of fish to prevent horizontal transmission, and because of the possibility that other marine species might serve as reservoirs for R. salmoninarum (7, 14, 50, 54).

To reduce the probability of vertical transmission of R. salmoninarum in cultured salmonids, brood stock segregation or culling is now used to select egg lots to retain as a source of juvenile fish for hatchery rearing (30, 43, 55). The selection process is aimed at rearing egg lots from mating pairs with undetectable or very low levels of R. salmoninarum. This requires the use of sensitive BKD detection methods for testing the prevalence and levels of R. salmoninarum in each parent fish. Elliott & Barila (16) believed that the membrane-filtration fluorescent antibody test (MF-FAT) would provide the sensitivity and quantification
necessary to investigate the relationship between the levels of *R. salmoninarum* in the female parent and the probability of transmitting the disease to the progeny. Pascho et al. (43) later demonstrated the usefulness of brood stock segregation for controlling losses from BKD by using the MF-FAT in conjunction with the enzyme-linked immunosorbent assay to segregate egg lots from chinook salmon parents infected with either very low or very high levels of *R. salmoninarum*. These researchers reported that the losses from BKD among the progeny of parents with very low levels of *R. salmoninarum* were significantly less than those among the progeny of parents with very high infection levels. The aquaculturist must be aware, however, that brood stock segregation may not completely eliminate BKD from an affected population. Because the broodstock used for commercial fish farming should be free of the kidney disease bacterium, it may be necessary to repopulate a contaminated facility with brood fish from a BKD-free population.

Crucial to the success of any BKD control programme is the application of reliable diagnostic methods that can detect low levels of *R. salmoninarum* in a variety of sample types. For that reason, fish health specialists and researchers have long been interested in developing methods for more rapid and reliable detection of *R. salmoninarum* infections (29, 41, 42, 48, 56). As each new test has been developed, however, there has been a tendency to reject older techniques. Nevertheless, no single ideal diagnostic test has yet been developed for the evaluation of multiple samples for the presence of BKD.

**DIAGNOSTIC PROCEDURES**

Bacteriological culture remains the benchmark method for determining the viability of *Renibacterium salmoninarum* in a sample, and may also be used to quantify the number of bacteria in samples. Immunodiagnostic procedures, however, have become the most widely used for screening large numbers of fish in aquaculture facilities or elsewhere in the field. The two principal immunodiagnostic methods are the enzyme-linked immunosorbent assay (ELISA) and the fluorescent antibody test (FAT). Nucleic-acid-based diagnostic tests, such as the polymerase chain reaction (PCR), are now acceptable for confirmatory identification of *R. salmoninarum* in bacteriological cultures and fish tissue or body fluid samples.

**Sampling procedures:** see Chapter I.1. Sections B and C.

1. **STANDARD SCREENING METHODS FOR *RENI BACTERIUM SALMONINARUM***

Screening for and diagnosis of bacterial kidney disease (BKD) should be based on the ELISA and the FAT. Confirmation of *R. salmoninarum* should be done by bacteriological culture on a KDM-2 medium (kidney disease medium), or by the PCR. Sample preparation and processing procedures are detailed below.

2. **DIAGNOSTIC METHODS FOR *RENI BACTERIUM SALMONINARUM***

2.1. **Enzyme-linked immunosorbent assay for testing tissue, plasma, and coelomic fluid**

The recommended procedure is based on the method of Pascho & Mulcahy (44) as modified by Pascho *et al.* (43). Other ELISA systems are also available commercially (46).

**a) Supplies and reagents**

i) **Sample tubes**

Recommended sample tubes are sterile, 2 ml microcentrifuge tubes with screen-printed graduations, a writing space, and a gasketed screw cap.

ii) **Test sample and control preparation diluent**

Phosphate buffered saline (PBS), pH 7.4, supplemented with 0.05% (v/v) Tween 20 (PBST), and 0.01% (w/v) thimerosal as a preservative. For 1 litre: 8.00 g NaCl, 0.20 g KH₂PO₄, 1.09 g Na₂HPO₄, 0.20 g KCl, 0.10 g thimerosal, confirm pH = 7.4, and 0.5 ml Tween 20.

iii) **96-well microplates**
For the ELISA a 96-well microplate that is designed for use in immunoassays must be used. The performance of these plates will vary, and for a given ELISA, microplates from several manufacturers should be tested to determine which is most suitable.

iv) Positive control antigen

*Renibacterium salmoninarum* cells (0.5% [w/v] wet packed cells) in PBS, pH 7.4, with 0.01% (w/v) thimerosal. The bacterial cells can be lyophilised for long-term storage; lyophilised bacteria normally contain a carrier compound, such as dextrose, for stability. Rehydrate lyophilised bacteria with 1.0 ml reagent-grade water and prepare necessary dilutions, 1/100, 1/500, 1/1000, and 1/5000 (v/v), in PBST and store at −70°C.

v) Coating buffer

Prepare sodium carbonate or purchase a commercial coating solution.

Sodium bicarbonate, pH 9.6: store at room temperature and discard after 30 days. For 1 litre: 1.59 g Na₂CO₃, 2.93 g NaHCO₃, and 0.10 g thimerosal, confirm pH = 9.6.

Commercial coating solution concentrate: store and dilute according to the manufacturer’s instructions.

vi) Wash solution

PBST, or a commercial wash solution that contains Tween 20. Prepare fresh wash solution for each ELISA. Either make PBST as described above, or dilute commercial wash solution concentrates according to the manufacturer’s instructions.

vii) Conjugate diluent

Prepare fresh for each ELISA. Use PBST as described above, or 2% (w/v) nonfat dry milk may be substituted for the Tween 20. Commercial products are also available as concentrates, often marketed as diluents or blocking solutions.

viii) ABTS–peroxidase–chromogen substrate

Commercial products are available, and often are provided as a two-part system: the ABTS chromogen – 0.6 g/litre ABTS (2,2’-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) in a glycine buffer, and a hydrogen peroxide substrate – 0.02% hydrogen peroxide in a citric acid buffer.

ix) Stop solution

5% (v/v) sodium dodecyl sulphate (SDS). Prepare in reagent-grade water immediately before use.

b) Coating antibody and conjugate

i) Coating antibody

Affinity purified immunoglobulin to *R. salmoninarum*: rehydrate lyophilised coating antibody by first mixing 1.0 ml glycerol + 1.0 ml reagent-grade water that contains 0.2% (w/v; 2×) thimerosal, and then transferring 1.0 ml of the 50% glycerol solution to each product vial. Rehydrate the contents of a sufficient number vials to test the anticipated number of fish that will be sampled for a given spawning season. Pool the contents of all vials, then dispense into several cryovials and store at −70°C.

ii) Conjugate

Affinity purified immunoglobulin to *R. salmoninarum* labelled with horseradish peroxidase (HRPO): rehydrate lyophilised coating antibody by first mixing 1.0 ml glycerol + 1.0 ml reagent-grade water that contains 0.2% (w/v; 2×) thimerosal, and then transferring 1.0 ml of the 50% glycerol solution to each product vial. Rehydrate the contents of a sufficient number of vials to test the anticipated number of fish that will be sampled for a given spawning season.
spawning season. Pool the contents of all vials, then dispense into several cryovials and store at –70°C.

iii) Working concentrations of the coating antibody and conjugate

Working concentrations for the purified goat anti-R. salmoninarum antibody (coating antibody), and the HRPO-conjugated goat anti-R. salmoninarum antibody must first be determined by checkerboard titration with the coating antibody and the HRPO-conjugate.

NOTE: The anti-R. salmoninarum antibody is typically applied to the microplate wells at 1 µg per ml; the dilution is made from a concentrated antibody preparation at 1 mg/ml, and the working dilution of the HRPO-conjugated antibody is normally about 1/2000 (v/v).

c) Sample collection and preparation

i) Adult fish

Kidney tissue, 1/4 (w/v): one part tissue + three parts PBST. Collect 2–5 g total; when sampling the kidney, it is recommended that this sample consist of a pool of small tissue pieces from the anterior, mid, and posterior kidney.

Ovarian fluid, 1/2 (v/v): one volume ovarian fluid + one volume PBST. Collect approximately 1 ml.

Plasma, 1/5 (w/v): one volume whole blood + four volumes PBST. Collect blood in a syringe or capillary tube, then dispense the correct volume of blood into the appropriate volume of PBST, remove the cellular fraction by low-speed centrifugation, decant and then test the supernatant.

Samples are usually taken following spawning. Care should be exercised to avoid cross-contamination between fish and contamination of tissue samples from body fluids. Keep tissue and body fluid samples on ice during collection. Store at –70°C.

ii) Juvenile fish

Kidney–spleen tissue pool, recommend 1/4 (w/v) dilution, but can use 1/8 (w/v): one part tissue + seven parts PBST. Juvenile fish are often collected as whole fish and dissected on return to the laboratory. Remove the entire kidney and spleen from each fish. It is preferable to test tissues from individual fish, but a tissue pool may be made if the fish are too small. Two-fish pools are recommended when an insufficient amount of tissue is recovered from an individual fish. Store at –70°C.

Plasma, recommend 1/5 (w/v) dilution, but can use 1/10 (w/v): one volume whole blood + nine volumes PBST. Collect blood in a syringe or capillary tube, then dispense the correct volume of blood into the appropriate volume of PBST, remove the cellular fraction by low-speed centrifugation, decant the supernatant, store fluid or heat at 100°C for 15 minutes, and test the supernatant. Store at –70°C.

d) Sample processing for the ELISA

i) Prepare fish tissue or body fluid samples for the ELISA as described above.

ii) Heat each sample at 100°C for 15 minutes, then centrifuge at from 8000 to 10,000 g for 10 minutes at 4°C. If the samples were prepared earlier and frozen, thaw before heating.

iii) Store processed and heated samples at 4°C, or freeze at –70°C for later testing.

e) ELISA day 1

NOTE: Before beginning the ELISA, users should review the controls and appropriate reagent applications described in Table 1.

i) Dilute the concentrated goat antibody to R. salmoninarum in coating buffer: carbonate/bicarbonate coating buffer, pH 9.6, or a commercial coating solution. When using the
coating solution, make a fresh preparation for each ELISA. The carbonate/bicarbonate buffer is normally discarded after 30 days. Use water of reagent grade or equivalent.

ii) The conjugate control and substrate/chromogen control wells receive no coating antibody. Place 200 µl of coating buffer in each of these wells.

iii) The blanks, negative controls wells, positive control wells, and the wells designated for the test samples receive 200 µl of coating antibody.

iv) Seal each plate with an adhesive plate sealer after addition of the buffer or coating antibody. Place each plate in a humid chamber and incubate at 4°C for 16 hours.

Table 1. Controls for the ELISA to detect Renibacterium salmoninarum

<table>
<thead>
<tr>
<th>Control group</th>
<th>Purpose</th>
<th>Step in the ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>Background absorbance levels in the absence of a test sample</td>
<td>Coating antibody: Yes; Sample addition: PBST only; Conjugate: Yes; Substrate/chromogen: Yes; Stop solution: Yes</td>
</tr>
<tr>
<td>Reference or positive controls</td>
<td>Internal control to insure predictable absorbance by certain levels of antigen</td>
<td>Yes; Yes; Yes; Yes; Yes</td>
</tr>
<tr>
<td>Negative control</td>
<td>Absorbance produced by sample from a negative control fish</td>
<td>Yes; Yes; Yes; Yes; Yes</td>
</tr>
<tr>
<td>Conjugate control</td>
<td>To ensure that there is no nonspecific binding of the conjugate to well surfaces or to the coating antibody</td>
<td>Coating buffer only; PBST only; Yes; Yes; Yes</td>
</tr>
<tr>
<td>Substrate/chromogen control</td>
<td>Test for nonenzymatic production of the colour reaction</td>
<td>Coating buffer only; PBST only; Diluent only; Yes; Yes</td>
</tr>
</tbody>
</table>

j) ELISA day 2

i) Prepare PBST wash solution or dilute a commercial wash solution concentrate in reagent-grade water, and store overnight at 4°C. The wash buffer should remain at room temperature during the ELISA.

ii) Remove the unbound coating antibody by washing each plate five times, with a 30-second soak each time the wells are refilled. Shake excess wash buffer out of each plate after the five washes are completed. Wash plates in numerical order.

iii) Place aliquots of controls and test samples into microplate wells. The following control wells receive 200 µl of the test sample diluent (PBS, pH 7.4, supplemented with 0.05% [v/v] Tween 20): Blank, conjugate control, substrate/chromogen control. Control tissue wells receive the appropriate tissue or body fluid.
iv) Place 200 µl aliquots of each positive control in the appropriate wells.

v) Place 200 µl aliquots of each test sample in the appropriate wells.

vi) Cover each plate with an adhesive plate sealer and incubate for 3 hours at 25°C in a humid chamber. Wash each microplate five times as described previously.

vii) Apply 200 µl of the diluted conjugate to the appropriate wells. Substrate/chromogen control wells receive an equivalent amount of diluent without conjugated antibody.

viii) Seal each plate with an adhesive plate sealer and incubate in a humid chamber for 2 hours at 25°C.

ix) Wash microplates five times as described previously.

x) Substrate/chromogen reaction.

The timing of the substrate/chromogen reaction is critical. The reaction must be stopped after exactly 20 minutes. The volume of stop solution is 50 µl to insure that the wells are not over-filled. The SDS stop solution is prepared from a 5% (v/v) concentrate as follows: four parts concentrate + one part water. Apply 200 µl of substrate/chromogen solution to each well. Fill all plates in numerical order. Immediately after all the wells have received the substrate/chromogen solution, seal the plate and put it in the humid chamber at 37°C.

xi) Stop the substrate/chromogen reaction and measure the absorbance. Begin to apply the stop solution immediately after the incubation period is complete. Wells should receive 50 µl of stop solution in the exact sequence used to apply the substrate/chromogen solution. Wipe any condensation off the bottom of the plate and immediately measure the absorbances at 405 nm.

2.2. Standard indirect and direct fluorescent antibody technique for testing tissue samples and bacteriological isolates

In general, there are two types of staining procedures that use fluorescein isothiocyanate (FITC)-labelled antibodies: the indirect and direct fluorescent antibody techniques (IFAT and DFAT, respectively). The principle and techniques are similar, but the indirect uses a second antibody that is often biotinylated for increased sensitivity. The direct FAT is used more commonly for bacterial corroboration testing and staining of *R. salmoninarum*.

There are three basic steps for DFAT: preparing and fixing bacterial cultures or kidney tissue on multiple-well glass slides; staining the slides with antibody reagents; and reading and interpreting the slides.

**a) Preparing the slides**

i) Pure bacterial cultures (corroboration testing of pure isolates *R. salmoninarum*): pure isolates of bacteria are diluted in sterile PBS and applied to a glass microscope slide. Air-dry and fix in absolute methanol for 5–10 minutes.

ii) Kidney smears from fish – DFAT for *R. salmoninarum*: after the tissue has completely dried, slides are fixed in methanol for approximately 5–10 minutes.

**b) Staining**

i) Positive and negative controls: slides containing both a non-cross-reacting bacterial species and a known positive control can be prepared in quantity and stored refrigerated for use as controls for DFAT staining. Positive controls are always used in corroboration testing to correctly identify morphology and fluorescence of the bacteria in question. The negative control is important in determining overall staining technique, background debris, and nonspecific fluorescence.

ii) Place slides in dark, humidified chamber, and place one drop of specific FITC-conjugated antibody on each slide.
iii) Incubate for 60 minutes at room temperature.
iv) Gently rinse the slides with PBS, pH 7.1 (see Section 2.3.).
v) Counterstain in Evan’s blue for 3–4 minutes for *R. salmoninarum*. (A counterstain is not necessary for corroboration testing of pure bacterial cultures, although Rhodamine can be used if desired.)
vi) Rinse with PBS, pH 7.1.
vii) Air-dry completely, add mounting medium and a cover-slip.

c) Reading and rating

Slides are read at ×1000 magnification on a compound fluorescence microscope (refer to the microscope manufacturer for correct wavelength and filters required for FITC epifluorescence microscopy). The positive and negative control slides are read first. This has two purposes: (1) quality assurance for the staining process (positive control should have myriad numbers of fluorescing bacteria, the negative control should have no fluorescence); and (2) to familiarise the reader with the correct bacterial size and shape, and the magnitude of the bacteria’s fluorescent halo in the positive control. The reader can refer back to the positive control as a reference, if needed, to confirm suspect bacteria in the sample wells.

i) Bacterial corroboration testing: positive bacterial isolates will fluoresce strongly and have the same morphology and size as the positive control.

ii) Kidney smears tested for *R. salmoninarum*: bacterium will have a distinctly apple-green fluorescent cell wall; be the appropriate size (1 µm long by 0.5 µm); and be the proper shape (bean shaped or pear shaped). Compare any suspect bacteria with the control slide to be sure all three of the above criteria are met for *R. salmoninarum*.

d) Fluorescent antibody technique rating guide

(Based on those described in Fish Pathology Section Laboratory Manual, Meyers, T.R., ed. Special Publication 11, Alaska Dept Fish and Game, P.O. Box 25526, Juneau, AK 99802 USA.) Standard rating criteria for interpretation of the FAT on tissue smears are based on a minimum of 60 fields examined at ×1000 magnification.

i) Negative (−): no organisms seen in thirty fields examined.

ii) Plus/minus (±): organisms observed with questionable fluorescence or morphology not typical of the target organisms. Total of one typical organism observed but suspected of not originating from the sample examined, i.e. wash over from a high-level positive sample.

iii) One plus (1+): one to five organisms observed. If only one organism is found, examination of up to 100 fields continues in an attempt to find a second organism that would confirm the 1+ status. If no other organism is detected the final ± or 1+ interpretation is at the discretion of the individual reader.

iv) Two plus (2+): 6 to 50 organisms observed in which some fields will be negative and some will typically contain several organisms.

v) Three plus (3+): 51 to 150 organisms observed with a typical field containing a dozen or more organisms.

vi) Four plus (4+): Greater than 150 organisms with no more than 200 organisms in an average field.

vii) Clinical (C5+): Greater than 200 organisms in an average field. Gross lesions are likely to be observed in the sampled kidneys from this category.

2.3. Membrane-filtration FAT for testing coelomic fluid
This procedure is based on the method of Elliott & Barila (16) as modified by Elliott & McKibben (17).

a) Reagents

i) 0.01 M PBS, pH 7.1
8.50 g NaCl, 1.07 g Na₃HPO₄ (anhydrous), 0.34 g Na₂H₂PO₄·H₂O (monohydrate), and distilled water to 1 litre. Preserve the solution by adding 0.01% (w/v) thimerosal.

ii) PBS/Triton
5.0 ml Triton X-100 (Bio-Rad), and PBS, pH 7.1, to 1 litre. Filter through a 0.2 µm bottle filter.

iii) Trypsin solution
1.0 g Trypsin powder (Difco) 1/250, and DH₂O to 1 litre. Mix trypsin with water at 4°C. Clarify the solution by filtration through Whatman No. 1 filter paper (or by centrifugation at 4000 g, followed by filtration through a 0.2 µm filter). Dispense in small aliquots and freeze at –20°C or colder. (Trypsin will retain activity after storage at –20°C for 1 or 2 months; longer storage should be at –70°C.) Thaw new aliquots of trypsin each day as needed; do not re-freeze.

iv) Eriochrome black T counterstain

1/2000 stock suspension
0.25 g Eriochrome black T, and PBS, pH 7.1, to 500 ml. Filter through Whatman No. 1, then Whatman No. 42 filter papers (the latter is optional) to remove large chunks of stain. Store the counterstain in a dark or foil-covered bottle.

NOTE: There are variations in dye content among lots of the compound. Eriochrome black T dilutions (w/v in PBS) ranging from 1/2000 to 1/20,000 may be required for appropriate staining, depending on the lot of stain used.

Working suspension
Check the stain quality on test filters. The prepared counterstain suspension should be a medium purple colour, and the filters should show a definite purple colour after counterstaining, but should not be so heavily counterstained that they are nearly black.

v) Glycerol mounting medium

0.01 M PBS, pH 7.4
3.36 ml solution A: 0.5 M KH₂PO₄, (anhydrous, 68.04 g/litre), 16.0 ml solution B: 0.5 M K₂HPO₄, (anhydrous, 87.09 g/litre), 8.5 g NaCl, and distilled water to 1 litre.

Mounting medium
90 ml glycerol, 2.5 g 1,4-diazobicyclo-(2,2,2)-octane (DABCO), and 10 ml 0.01 M PBS, pH 7.4. Add the DABCO to the glycerol and dissolve by heating the mixture gently in a water bath. Then, add the PBS. Adjust to pH 8.6–9.0 by adding 0.1 N HCl or 0.1 N NaOH as necessary. Store at room temperature in a dark or foil-covered bottle.

b) Sample preparation

i) Mix 0.5 ml of ovarian (coelomic) fluid with 0.5 ml of PBS/Triton and 0.5 ml of trypsin solution in a small centrifuge tube. Mix vigorously for 20–30 seconds.

ii) Heat the mixture at 50°C for 10 minutes.

iii) Withdraw the sample from the centrifuge tube with a 3 ml syringe equipped with a needle (22 g × 1.5 inch or similar). Triturate the sample a few times with a syringe to break up any remaining clumps of material.

c) Membrane filtration
i) Attach the syringe containing the sample to a Swinney-type filter holder or a disposable pop-top holder. The filter holder should contain a 13 mm diameter, 0.2 µm pore size polycarbonate filter (Whatman Nuclepore, Cambridge, Massachusetts, USA) and a supporting 13 mm diameter, 5.0 µm (or larger) pore size nylon membrane filter (Osmonics, Minnetonka, Minnesota, USA).

NOTE: The polycarbonate filter should be placed shiny side up (toward the syringe) in the filter holder. Polycarbonate filters are thin, and frequently develop ‘ridges’ identical to the ridges on the support screens of the filter holders. Placing a thicker nylon filter between the polycarbonate filter and the support screen helps to reduce the formation of these ridges and therefore makes the filter surface flatter for easier observation and counting of bacteria.

ii) Force the sample through the filter.

iii) Rinse each filter with 3 ml of PBS/Triton (force through the filter with a syringe; use a separate syringe for each sample).

iv) Leave the filter in the holder, and drop on 100 µl of FITC-conjugated anti-
\( R. \text{salmoninarum} \) serum at the optimum working dilution. Cover the top of the filter holders with paraffin film or foil (alternatively, place them in a humid chamber), and incubate upright in the dark at room temperature for 1 hour.

v) After incubation, rinse each filter with 3 ml of PBS/Triton by forcing the rinse through the filter with a syringe.

vi) Counterstain by forcing 1 ml of Eriochrome black T suspension through the filter with a syringe.

vii) Remove the polycarbonate filters from the holders and place them on microscope slides to air-dry. Discard the support filters. When the filters are dry, place a drop or two of glycerol mounting medium, pH 9, on the centre of each filter, and mount with coverslips. Examine at ×1000 magnification with a microscope equipped for FITC epifluorescence.

viii) Filter counts of the number of \( R. \text{salmoninarum} \) in a given number of microscope fields can be converted to cells/ml of the original ovarian fluid sample according to the formula:

\[
\text{Cells/ml} = \frac{\text{(conversion factor) \ (dilution factor) \ (total number of cells counted)}}{\text{Total number of fields examined}}
\]

Where the conversion factor is the filtering surface area divided by the area of a single field at the magnification used. One can calculate the theoretical sensitivity of the technique for any desired number of fields to be examined by entering ‘1’ in the equation for the total number of bacteria counted. In general, 50 or more fields are examined per filter.

ix) To confirm MF-FAT results, a procedure such as a nested PCR for \( R. \text{salmoninarum} \) can be used (11, 41).

2.4. Bacteriological culture

a) Sampling

Tissue samples for diagnosis and identification of \( R. \text{salmoninarum} \) should be taken aseptically from kidney lesions or from lesions in other organs. When no lesions are present, the kidney is the preferred organ for sampling. In mature females, the coelomic fluid may also represent convenient material.

For routine controls for detecting infected individuals in a population, a sufficient number of fish must be sampled (see Chapter I.1., Section B.1.).
b) Isolation

*Renibacterium salmoninarum* is a fastidiously growing organism that requires prolonged incubation (2–3 weeks, sometimes more, at 15°C) to produce colonies. L-Cysteine and serum or serum substitutes are requisite factors, and different media or ingredients have been proposed to improve its growth or reduce the development of associated microorganisms. The following two special media are currently used:

- Kidney disease medium enriched with serum (KDM-2) or charcoal (KDM-C) (15, 19): 0.1 g L-cysteine (chlorhydrate), 1 g tryptone, 0.05 g yeast extract, 1.5 g agar and 100 ml distilled water. Adjust the pH to 6.5–6.8 with NaOH, distribute into flasks or tubes and autoclave for 20 minutes at 120°C. Can be stored for 1 month at 4°C. Regenerate prior to use and add 5–10% fetal calf serum (FCS), or 0.1% activated charcoal.

- Selective kidney disease medium (SKDM-2) (3): 0.1 g L-cysteine (chlorhydrate), 0.005 g cycloheximide, 1 g tryptone, 0.05 g yeast extract, 1 g agar and 100 ml distilled water. Adjust the pH to 6.8 with NaOH and autoclave for 20 minutes at 120°C. Cool to approximately 48°C, and add 10% FCS and the following components, previously filter sterilised (0.22 µm): 0.00125 g D-cycloserine, 0.00025 g oxolinic acid and 0.0025 g polymyxin B sulfate (all final concentrations). Dishes with isolation medium are dried at room temperature for 24–48 hours, inoculated by streaking a 0.1–0.2 ml drop of infectious material across the agar surface, and incubated at 15°C in plastic bags or humid chambers when the absorption is complete. The effect of storage of SKDM-2 plates on the effectiveness of the antimicrobial supplements has not been reported. It is recommended that the SKDM-2 dishes before stored at 4°C and used shortly after preparation.

It seems that serum and charcoal are more likely to act as detoxifying agents than as sources of essential nutrients, and their efficacy has been proved comparable (15), and even optional (53). Supplementing KDM-2 medium with antibiotics may reduce the problem of fast-growing organisms (bacteria and fungi), but there is some evidence that they may also inhibit *R. salmoninarum* itself (40). Another possibility is to inspect the dishes regularly at intervals of 2–3 days, and to remove aseptically the colonies produced by fast-growing organisms. In order to maintain the viability of the *R. salmoninarum*, Evelyn (20) recommends the preparation of tissue suspensions in isotonic saline enriched with peptone (0.1 % [w/v]).

When a stock culture of *R. salmoninarum* is already available, it is possible to take advantage of the 'satellitism' phenomenon described by Evelyn et al. (24) for accelerating the growth of the isolates. A heavy suspension of the laboratory feeding strain is dropped on to 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. Growth enhancement may also be achieved by adding 1.5% (v/v) sterile spent KDM-2 broth to the medium (24).

c) Characteristics (28, 35)

After a sufficiently long incubation period on KDM-2, KDM-C or SKDM-2, *R. salmoninarum* produces white or creamy, shiny, smooth, round, raised, entire colonies that are pinpoint to 2 mm in size. Bacteria from diseased fish will produce visible colonies after 2–3 weeks on average, however up to 8 weeks have been reported for initial growth on KDM-2, and 12 weeks on the selective medium SKDM-2. 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. Growth does not occur on blood agar medium without cysteine supplement or on trypticase–yeast agar. For some strains a uniformly turbid growth occurs in broth, but for others a sediment may develop. *Renibacterium salmoninarum* appears as small (0.3–1.5 × 0.1–1 µm) Gram-positive, PAS-positive, asporogenous, nonmotile, nonacid-fast rods, frequently in pairs, short chains or pleomorphic forms as ‘Chinese letters’, especially in fish tissue.
Renibacterium salmoninarum is catalase positive and oxidase negative. Its phenotypic characteristics have been established using API-Zym systems and conventional tests (Table 2). The API-Zym profile for R. salmoninarum is: – + – + – + – + – + – – – + – – – + – – – + – + (Austin & Austin 1999). 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 2. Characteristics of Renibacterium salmoninarum (18, 28)**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Response</th>
<th>Characteristic</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Production of:</strong></td>
<td></td>
<td><strong>Degradation of (continued):</strong></td>
<td></td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>+</td>
<td>DNA</td>
<td>–</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>+</td>
<td>Elastin</td>
<td>–</td>
</tr>
<tr>
<td>Butyrate esterase</td>
<td>–</td>
<td>Gelatin</td>
<td>–</td>
</tr>
<tr>
<td>Caprylate esterase</td>
<td>+</td>
<td>Guanine</td>
<td>–</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>Hyaluronic acid</td>
<td>–</td>
</tr>
<tr>
<td>Chymotrypsinase</td>
<td>–</td>
<td>Hypoxanthine</td>
<td>–</td>
</tr>
<tr>
<td>Cystine arylamidase</td>
<td>–</td>
<td>Lecithin</td>
<td>–</td>
</tr>
<tr>
<td>α-fucosidase</td>
<td>–</td>
<td>RNA</td>
<td>–</td>
</tr>
<tr>
<td>α-galactosidase</td>
<td>–</td>
<td>Starch</td>
<td>–</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>–</td>
<td>Testosterone</td>
<td>–</td>
</tr>
<tr>
<td>β-glucosaminidase</td>
<td>–</td>
<td>Tributyrin</td>
<td>+</td>
</tr>
<tr>
<td>α-glucosidase</td>
<td>+</td>
<td>Tween 40</td>
<td>+</td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>–</td>
<td>Tween 60</td>
<td>+</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>–</td>
<td>Tween 80</td>
<td>–</td>
</tr>
<tr>
<td>Leucine arylamidase</td>
<td>+</td>
<td>Tyrosine</td>
<td>–</td>
</tr>
<tr>
<td>α-mannosidase</td>
<td>+</td>
<td>Xanthine</td>
<td>–</td>
</tr>
<tr>
<td>Myristate esterase</td>
<td>–</td>
<td>Acid production from sugars</td>
<td>–</td>
</tr>
<tr>
<td>Oxidase</td>
<td>–</td>
<td><strong>Growth on/at:</strong></td>
<td></td>
</tr>
<tr>
<td>Trypsinase</td>
<td>+</td>
<td>pH 7.8</td>
<td>+</td>
</tr>
<tr>
<td>Valine arylamidase</td>
<td>–</td>
<td>Bile salts, 0.025%, (w/v)</td>
<td>–</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>Crystal violet, 0.0001% (w/v)</td>
<td>+</td>
</tr>
<tr>
<td><strong>Degradation of:</strong></td>
<td></td>
<td><strong>Methylene blue, 0.001% (w/v)</strong></td>
<td>–</td>
</tr>
<tr>
<td>Adenine</td>
<td>–</td>
<td>Nile blue, 0.00001% (w/v)</td>
<td>+</td>
</tr>
<tr>
<td>Aesculin</td>
<td>–</td>
<td>Phenol, 0.025% (w/v)</td>
<td>–</td>
</tr>
<tr>
<td>Arbutin</td>
<td>–</td>
<td>Potassium thiocyanate, 1% (w/v)</td>
<td>–</td>
</tr>
<tr>
<td>Casein</td>
<td>+</td>
<td>Sodium chloride, 1%(w/v)</td>
<td>+ (poor)</td>
</tr>
<tr>
<td>Chitin</td>
<td>–</td>
<td>Sodium selenite, 0.01% (w/v)</td>
<td>–</td>
</tr>
<tr>
<td>Chondroitin</td>
<td>–</td>
<td>Thallous acetate, 0.001% (w/v)</td>
<td>–</td>
</tr>
</tbody>
</table>
Table 2 (cont.) Characteristics of Renibacterium salmoninarum (18, 28)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Response</th>
<th>Characteristic</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Use of: 4-umbelliferyl (4MU)-acetate</td>
<td>+</td>
<td>Use of: 4PU-heptanoate</td>
<td>+</td>
</tr>
<tr>
<td>4MU-butyrate</td>
<td>+</td>
<td>4PU-laurate</td>
<td>+</td>
</tr>
<tr>
<td>4MU-β-D-cellobiopyranoside monohydrate</td>
<td>–</td>
<td>4PU-nonanoate</td>
<td>+</td>
</tr>
<tr>
<td>4MU-elaidate</td>
<td>–</td>
<td>4MU-oleate</td>
<td>+</td>
</tr>
<tr>
<td>4MU-α-L-arabinopyranoside</td>
<td>–</td>
<td>4MU-palmitate</td>
<td>–</td>
</tr>
<tr>
<td>4MU-2-acetamido-2-deoxy-β-D-galactopyranoside</td>
<td>–</td>
<td>4MU-propionate</td>
<td>+</td>
</tr>
<tr>
<td>4MU-β-L-fucopyranoside</td>
<td>–</td>
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<td></td>
</tr>
</tbody>
</table>

2.5. Nested polymerase chain reaction for testing tissue samples, whole blood, and coelomic fluid

This procedure is based on the nested PCR method described by Chase & Pascho (11), and Pascho et al. (41). The nested design must only be used with rigorous controls to ensure the accuracy of each diagnosis, and to avoid cross-contamination between samples. Other designs may prove desirable when this can be difficult to achieve, as false-positive results may have serious consequences such as the unnecessary culling of an entire broodstock. Other molecular detection methods have also been reported for diagnosing R. salmoninarum infections (8, 13, 32, 37, 47).

a) General considerations

i) DNA extractions should be done in an area that is free from amplified PCR product to reduce the risk of contamination.

ii) To prevent false-positive results from carry-over of amplified DNA sequences, first round reaction mixtures should also be set up in a separate area, or under a UV hood.

iii) Each work area should include a separate set of supplies, reagents and pipetting devices.

iv) For each PCR analysis include multiple reagent controls without DNA to test for contamination, and select a weak positive control that will be indicative of PCR performance.

b) Primer design

i) Two pairs of oligonucleotide primers are used in the nested PCR protocol. The primers were designed from the published sequence of the p57 protein of R. salmoninarum (12).

ii) The primers used in the first round were: forward 75–93 (5’-AGC-TTC-GCA-AGG-TGA-AGG-G-3’; P3) and reverse 438–458 (5’-GCA-ACA-GGT-TTA-TTT-GCC-GGG-3; M21). Primer location number corresponds to the nucleotide sequence of the open reading frame.

iii) The primers used in the second round of amplification reaction were: forward 95–119 (5’-ATT-CTT-CCA-CTT-CAA-CAG-TAC-AAG-G-3’; P4) and reverse 394–415 (5’-CAT-TAT-GTG-TAC-ACC-CCA-AAC-C-3’; M38). Primer location number corresponds to the nucleotide sequence of the open reading frame.
Chapter 2.1.11. - Bacterial kidney disease (Renibacterium salmoninarum)

c) **DNA extraction and purification**

To purify nucleic acids from tissue use a DNA recovery kit and follow the manufacturer’s instruction for DNA recovery from tissue. DNA extraction protocols typically do not account for the rigid cell wall of a Gram-positive bacterium, and the bacteria could remain intact during the lysis steps. Treatment of tissue and body fluid samples with lysozyme, however, has been reported to be effective and essential for recovery of high-quality bacterial DNA and rRNA (11, 47). After the initial cell lysis, add 50 µl of 4× lysozyme buffer and incubate at 37°C for 1 hour. Consult the manufacturer for specific instructions regarding this lysis step.

Manufacturers of DNA recovery kits from tissue include: DNeasy Tissue Kit from Qiagen, Chatsworth, California (CA), USA; Nucleospin Tissue Kit from Clonetech Palo Alto, CA, USA, or Clinipure Genomic DNA Kit from GeneMate, Kaysville, Utah, USA.

i) Kidney tissue: cut 25–50 mg of tissue into small pieces and place in a 1.5 ml microfuge tube. Add tissue lysis buffer and incubate according to the manufacturer’s instructions. After the initial tissue lysis, add 50 µl of 4× lysozyme buffer (80 mg/ml lysozyme, 80 mM Tris/HCl, pH 8.0, 8 mM EDTA [ethylene diamine tetra-acetic acid]; 4.8% Triton) and incubate for 1 hour at 37°C. Continue with DNA purification as instructed.

ii) Ovarian fluid: pipette 50 µl of ovarian fluid into a 1.5 ml microfuge tube, add tissue lysis buffer and incubate according to the manufacturer’s instructions. After the initial tissue lysis, add 50 µl of 4× lysozyme buffer and incubate for 1 hour at 37°C. Continue with DNA purification as instructed.

iii) Whole blood: pipette 50 µl of whole blood into a 1.5 ml microfuge tube, add tissue lysis buffer and incubate according to the manufacturer’s instructions. After the initial tissue lysis, add 50 µl of 4× lysozyme buffer and incubate for 1 hour at 37°C. Continue with DNA purification as instructed.

iv) **Renibacterium salmoninarum** cells: Pellet bacteria solution by centrifugation at 7000 g for 15 minutes. Pour off supernatant, resuspend pellet in lysis buffer and incubate according to the manufacturer’s instructions. After the initial tissue lysis, add 50 µl of 4× lysozyme buffer and incubate for 1 hour at 37°C. Continue with DNA purification as instructed.

d) **Determination of yield and purity of nucleic acid samples**

On the basis of their absorbance value at 260 nm, adjust the DNA samples with molecular biology-quality water to a concentration between 0.01 and 0.1 ng/µl. Determine the purity of each DNA sample by calculating the ratio of the readings at 260 nm and 280 nm. Pure DNA samples have an $A_{260}/A_{280}$ ratio of 1.8–2.0.

e) **First round PCR protocol**

i) Preparation of first round PCR reaction mixture.

Total volume of the first round PCR is 50 µl: 10 µl of the nucleic acid sample and 40 µl of the reaction mixture. To prepare the reaction mixture, combine 0.2 mM of each nucleotide, 50 mM KCl, 10 mM Tris/HCl, pH 8.3, 1.5 mM MgCl$_2$, 0.2 mM each of primers P3 and M21 and 2 U of Taq polymerase.

ii) Add the PCR reagents except the template DNA into the ‘Master Mix’ tube.

iii) In the PCR tubes, aliquot 40 µl of Master Mix and overlay samples with one drop of mineral oil. Close the cap tightly after each addition.

iv) Add 10 µl of extracted DNA to the PCR tubes.

v) In each reaction well of the thermal cycler, add two drops of mineral oil. Load the samples into the thermal cycler.

vi) Programme the thermal cycler for 30 cycles as follows: denaturing at 94°C for 30 seconds; annealing at 60°C for 30 seconds; and extending at 72°C for 1 minute.
Chapter 2.1.11. - Bacterial kidney disease (Renibacterium salmoninarum)

f) Second round PCR protocol
   i) Preparation of the second round PCR reaction mixture.
      Total volume of the reaction mixture is 50 µl; this will include 1 µl of amplified DNA from the first round as template DNA. Use the same reaction mixture as described for the first round except that primers P4 and M38 are used.
   ii) Add the PCR reagents except the template DNA into the Master Mix tube.
   iii) In nested PCR tubes, aliquot 49 µl of Master Mix and overlay the samples with one drop of mineral oil. Close the cap tightly after addition.
   iv) Add 1 µl of first round PCR product to the nested PCR tubes.
   v) In each reaction well of the thermal cycler, add two drops of mineral oil. Load the samples into the thermal cycler.
   vi) Programme the thermal cycler for 30 cycles as follows: denaturing at 94°C for 30 seconds; annealing at 60°C for 30 seconds; and extending at 72°C for 1 minute.

g) Visualisation of amplified DNA
   i) Use 10 µl of the second round PCR product for gel electrophoresis on a 2% agarose gel. Each electrophoresis gel should include a 1 kb DNA ladder.
   ii) Stain gels for 30 minutes in a solution of 5 µg/ml ethidium bromide, 0.02 M hydroxymethyl aminomethane, 0.02 M glacial acetic acid, and 0.5 mM EDTA.
   iii) Examine the gels under UV transillumination. Samples are considered positive for R. salmoninarum if the anticipated 320 base pair product is observed.

REFERENCES

Chapter 2.1.11. - Bacterial kidney disease (Renibacterium salmoninarum)


Chapter 2.1.11. - Bacterial kidney disease (Renibacterium salmoninarum)


Chapter 2.1.11. - Bacterial kidney disease (Renibacterium salmoninarum)


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NB: There is an OIE Reference Laboratory for Bacterial kidney disease (*Renibacterium salmoninarum*) (see Table at the end of this Aquatic Manual or consult the OIE Web site for the most up-to-date list: [www.oie.int](http://www.oie.int)).
NB: This disease is no longer listed in chapter 1.2.3 of the Aquatic Code. This chapter has not been updated since 2003

CHAPTER 2.1.12.

ENTERIC SEPTICAEMIA OF CATFISH
(Edwardsiella ictaluri)

SUMMARY

Enteric septicaemia of catfish (ESC) is caused by the bacterium Edwardsiella ictaluri, which belongs to the Enterobacteriaceae family (11). ESC is one of the most important infectious disease problems in the commercial catfish industry in the United States of America (USA). Most reported cases of disease caused by E. ictaluri are in channel catfish (Ictalurus punctatus), but the bacterium has been isolated from related North American catfish including blue catfish (I. furcatus), white catfish (Ameiurus carus), and brown bullhead (A. nebulosus) (11). ESC has also been reported from Clarias batrachus in Thailand (12) and from several ornamental species (13, 37). The susceptibility of other species including salmonids has been shown experimentally (4). Edwardsiella ictaluri should not be confused with E. tarda, another member of the same genus that is frequently found in aquatic animals and is responsible for opportunistic infections in fish and mammals, including humans.

Several studies have shown that E. ictaluri is a very biochemically and antigenically homogeneous species (5, 21, 33, 38).

Acute outbreaks of ESC occur within a limited temperature range, from 18 to 28°C. This critical temperature window makes spring and autumn the most common periods for outbreaks in regions where channel catfish are normally cultured. However, low-level mortality due to ESC can occur in carrier populations outside of this temperature range. Other environmental factors (poor water quality, high stocking density and other stressors) predispose the host to ESC. Edwardsiella ictaluri is considered to be a true obligate pathogen.

Two clinical forms of ESC occur in channel catfish, a chronic encephalitis and an acute septicaemia (20, 22, 30). In the chronic form the bacterium infects the olfactory sacs, and migrates along the olfactory nerves to the brain, generating granulomatous inflammation. This meningo-encephalitis causes abnormal behaviour, with alternating listlessness and chaotic swimming. In late stages of this disease, swelling develops on the dorsum of the head as the inflammatory process erodes the connective tissue in this region. This swelling ulcerates exposing the brain. This has lead to the term 'hole in the head disease', used in the industry. In the acute form of ESC the bacterium is thought to infect through the intestinal mucosa (3), and then to establish a bacteraemia. The affected fish display petechial haemorrhages around the mouth, on the throat, the abdomen and at the base of the fins. Multifocal distinct 2 mm diameter raised haemorrhagic cutaneous lesions that progress to depigmented ulcers also occur. Anaemia, moderate gill inflammation and exophthalmia are common signs. Internally, haemorrhages and necrotic foci are scattered in the liver and other internal organs. Haemorrhagic enteritis, systemic oedema, accumulation of ascitic fluid in the body cavity and enlargement of the spleen are nonspecific signs. Histological examination reveals a systemic infection of all organs and skeletal muscles, with the most severe changes being diffuse interstitial necrosis of the anterior and posterior kidney. Focal necrosis in the liver and spleen are also generally seen.

Fish from a population that has recovered from the disease are considered to be carriers. These fish will have protective immunity and may have high levels of E.-ictaluri-specific antibodies. Occasional losses due to recurrent ESC will occur in these populations, especially after a stress is induced. Edwardsiella ictaluri has been detected in the kidney of such fish well over 4 months after exposure (2, 14), suggesting that carrier fish act as the natural reservoir for the organism. It is believed that shedding with faeces is the main means of dissemination into the environment. The pathogen persistence and the common practice of continual
Chapter 2.1.12. - Enteric septicaemia of catfish (Edwardsiella ictaluri)

Partial harvest and stocking within a production pond have contributed to the success of this pathogen and the prevalence of ESC in the industry. Moreover, the agent can survive in pond sediments for an extended period of time (27), and this may be another important factor in disease recurrence in given areas. Researchers have found the bacterium in the gut of fish-eating birds by performing fluorescent antibody tests on ingesta, but generally no E. ictaluri could be cultured indicating that the bacteria were not viable (34, 40). This suggests that birds are not an important means of disseminating this pathogen.

ESC may be controlled through chemotherapy and/or prophylactic measures. The most common antimicrobial treatments are oral application of potentiated sulfonamide sulfadimethoxine or oxytetracycline, but plasmid-mediated resistance to these antibiotics does occur (6). Many producers are now focusing on alternative methods to reduce losses. This relies on management to reduce stress in fish, the cessation of feeding when ESC-induced losses are detected (41) and on vaccination.

**DIAGNOSTIC PROCEDURES**

1. **IDENTIFICATION OF THE AGENT**

The identification of Edwardsiella ictaluri is based on the isolation of the causative agent and characterisation by biochemical tests. Edwardsiella ictaluri can easily be differentiated from E. tarda by its inability to produce indole and hydrogen sulfide (E. tarda produces both). Additionally, the two species do not cross-react serologically.

1.1. **Isolation and bacteriological identification**

   **a) Sampling and isolation of the agent**

   Bacteriological samples from freshly dead or moribund fish should be taken aseptically from the brain and kidney tissue. The samples should be streaked for isolation on to blood agar plates, brain–heart infusion (BHI) agar or nutrient agar plates. The bacterium grows slowly but does not require special nutrients. In mixed cultures E. ictaluri can be overgrown by more rapidly growing bacteria, but E. ictaluri is present in very high numbers in fish affected by ESC. A selective medium (EIM) has been developed (31) that may prove useful when samples are taken from heavily contaminated environments, but it is not essential under normal conditions. For detecting a carrier state in a healthy population, kidney tissue has been homogenised in 0.5% triton-X 100, filtered on to 0.45 µm nitrocellulose and grown on EIM agar medium (8) or homogenised kidney tissue was cultured overnight in liquid EIM and 100 µl of this sample was plated on to BHI agar plates (14). Intraperitoneal administration of suspect carrier fish with 0.8 mg/g of Kenalog (triamcinolone acetonide) 2 weeks before attempted culture enhances the detection of the bacterium (2). Optimal temperature for incubation is 28–30°C.

   For routine sampling of fish populations, see Chapter I.1. Section B.1.

   **b) Characteristics**

   Following incubation for 36–48 hours, E. ictaluri appears as smooth, circular (1–2 mm diameter), slightly convex nonpigmented colonies with entire edges. It is a Gram-negative rod, measuring 0.75–2.5 µm, and is weakly motile by means of a peritrichous flagellation and is cytochrome oxidase negative. This bacterium grows slowly or not at all at 37°C.

   After isolation, the bacterium should be identified by biochemical and serological characteristics (10, 11). Table 1 shows some of the characteristics of the species and biogroups of the genus Edwardsiella and similar bacteria that can be isolated from fish as given in Bergey’s Manual of Determinative Bacteriology (9). The optimal growth temperature 28–30°C should be used for evaluating biochemical characteristics. Edwardsiella ictaluri is biochemically less active than the other Edwardsiella species, but it appears to be homogeneous (38). A clear-cut biotype variation is not detected. Edwardsiella ictaluri and E. tarda may be differentiated from each other biochemically by the production of indole and hydrogen sulfide (E. tarda produces both,
while *E. ictaluri* does not). Also *E. tarda*, *Yersinia ruckeri*, *Hafnia alvei* and *E. hoshinae* grow well at 37°C whereas *E. ictaluri* does not. *Edwardsiella ictaluri* degrades chondroitin sulfate and this may be an important virulence factor (7, 32, 38).

### Table 1. Differentiation of the species and biogroups of the genus *Edwardsiella* and other Enterobacteriaceae found in fish

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>Yersinia ruckeri</em></th>
<th><em>Hafnia alvei</em></th>
<th><em>E. tarda</em></th>
<th><em>E. hoshinae</em></th>
<th><em>E. ictaluri</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>(–)</td>
</tr>
<tr>
<td>Malonate utilisation</td>
<td>–</td>
<td>d</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Indole production</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Hydrogen sulfide production in</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>triple sugar iron (agar)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motility</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Citrate (Christensen’s)</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–**</td>
</tr>
</tbody>
</table>

* Bergey’s Manual of Determinative Bacteriology.
** Weakly motile at 28°C. Hawke, 1979.
*** Citrate may be negative on rapid test strips (API 20E). Assay should be run at 25°C.

### 1.2. Detection of the bacterial antigen by serological methods

In addition to well defined biochemical characteristics, there is antigenic homogeneity of the species and serology can easily differentiate *E. ictaluri* from other Enterobacteriaceae (5, 26, 29). Slide agglutination with specific antisera against *E. ictaluri*, fluorescent antibody techniques (FATs), enzyme-linked immunostaining and enzyme-linked immunosorbent assays (ELISAs) have been used to provide confirmatory diagnosis. Characterised specific monoclonal antibodies (MAbs) are generally used in these assays, but polyclonal antisera obtained using formalin-killed bacterins to immunise rabbits according to classical standards may also be used (1, 16, 28).

**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 by comparison with a similar suspension in normal rabbit serum (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 test (IFAT) may be employed on bacterial smears, or smears from infected organs, for rapid confirmation of a clinical diagnosis (28). Smears are air-dried and heated for 2 minutes at 60°C before being flooded and incubated for 5 minutes with specific rabbit antibody. They are washed in phosphate buffered saline (PBS), pH 7.2, flooded for 5 minutes with the rabbit-Ig-specific secondary antibody conjugated with fluorescein isothiocyanate (FITC). After rinsing, the slides are mounted with cover-slips using phosphate-buffered mounting medium and observed microscopically for bright green fluorescence under blue epi-illumination. Use undiluted cell culture supernate when using MAbs that are produced from cell culture in the first step, and a commercially available FITC-conjugated mouse-Ig-
specific secondary antibody at the suggested working concentration (1). Smears of bacterial suspensions must be very thin. Positive and negative controls (such as *E. tarda*) should be stained on separate slides.

c) **Enzyme-linked immunostaining**

An enzyme-linked immunostaining technique to directly identify *E. ictaluri* in tissue smears from infected fish has been described (28). Smears are prepared as for IFAT – the first steps are similar, but the second incubation step uses heterospecific immunoglobulin against rabbit antiserum, conjugated to horseradish peroxidase. A third incubation step with a substrate (DMOB, Sigma) is performed for 10 minutes, and after washing and drying, the smears are mounted in buffered glycerine and observed microscopically under normal trans-illumination. If smears are too thick, they may produce nonspecific retention of the stain. Rinsing the smear again for 1 or 2 minutes in 1 N HCl can solve this problem.

1.3. **Nucleic-acid-based diagnosis**

Assays based on PCR amplification of structural RNA sequences from bacterial colonies and direct sequencing the products are being adapted by several diagnostic bacteriology laboratories and some of these assays are commercially available (MicroSeq, Applied Biosystems). Species confirmation can be done by amplifying and sequencing the 16S portion of the ribosomal RNA operon and comparing the sequence with GenBank accession AF310622.

2. **STANDARD SCREENING METHODS FOR ESC**

2.1. **Detection of the agent**

The techniques are the same as described in Section 1. Unless performed on bacteria isolated and purified first, any positive result obtained using a detection method will have to be confirmed, preferably by plating and isolation of the bacteria.

2.2. **Sero logical tests**

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

a) **Microagglutination test**

Direct microagglutination, performed in 96-well round-bottom microplates as described for other bacterial pathogens (19) can provide quick quantitative data at minimal cost when high sensitivity is not required. All that is needed is a formalin-killed bacterial suspension prepared according to the usual techniques (i.e. formalin [0.35% (v/v)] overnight) and adjusted to an optical density of 0.8 at 525 nm (about 5 × 10⁸ colony-forming units/ml). Twofold dilutions of the sera are made in isotonic saline, so that the final volume is 25 µl/well. Antigen is added (75 µl/well) and the plates are incubated for 2 hours at 37°C and overnight at 4°C before being read. Controls include a rabbit standard serum of previously established titre and antigen incubated in saline.

b) **Passive haemagglutination**

This technique has been described using *E. ictaluri* lipopolysaccharide (1 mg/ml in PBS, pH 7.2) passively coated on human Group O red blood cells at 4% (29). Tested sera must be heated for 30 minutes at 45°C to inactivate complement, and absorbed with Group O human red blood cells to remove nonspecific agglutinins before dilutions are done in buffered saline. Coated blood cells adjusted to 1% concentration are used. Incubation is for 6 hours at room
Enteric septicaemia of catfish (Edwardsiella ictaluri)

232 Manual of Diagnostic Tests for Aquatic Animals 2006

temperature and overnight at 4°C. Controls include coated and uncoated red cells in buffer and coated cells in serum.

c) Indirect enzyme-linked immunosorbent assay

ELISA methods have been developed to detect catfish antibodies to E. ictaluri and are the most widely used assays in research (15, 39). The first method uses whole heat-killed bacteria at 4 × 10⁸ cells/ml PBS, 50 µl/well to coat poly-L-lysine-treated ELISA plates (39). The plates are washed with PBS and then blocked by the addition of 100 µl of 100 mM glycine and 1% bovine serum albumin in PBS for 30 minutes. The plates are then incubated for 30 minutes with dilutions of the sera to be tested, washed, and incubated with anti-fish-species immunoglobulin serum (MAb can be used), washed, and incubated with an antibody conjugate (either horseradish peroxidase or alkaline phosphatase) specific to the secondary antibody. Then the plate is washed and the chromogenic enzyme substrate is added, the colour is allowed to develop and the plate is read. The second method is similar but uses a soluble major antigen (16) obtained by sonicating the bacteria, or merely by dialysing the supernatants of 24-hour broth cultures then concentrating the sample to 25 µg/ml of protein content. Approximately 100 µl is used to coat the wells of the microplates. Optimal working concentrations must first be determined for each reagent used in the test. These techniques have proven useful for investigating the immune response of channel catfish to E. ictaluri, and may be applicable for screening populations of fish for previous exposure.

REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

The possibility of protecting channel catfish populations by vaccination has been reviewed by Plumb in 1988 (25) and Thune et al. in 1997 (36). Edwardsiella ictaluri is known to induce an antibody response after natural disease or immunisation. A commercial vaccine consisting of an inactivated bacterin was provisionally licensed in the USA against ESC and marketed in 1991. The effectiveness of this vaccine was low and it is no longer marketed. Age-related factors and the induction of a cellular immune response could be of critical importance in inducing strong anti-E.-ictaluri defences. Recent studies by Petrie-Hanson and Ainsworth show that catfish fry under 3 weeks of age are immunologically unresponsive to E. ictaluri (23), and this is thought to be due to poorly developed lymphoid organs in the young fish (24). New attenuated agents show promise in inducing more effective immune responses (7, 17, 18, 35). One such mutant is commercially available (17) and when administered at a high dose to 12-day-old fry, persists long enough to induce protective immunity (42). Additional research in developing genetically resistant strains of catfish show promise (43) and may help to reduce losses caused by this important disease.

REFERENCES


Chapter 2.1.12. - Enteric septicaemia of catfish (*Edwardsiella ictaluri*)


Chapter 2.1.12. - Enteric septicaemia of catfish (*Edwardsiella ictaluri*)


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**NB:** There is an OIE Reference Laboratory for Enteric septicaemia of catfish (*Edwardsiella ictaluri*) (see Table at the end of this Aquatic Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
NB: This disease is no longer listed in chapter 1.2.3 of the Aquatic Code. This chapter has not been updated since 2003

CHAPTER 2.1.13.

PISCIRICKETTTSIOSIS
(Piscirickettsia salmonis)

SUMMARY

Piscirickettiosis is a disease of salmonids caused by Piscirickettsia salmonis that was first reported in farmed coho salmon (Oncorhynchus kisutch). The disease was initially described in 1989 from fish in Chile. Piscirickettsia salmonis is a Gram-negative, highly fastidious, intracellular bacterial pathogen of fish. It is distantly related to the genera Coxiella and Francisella and is grouped with the gamma subdivision of the Proteobacteria.

The identification of P. salmonis is based on isolation of the causative agent with subsequent testing for characteristics of this intracellular pathogen. Piscirickettsia salmonis occurs in cytoplasmic vacuoles in the host cell. The organism may be distinguished from Chlamydia as it does not possess the characteristic chlamydial developmental cycle, and does not contain the group-specific lipopolysaccharide chlamydial antigen. The identity is confirmed by means of serological tests or polymerase chain reaction (PCR).

For a rapid result, the identity of P. salmonis isolated in cell culture or observed in smears from diseased tissue may be confirmed by means of the fluorescent antibody test or immunohistochemical methods with polyclonal or monoclonal antibodies, or by PCR with P.-salmonis-specific primers.

The implementation of hygienic measures and management policy are the only control methods currently available. Antibiotics have been used, but their value is questionable. Intensive efforts are underway by various groups to develop an effective vaccine.

INTRODUCTION

Piscirickettiosis is a septicemic condition of salmonids. The causative agent of the disease is Piscirickettsia salmonis (ATCC VR-1361), and the type strain is LF-89 (13, 15). Thus far the disease has been described from Chile (5, 12), Ireland (22, 23), Norway (21), and both the West (6, 11) and East (16) coasts of Canada.

Piscirickettsia salmonis has been detected in coho salmon (Oncorhynchus kisutch), chinook salmon (O. tshawytscha), sakura salmon (O. masou), rainbow trout (O. mykiss), pink salmon (O. gorbuscha) and Atlantic salmon (Salmo salar). Coho salmon are believed to be most susceptible (13). Mortality in seawater net pens was reported to be 30–90% among coho salmon reared in Chile (5). Piscirickettssia-salmonis-like organisms have recently been isolated from non-salmonid fish (7–9). The relationships of most of these organisms to P. salmonis have not been fully elucidated, but the organism isolated from white sea bass (Atractoscion nobilis) (7) is genetically indistinguishable from P. salmonis.

The mechanisms of transmission are still under investigation. The disease has been primarily reported in marine fish farms, and has also been observed in freshwater facilities (4, 14). Horizontal transmission occurs in saltwater and freshwater (2, 10). Transmission by vectors remains a consideration, and the role of vertical transmission is obscure.

Although antibacterial treatment provides some benefit, it is not entirely effective as a means of controlling the disease. Currently, oxalinic acid appears to be the drug of choice. Eggs may be disinfected as part of good hatchery practice.
The disease is a chronic, systemic infection that generally affects salmonids reared in sea water. All ages are susceptible, from smolts to market size fish. Signs of the disease both external and internal are well documented (10, 12, 21, 23). The disease begins approximately 1 month after fish are introduced into the seawater net pens, and the organism was thought to be a marine bacterium.

A range of gross signs of infection may be present in salmonids infected with *P. salmonis*. Severely affected fish are dark, anorexic and lethargic. They often swim near the surface or edges of the cages. Fish with milder infections often show no abnormal external signs. Infections of the brain may cause erratic swimming behaviour (25). Skin lesions, appearing as small white patches that can progress to shallow ulcers, may also be present on some fish. Perhaps the most consistent external signs observed during *P. salmonis* infections are pale gills resulting from a significant anaemia, but this is not pathognomonic for the disease.

Consistent with many systemic, chronic inflammatory diseases of salmonids, the internal signs are a swollen and discoloured kidney and an enlarged spleen. Ascites in the peritoneum may be present and haemorrhages on the visceral fat, stomach, swim bladder, and body musculature can also occur (10, 24). Hallmark internal lesions of the disease are found in the liver, which may exhibit large, whitish or yellow, multifocal, coalescing, pyogranulomatous nodules. These lesions often rupture, resulting in shallow crater-like cavities in the liver. Whereas these liver lesions are somewhat unique to piscirickettsiosis, many fish with the disease do not exhibit them.

The most prominent histological changes are found in the liver, kidney, spleen and intestine, but pathological changes in the brain, heart, ovary and gill can also be observed (3, 7, 10, 22, 24). Multifocal necrosis of hepatocytes, accompanied by a chronic inflammatory infiltrate of mononuclear cells, is observed in the liver. Vascular and perivascular necrosis are also evident in the liver, and intravascular coagulation resulting in fibrin thrombi within major vessels is a common finding. The focal areas of necrosis underlie the pale circular lesions observed grossly in more chronically infected fish. In more acute infections, the coalescence of areas of necrosis results in a more mottled appearance to the organ rather than discrete nodules. Granulomatous inflammation also occurs in the interstitium and parenchyma of the kidney and spleen, respectively. Vascular changes similar to those in the liver may also be observed in the kidney and spleen. Meningitis, endocarditis, peritonitis, pancreatitis, and branchitis may be observed with accompanying chronic inflammatory and vascular changes similar to those in the liver and haematopoietic organs. The ovary was reported to be involved in certain infections in coho salmon (10).

High magnification examination of lesions reveals aggregates of the organism in the cytoplasm of degenerated hepatocytes and in macrophages. Infected macrophages are usually hypertrophied and replete with cellular debris. In tissue sections stained with haematoxylin and eosin (H&E), the organism appears as basophilic or amphophilic spheres, about 1 µm in diameter.

**DIAGNOSTIC PROCEDURES**

Gross and microscopic changes resulting from piscirickettsiosis are not unique enough to allow for definitive diagnosis of the disease. Therefore, screening for and diagnosis of piscirickettsiosis is based on detection of the causative agent. Presumptive diagnosis can be achieved by the visualisation of the causative agent within macrophages or hepatocytes in histological sections or tissue imprints. Confirmatory diagnosis is achieved by isolation of *Piscirickettsia salmonis* in cell culture, but it does not grow on any known artificial bacteriological media. Confirmation of *P. salmonis* in culture may be made by indirect fluorescent antibody test (IFAT) or polymerase chain reaction (PCR) assay.

PCR assays can also be conducted directly on tissues (19, 20), and thus PCR assays on tissues along with the observation of suspect organisms within macrophages or hepatocytes are also suitable methods for confirmatory diagnosis. Alternatively, *P. salmonis* can be detected with Giemsa-stained tissue smears, followed by IFAT for positive identification. An enzyme-linked immunosorbent assay for detecting *P. salmonis* is commercially available, although there is no published information using this method.
**Piscirickettsia salmonis**-infected fish tissues suitable for examination in cell culture, PCR, tissue imprints and histology are kidney, liver and blood, collected from diseased fish during either overt or covert infections (18). Due to sensitivity of *P. salmonis* to antibiotics *in vitro*, none should be used in media during collection of tissue or the culture of cells.

**Sampling procedures:** See Chapter I.1 Section B.

1. **STANDARD MONITORING METHODS FOR PISCIRICKETTSIOSIS**

   1.1. Isolation of *Piscirickettsia salmonis* in cell culture (18)

   **Cell line to be used:** CHSE-214 or EPC (without antibiotics added)

   a) **Preparation of tissue**

   i) The kidney must be aseptically removed and transferred to a sterile container. Antibiotics must not 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), and then, without centrifugation, further diluted 1/5 and 1/50 in antibiotic-free BSS for inoculation on to 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 cell monolayers and 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 *P. salmonis* CPE consists of plaque-like clusters or rounded cells. With time, the CPE progresses until the entire cell sheet is destroyed.

   iv) If CPE does not occur (except in positive controls), cultures should be incubated at 15–18°C for an additional 14 days.

1.2. **Giemsa stain and fluorescent antibody test of cell culture supernatant**

Fluid from cell cultures showing extensive CPE can be spotted directly on to microscope slides, and stained with Giemsa or tested in the IFAT (17) as described in the following section.

2. **DIAGNOSTIC METHODS FOR PISCIRICKETTSIOSIS**

   2.1. **Giemsa stain**

   i) Preparations of tissue culture supernatant, smears or impressions of the kidney, liver, and spleen should be prepared, air-dried, and fixed for 5 minutes in absolute methanol.

   ii) Immerse slides in a working solution of Giemsa stain for 30 minutes.

   **Stock solution:** 0.4 (w/v) in buffered methanol solution, pH 6.9 (commercially available).

   **Working solution:** diluted 1/10 in phosphate buffer pH 6.0 (0.074 M NaH₂PO₄, 0.009 M Na₂HPO₄).

   iii) Destain with tap water.

   iv) Observe slides under oil immersion. Tissue smears from infected organs show darkly stained pleomorphic organisms occurring in coccoid or ring forms, frequently in pairs, with a diameter of 0.5–1.5 µm.
2.2. Fluorescent antibody test of tissue smear (17)

i) The positive identity of \( P. \) \( \text{salmonis} \) isolated in cell culture or observed in Giemsa-stained smears may be determined by serological methods, for example IFAT.

ii) Smears or impressions of the kidney, liver, and spleen should be prepared, air-dried, and fixed for 5 minutes in absolute methanol.

iii) Tissues smears to be examined by IFAT must be freshly prepared or stored at \(-20^\circ\text{C}\).

iv) The sample is first incubated with anti-\( P. \text{-salmonis} \) polyclonal or monoclonal antibody, then washed and incubated with a secondary antibody conjugated with fluorescein isothiocyanate.

v) Following washing, apply glycerol-based mounting media and cover-slip, then examine under a fluorescence microscope.

2.3. Histology

i) Preserve visceral organs in formalin-based fixative and process for routine histology.

ii) Stain histological slides with H&E or Giemsa.

iii) Examine macrophages within the kidney interstitium, spleen or blood, or hepatocytes within liver lesions, for the presence of multiple, spherical, basophilic or amphophilic bodies (by H&E) or dark blue (by Giemsa), approximately 1 \( \mu \text{m} \) in diameter in the cytoplasm.

2.4. Immunohistochemistry of tissue section (1)

i) Sections (5 \( \mu \text{m} \)) of formalin-fixed, paraffin-embedded tissues are deparaffinised, and treated to eliminate endogenous peroxidase activity.

ii) The tissue is first incubated with anti-\( P. \text{-salmonis} \) polyclonal or monoclonal antibody, then washed and incubated with a secondary antibody conjugated with horseradish peroxidase.

iii) Following washing, the tissue is exposed to a chromogen, counterstained with haematoxylin, dehydrated and prepared for examination under a light microscope.

2.5. Polymerase chain reaction amplification (20)

A nested PCR has been developed to detect genomic DNA of \( P. \text{salmonis} \) using general bacterial 16S rDNA primers in the first amplification and \( P. \text{-salmonis} \)-specific primers in a second reaction. The two general eubacterial primer sequences are EubA (1518R) 5’-AAG-GAG-GTG-ATC-CAN-CCR-CA-3’ and EubB (27F) 5’-AGA-GTT-TGA-TCM-TGG-CTC-AG-3’. The two specific \( P. \text{salmonis} \) primers PS2S (223F) and PS2AS (690R) have the following sequences: 5’-CTA-GGA-GAT-GAG-CCC-GCG-TTG-3’ and 5’-GCT-ACA-CCT-GAA-ATT-CCA-CTT-3’, respectively. These primers yield a specific 467 \( \text{bp} \) product. The PS2AS primer sequence has been updated to correspond to corrections in the target DNA sequence (GenBank accession number U36941).

a) Preparation of infected cell culture supernatant or tissue

Use of a commercially available spin column to purify DNA from cell culture supernatant or tissue is recommended for PCR sample preparation. In addition to following the manufacturer’s instructions on the use of the columns, initial digestion of the sample in lysis buffer (20 mM Tris/HC, pH 8.0, 2 mM EDTA (ethylene diamine tetra-acetic acid), 1.2% Triton X-100, 4 mg/ml lysozyme) at 37\(^\circ\text{C}\) for 30 minutes is suggested.

b) Nested polymerase chain reaction

i) Add 5 \( \mu \text{l} \) of the DNA preparation to the 45 \( \mu \text{l} \) of reaction mixture consisting of PCR buffer (10 mM Tris/HCl, pH 9, 1.5 mM MgCl\(_2\), 50 mM KCl, and 0.1% Triton X-100), 200 \( \mu \text{M} \) each of dATP, dCTP, dTTP and dGTP, 1 \( \mu \text{M} \) EubA primer, 1 \( \mu \text{M} \) EubB primer,
and 2.5 units Taq DNA polymerase. The mixture is covered with 50 µl of mineral oil. Denature the mixture at 94°C for 2 minutes and then amplify by 35 cycles at 94°C for 1 minute, 50°C for 2 minutes, and 72°C for 3 minutes.

ii) The second amplification is performed by adding 2 µl of the first PCR products to 48 µl of reaction mixture containing 2 µM each of the PS2S and PS2AS primers instead of EubA and EubB under the following reaction conditions: denature the mixture at 94°C for 2 minutes and then amplify by 35 cycles at 94°C for 1 minute, 65°C for 2 minutes and 72°C for 3 minutes.

Amplified DNA (476 bp) (10 µl of the PCR reaction mixture) is analysed by electrophoresis in 2% agarose TAE gel (40 mM Tris acetate/1 mM EDTA) containing 1 mg per 50 ml ethidium bromide. To confirm the identification, 10 µl aliquots of the PCR amplification mixture can be digested with EcoR1 and Pst1 according to the manufacturer’s instructions (expected products: EcoR1 994, 546; Pst1 541, 519, 480). If a product is obtained with the P.-salmonis-specific primers but does not cut as expected, further confirmation of the isolate should be obtained by sequencing.

c) Direct polymerase chain reaction

Add 5 µl of the DNA preparation to the 45 µl of reaction mixture consisting of PCR buffer (10 mM Tris/HCl, pH 9, 1.5 mM MgCl₂, 50 mM KCl, and 0.1% Triton X-100), 200 µM each of dATP, dCTP, dTTP and dGTP, 2 µM each of PS2S primer and PS2AS primer, and 2.5 units Taq DNA polymerase. The mixture is covered with 50 µl of mineral oil if the thermocycler does not have a hot bonnet. The direct PCR is performed under the same reaction conditions as for the second step of the nested PCR.

OTHER PCR assays have been developed to detect P. salmonis (19). These primer sequences and reaction conditions would also be suitable for confirmation of the presence of P. salmonis.

REFERENCES


Chapter 2.1.13. - Piscirickettiosis (Piscirickettsia salmonis)


CHAPTER 2.1.14.

GYRODACTYLOSIS

(Gyrodactylus salaris)

1. CASE DEFINITION

Gyrodactylosis is a disease of Atlantic salmon (Salmo salar) caused by the viviparous freshwater parasite Gyrodactylus salaris (Platyhelminthes; Monogenea). All stages of salmon, including adult spawners, in freshwater, can be infected, but disease and mortality has only been observed in pre-smolt stages. In the early disease phase, increased flashing (fish scratch their skin on the substrate) is typical. Later, fish may become greyish due to increased mucus production and the fins may be eroded. Diseased fish are lethargic and are usually found in slower-moving water. Mortalities in farmed fish may be 100% if not treated while population reductions as high as 98% of salmon have been observed in rivers. Corroborative diagnostic criteria are summarised in Section 5 of this chapter.

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

a) Agent factors

- Several strains or clades of Gyrodactylus salaris have been identified based on phenotyping or genotyping (mainly the latter). All strains of this parasite from Atlantic salmon and rainbow trout (Oncorhynchus mykiss) that have been studied in laboratory experiments so far are highly pathogenic to Atlantic salmon. Recently, strains non-pathogenic to salmon were found on non-anadromous char in Norway and rainbow trout in Denmark.

- Gyrodactylus salaris is an obligate parasite with a direct life cycle. Parasites give birth to offspring; there are no eggs, resting stages, specialised transmission stages or intermediate hosts.

- Survival of detached parasites is temperature dependant, e.g. about 24 hours at 19°C, 54 hours at 13°C, 96 hours at 7°C and 132 hours at 3°C.

- Gyrodactylus salaris survives all temperatures between 0°C and 25°C. Tolerance to temperatures above 25°C is unknown. It is not resistant to freezing.

- Gyrodactylus salaris is not drought resistant and must be surrounded by water for survival.

- Gyrodactylus salaris dies after few days at pH≤5. It is more sensitive to low pH (5.1<pH<6.4) in association with aluminium and zinc than the host Atlantic salmon.

b) Host factors

- Gyrodactylus salaris is mainly an ectoparasite on Atlantic salmon (Salmo salar) but can survive and reproduce on several salmonids, such as rainbow trout (Oncorhynchus mykiss), Arctic char (Salvelinus alpinus), North American brook trout (Salvelinus fontinalis), grayling (Thymallus thymallus), North American lake trout (Salvelinus namaycush) and brown trout (Salmo trutta) (in declining order of susceptibility).

- All stages of the host are susceptible, but mortality has only been observed in fry and parr stages.
Strains of Atlantic salmon have shown variable susceptibility to *G. salaris*. The Baltic strains have been considered resistant. However, this has only been shown for salmon from the Russian River Neva. Salmon from the Baltic Swedish River Indalsälven are almost as susceptible as the Norwegian salmon and salmon from the Scottish River Conon. Salmon from other Baltic rivers have shown intermediate susceptibility.

*Gyrodactylus salaris* occurs on the fins on most infected Atlantic salmon. Parasites are also commonly found on the body and less commonly on the gills. On other hosts the distribution may be different; on some species the parasite is relatively less abundant on the fins and relatively more common on the body compared with salmon.

c) Disease pattern

*Gyrodactylus salaris* has spread between rivers and farms mainly by the transport/restocking of live fish. Fish swimming through brackish water can also cause the parasite to be spread between rivers. Although *G. salaris* mainly lives in fresh water, it reproduces normally at salinities up to 5–6 parts per thousand (ppt). Survival at higher salinities is temperature dependent. For example at 1.4°C, *G. salaris* may survive for 240 hours, 78 hours and 42 hours at 10 ppt, 15 ppt and 20 ppt salinity, respectively, while at 12°C it may survive for 72 hours, 24 hours and 12 hours at the same three salinities, respectively (19).

Prevalence in susceptible strains of Atlantic salmon in rivers and farms soon become 100%. Prevalence in resistant strains in rivers and farms is unknown. Prevalence in other susceptible species is usually much lower and can be below 10%, e.g. in farmed rainbow trout.

*Gyrodactylus salaris* is restricted in its distribution to Europe. It has been found in farmed Atlantic salmon or farmed rainbow trout in several (mainly northern) European countries. The parasite has been found in wild salmonids, mainly Atlantic salmon parr, from rivers in Russia, Sweden and Norway. *Gyrodactylus salaris* is much more common in farmed rainbow trout than previously thought, and is likely to be present in more countries than those currently known. Great Britain and Ireland appear to be free from the parasite.

Mortality can be 100% in susceptible farmed Atlantic salmon if not treated. Mortality in Norwegian rivers can be as high as 98%, with an average of about 85%. Mortality in other susceptible host species is usually low or not observed.

Economic loss in farmed fish should be relatively low as treatment will easily keep the number of parasites to an acceptable level. The economic loss due to *G. salaris* in Norwegian rivers is estimated to be more than 1 billion NOK over the past 25 years.

d) Control and prevention

Vaccines are not feasible.

*Gyrodactylus salaris* is sensitive to changes in the chemical composition of the water. It is sensitive to most chemicals used for bath treatment of farmed salmon parr and its eggs, e.g. high salinity salt water, formaldehyde and compounds containing chlorine and iodine.

Immunostimulation is not feasible.

Resistance breeding has not been tried.

Restocking with resistant strains of Atlantic salmon (e.g. Baltic Neva strain) in affected rivers is not compatible with existing strain management of Atlantic salmon.

The general recommended husbandry practices for avoiding the spread of infective agents between units in freshwater fish farms apply to *G. salaris*. Equipment used in one unit should not be used in another without adequate disinfection.
Chapter 2.1.14. - Gyrodactylosis (Gyrodactylus salaris)

3. DIAGNOSTIC METHODS

a) Field diagnostic methods

- Usually there are no clinical signs in fish with a few tens of parasite specimens.
- Flashing is common among moderate to heavily infected farmed fish as they scratch their skin on the bottom or wall of a tank or pond.
- Heavily infected fish may have reduced activity and stay in low current areas.

b) Clinical methods

- When present on wild and farmed susceptible Atlantic salmon parr and smolt, G. salaris usually causes gyrodactylosis. Heavily infected fish may become greyish as a result of increased mucification; at a later stage the dorsal and pectoral fins may become whitish as a result of increased thickness (mainly hypertrophy) of epidermis.
- Heavily infected fish may have eroded fins, especially dorsal, tail and pectoral fin, due to parasite feeding.
- Secondary fungal infections (Saprolegnia spp.) are commonly observed in fish with gyrodactylosis.
- Disease outbreaks may occur at any time and at any water temperature, but are most common in spring and in periods when the water temperature is 7–17°C.
- Scrapings (wet mounts) from skin or fins can be used to detect Gyrodactylus specimens on fish with gyrodactylosis. In these cases, with high intensity infestation, hundreds or thousands of Gyrodactylus specimens are present all over the body and fins. Wet mount preparations are usually not suitable for identification of Gyrodactylus to the species level and other preparations for morphological or DNA analysis must be made (see below). If the number of Gyrodactylus specimens is low, the chances of detecting the parasites by scrapings are limited.

c) Agent detection and identification methods

- Detection of Gyrodactylus and identification of G. salaris is a two-step process. Firstly, parasite specimens are observed using optical equipment and secondly, parasites are identified, usually on an individual basis using other equipment and methods.
- Optical equipment must be used to detect Gyrodactylus. If in the case of a suspected gyrodactylosis outbreak, only light microscopy is available, wet mounts can be used to detect Gyrodactylus specimens. However, it is strongly advised not to use this method in a surveillance programme as the specificity is very low (value not known) and therefore, the number of fish examined needs to be unreasonably high.
- Fish can be examined as whole specimens alive (under anaesthesia), freshly killed or preserved/fixed. The same examination method (see below) is used in all cases. Examination of live, anaesthetised fish is very time-consuming and not recommended. Examination of formaldehyde-fixed fish is not recommended for reasons of operator safety. Fixed Gyrodactylus specimens are also very difficult to identify.
- Instead of examining the whole fish, the fins can be examined (by the method described below). When a Norwegian salmon parr is infested, almost all fish have at least one G. salaris on one of the fins (6, 17). On some fish, G. salaris specimens may occur on the body or head, including the nostrils, the gills and the mouth cavity. The distribution of G. salaris on fins and other parts of the fish varies between fish species and seems to vary between salmon strains.
- Fish or fins only should be examined under a binocular dissecting microscope with good illumination. The fish should be placed in a box and completely covered in water, preferably in the water in which they were transported. Living parasites are more easily detected by their
movements, so disturbing light refraction on the skin of the fish should be avoided. Live *Gyrodactylus* are colourless. Fish should be killed humanely ensuring that blood does not enter the water. If the dissecting microscope is illuminated from above, the bottom of the microscope stage should be black. This will increase the contrast and the parasites will be detected more easily. The whole surface of the fish, including gills and mouth cavity, must be examined. It is best to use two forceps for this process. The fins of relatively small fish, usually less than 10 cm, can also be studied using illumination through the bottom of the microscope stage. This way, *Gyrodactylus* specimens on the fins can usually be easily observed. Fins, especially larger ones, should be examined on both sides using a forceps to turn them.

- Preserved fish or fins can be studied in a similar way under a dissection microscope with illumination from above. Ethanol-preserved *Gyrodactylus* specimens are usually only slightly opaque. Before examination, the fish should be rinsed in tap water. This can be done by placing the transport container under a tap and letting the water flow slowly (gently) through the container for some time to allow the fish to sink. Before rinsing, however, the preservative, including the bottom sediment in the container, should be examined separately for the presence of *Gyrodactylus* specimens that may have washed off during preservation and transportation. For operator protection purposes, the dissecting microscope could be placed on a suction bench with a downwards outlet to avoid inhalation of evaporated preservative.

- Whole fish should be preserved in relatively large bottles that provide excess space and preservative. The concentration of ethanol after conservation should be about 70%. If the concentration is lower, the mucus and epidermis may disintegrate and *Gyrodactylus* specimens, even if they are preserved, may drop off. Because water is released from the fish during preservation, the concentration of ethanol may be as high as 96% before the fish are added. The opening of the bottles should be wide to avoid the possibility of scraping off *Gyrodactylus* specimens when the fish are taken out for examination. Bottles should be stored in a horizontal position, as should the fish, during fixation/preservation to prevent the fish curling. This facilitates later examination of the fish as they can easily be turned with a pair of forceps under the microscope. When preservation of the fish is complete, the bottles can be stored in a vertical position.

- If live fish need to be transported to a laboratory for examination, the fish should be transported in the same water as that in which they lived. Because excretory (and other) products from the fish may change the water quality, the number of fish/fish volume should be relatively small compared with the volume of water in the container. Transportation time should also be considered. The container should carry one fish species only because experiments and experience have shown that mixing fish species may initiate detachment of *Gyrodactylus* specimens from their hosts.

- *Gyrodactylus* specimens may die or detach from the host if the water quality/chemistry is changed. Furthermore, *Gyrodactylus* specimens die rapidly if not covered with water, and often leave a host soon after it dies.

- Dead fish, transported on ice, are not acceptable for *Gyrodactylus* examination, even if the fish are sent separately in plastic bags, etc. The parasites soon die if not covered in water, and as these parasites do not have an exoskeleton, dead parasites disintegrate as quickly as the mucus and epidermis cells of the fish. If such dead fish are rinsed in water, *Gyrodactylus* specimens may be found in the sediment. However, if specimens are not found in the sediment, it cannot be concluded that the fish were uninfected.

- *Gyrodactylus salaris* identification based on morphology and morphometry of sclerites in the attachment organ

- Identification of *Gyrodactylus* species is based on morphology and morphometry of marginal hooks, anchors (hamuli) and bars in the opisthaptor (the attachment organ). Good preparation of specimens is a prerequisite for discrimination of species (10).
• Malmberg’s ammonium picrate glycerine (APG) method is commonly used for preparing whole mounts of small Monogenea (9). According to this method, a drop of water is placed on a slide, a worm is transferred to the water and a cover-slip is gently placed on top. Using a piece of filter paper, as much water as possible is absorbed at the edge of the cover-slip so that the worm is very depressed but not squashed. A small drop of APG is added to the edge of the cover-slip. The parasite will be fixed as the yellow APG solution penetrates the space between the slide and the cover-slip. The slide should be labelled with the host species, location, locality, date of collection and water temperature. If the slide is to be transported, a small drop of nail-polish or a similar substance should be added to each corner of the cover-slip for ‘permanent’ attachment to the slide. Live worms are preferable for preparing APG whole-mounts because they can be depressed under cover-slip pressure. Fixed/preserved worms can be used, however they are more difficult to depress, resulting in inferior specimens that may be difficult to identify.

• The APG solution is made by mixing one part saturated ammonium picrate solution and one part glycerine/glycerol (puriss). Identification of *G. salaris* should be in accordance with refs 4, 9, 11, 14–16, 18, 20.

• Alternatively to the APG-method, live or ethanol-preserved specimens can be placed in a drop of proteinase K on a slide and covered with a cover-slip for a few hours (depending on the temperature). After digestion of the parasite soft parts, the opisthaptoral sclerites are suitable for species identification.

• Morphology and morphometry in *G. salaris* are presented in Table 1 and Figures 1 and 2.

• *Gyrodactylus salaris* is morphologically similar to *G. teuchis* from brown trout, Atlantic salmon, and rainbow trout, and to *G. thymalli* from grayling. The species can be differentiated on the basis of the shape of the marginal hook sickle. *Gyrodactylus teuchis* has a longer and more constantly curved sickle blade while *G. thymalli* has a small angle on the shaft of the sickle (4, 11, 18, 20).

**Table 1.** Total range of variation in 14 characters measured on marginal hooks, anchors, and ventral bars of *Gyrodactylus salaris* on Atlantic salmon parr and rainbow trout. The water temperature at the time of measuring varied between 0.0°C and 20.0°C. All measurements are in µm (*n* = number of specimens measured)
Chapter 2.1.14. — Gyrodactylosis (*Gyrodactylus salaris*)

Figure 1. Morphological variability/variation in opisthaptoral hand parts of *Gyrodactylus salaris*. 
*a) Marginal hooks; b) Anchors; c) Ventral bar. Scale bars: 40 µm, 50 µm and 30 µm, respectively."

Figure 2. Fourteen characters measured on marginal hooks, anchors and ventral bars of *Gyrodactylus salaris*.

\[
\begin{align*}
\text{lmh} &= \text{total length of marginal hook} \\
\text{lh} &= \text{length of marginal hook handle} \\
\text{lsi} &= \text{length of marginal hook sickle} \\
\text{la} &= \text{total length of anchor} \\
\text{las} &= \text{length of anchor shaft} \\
\text{lap} &= \text{length of anchor point} \\
\text{lar} &= \text{length of anchor root} \\
\text{mdpvd} &= \text{maximum distance between processes of ventral bar}
\end{align*}
\]

\[
\begin{align*}
\text{lvm} &= \text{length of ventral bar} \\
\text{tbwvb} &= \text{total basal width of ventral bar} \\
\text{bwvb} &= \text{basal width of ventral bar} \\
\text{tmwvb} &= \text{total median width of ventral bar} \\
\text{mwvb} &= \text{median width of ventral bar} \\
\text{lvbm} &= \text{length of ventral bar membrane} \\
\text{tmwvb} &= \text{mwvb} + \text{lvbm}
\end{align*}
\]
• *Gyrodactylus salaris* identification based on DNA analysis

*a) Preparation of samples*

Template DNA should be prepared from live/fresh or ethanol-preserved specimens using a suitable DNA preparation protocol. A DNA extraction kit may be used in accordance with the manufacturer’s recommendations.

*b) Analysis of the ribosomal RNA gene internal transcribed spacer region (2, 4)*

*i) PCR amplification of the internal transcribed spacer (ITS)*

For amplification of a 1300 base pair product of the ITS-region, primers 5'-TTT-CCG-TAG-GTG-AAC-CT-3' and 5'-TCC-TCC-GCT-TAG-TGA-TA-3', may be used. Annealing temperature should be 50°C.

*ii) ITS sequencing and sequence analysis*

Amplified ITS prepared as in Section b.i above may be sequenced and compared with those of *G. salaris*, *G. derjavini*, *G. truttae* and *G. teuchis* EMBL nucleotide database accession numbers Z72477, AJ132259, AJ132260 and AJ249350, respectively. *Gyrodactylus salaris* and *G. thymalli* have the identical sequence in this region of the genome and thus cannot be distinguished by this method.

*c) Analysis of the ribosomal RNA gene intergenic spacer region (1, 3)*

*i) PCR amplification of the intergenic spacer (IGS)*

For amplification of the IGS-region, primers 5'-CTG-GCT-ATA-ATC-ACG-TAA-GAC-TGC-3' and 5'-AAG-ATA-CTC-ATT-TGA-CTC-GGT-GTG-3' may be used. Annealing temperature should be 55°C.

*ii) IGS sequencing and sequence analysis*

Amplified IGS prepared as in Section c.i above may be sequenced and compared with other IGS sequences. Sequences are aligned using letter codes for the 23 bp repeats in the IGS, or the nucleotide sequences. Identical sequences from the same location and sequences that results in ambiguous alignments are removed, as are invariant positions in the alignment (1, 3). Phylogenetic analysis is carried out using appropriate methods such as parsimony or distance analysis (21), with re-sampling of data. Many strains of *G. salaris* and *G. thymalli* can be distinguished with this method.

*d) Analysis of the mitochondrial cytochrome oxidase I gene (5, 12, 13)*

*i) PCR amplification of the mitochondrial cytochrome oxidase I (COI) gene* 

For amplification of the COI-gene, primers LA and HA (5'-TAA-TCG-GCG-GGT-TCT-GTA-A-3' and 5'-GAA-CCA-TGT-ATC-GTG-TAG-CA-3'), or LB and HB (5'-TAA-TTG-GTG-GGT-TTG-GTA-A-3' and 5'-AGC-TAC-CAC-GAA-CCA-TGT-AT-3') may be used. Annealing temperature should be 50°C.

*ii) COI sequencing and sequence analysis*

Amplified COI prepared as in Section d.i above may be sequenced and compared with other COI sequences. Sequences are aligned and phylogenetic relationships inferred using suitable algorithms such as neighbour-joining and maximum parsimony (7, 8), with appropriate re-sampling. Different clades of *G. salaris* and *G. thymalli* can be distinguished with this method.

• Indirect detection methods

Not available.
4. RATING OF TESTS AGAINST PURPOSE OF USE

The same methods to detect and identify *Gyrodactylus salaris*, respectively, must be used independently of purpose.

5. CORROBORATIVE DIAGNOSTIC CRITERIA

a) Definition of suspect case

Observation of *Gyrodactylus* specimen(s) on fins or skin of Atlantic salmon or rainbow trout in skin scrapings or by stereo-microscopic examination.

b) Definition of confirmed case

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

6. DIAGNOSTIC/Detection METHODS TO DECLARE FREEDOM

Diagnostic/detection methods to declare freedom are the same as those mentioned in for Sections 4 and 5 above.

REFERENCES


Chapter 2.1.14. - Gyrodactylosis (Gyrodactylus salaris)


*  *

NB: There is an OIE Reference Laboratory for Gyrodactylosis (Gyrodactylus salaris) (see Table at the end of this Aquatic Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.1.15.

RED SEA BREAM IRIDO VIRAL DISEASE

1. CASE DEFINITION

For the purpose of this chapter, red sea bream iridoviral disease (RSIVD) (8) is considered to be infection with red sea bream iridovirus (RSIV). RSIV is a significant cause of mortality among red sea bream (*Pagrus major*) and more than 30 other species of cultured marine fish (15, 21) belonging mainly to the orders Perciformes and Pleuronectiformes. The first outbreak of RSIVD was recorded in cultured red sea bream in Shikoku Island, Japan in 1990 (8). Since 1991, the disease has caused mass mortalities in cultured fish populations in the western part of Japan, mainly among juvenile red sea bream. Affected fish become lethargic, exhibit severe anaemia, petechiae of the gill, and enlargement of the spleen (8, 12, 24). The disease is characterised by the appearance of enlarged cells stained deeply with Giemsa solution on microscopic observation of tissue sections of spleen, heart, kidney, intestine and gill of infected fish (8).

Recently, it has been proved that the disease is caused not only by RSIV (8, 9, 10, 18, 20, 29) and its synonyms (2–6, 12, 13, 16, 19, 22, 40), but also by infectious spleen and kidney necrosis virus (ISKNV) (7, 31), and that the distribution of the disease is not restricted to Japan but that the disease is found widely in East and South-East Asian countries (2–6, 9, 10, 12, 13, 16, 19, 22, 31, 40).

Monoclonal antibodies (MAbs) against RSIV (30) can detect both RSIV and ISKNV whereas they do not recognise fish ranaviruses (family: Iridoviridae) by immunofluorescent antibody tests (IFAT) (28, 31). A number of useful diagnostic methods are in use, such as the observation of stained impression smears or tissue sections, an IFAT using a MAb, and a polymerase chain reaction (PCR) (11, 17, 25, 28, 32, 33). A formalin-killed vaccine for RSIVD is effective and is commercially available in Japan (26–27).

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

a) Agent factors

- Aetiological agent, agent strains: the disease is caused by red sea bream iridovirus (RSIV) (8-10, 18, 20, 29) Ehime-1 strain and other genotypes of RSIV including many viruses that are considered to be synonyms of RSIV (2–6, 12, 13, 16, 22, 40). The disease is also caused by infectious spleen and kidney necrosis virus (ISKNV) (7, 31), which is one of the viruses related to, but distinct from, RSIV. A number of other iridoviruses that cause similar diseases in ornamental freshwater fish have been reported (34, 39). These viruses are difficult to distinguish genetically from ISKNV; it has not been determined whether these diseases should be included in RSIVD. Recently, turbot reddish body iridovirus (TRBIV) (37) and its probable synonyms (4, 10), which are related to, but distinct from, RSIV and ISKNV, were reported from the People’s Republic of China and the Republic of Korea. Further investigation is necessary before it can be determined whether or not the disease caused by TRBIV should also be included in RSIVD. All these agents belong to the fifth genus of the family Iridoviridae, and they are distinguishable genetically and biologically from fish ranaviruses such as epizootic haematopoietic necrosis virus (EHNV), European catfish iridovirus (ECV) and grouper iridovirus (GIV) (14, 23, 35-37), all of which are not pathogenic to red sea bream (24).
- Survival outside the host: unknown.
Chapter 2.1.15. - Red sea bream iridoviral disease

- Stability of the agent: inactivated at 56°C for 30 minutes; sensitive to ether and chloroform; inactivated by formalin (0.1%); stable in tissue at −80°C.
- Life cycle: unknown.

b) Host factors

- Susceptible host species
  In the case of ISKNV infection: Chinese perch (*Siniperca chuatsi*), barramundi or sea bass (*Lates calcarifer*), red drum (*Sciaenops ocellatus*), Flathead mullet (*Mugil cephalus*), orange-spotted grouper (*Epinephelus coioides*), *Epinephelus* sp. are known.
- Susceptible stages of the host: juvenile to adult (susceptibility of juvenile is generally higher than adult).
- Species or sub-population predilection (probability of detection): in the case of RSIV infection, fish belonging to the genus *Oplegnathus* are more sensitive than others. Protection of these fish by vaccination is difficult.
- Target organs and infected tissue: infected cells are observed in the spleen, kidney, heart, intestine and gill.
- Persistent infection with lifelong carriers: unknown.
- Vectors: unknown.

c) Disease pattern

- Transmission mechanisms: the principal mode of transmission of RSIV is horizontal via the water. Vertical transmission of RSIV has not yet been investigated.
- Prevalence: unknown
- Geographical distribution: RSIVD caused by RSIV and ISKNV has been reported not only from Japan but also widely in other East and South-East Asian countries (Republic of Korea, People’s Republic of China, Taipei China, Hong Kong, Thailand, Singapore, Malaysia and the Philippines) (2–6, 9, 10, 12, 13, 15, 19, 22, 31, 40). The disease caused by ISKNV has not yet been confirmed in Japan and Republic of Korea.
• Mortality and morbidity: depending on host fish species, fish age, water temperature, and other culture conditions, mortality rates range between 0% and 100%. Morbidity is unknown.
• Economic and/or production impact of the disease: severe.

d) Control and prevention
• Vaccination: an effective formalin-killed commercial vaccine for RSIVD is now available for red sea bream, striped jack and other fish species belonging to the genus *Seriola* in Japan.
• Chemotherapy: not available.
• Immunostimulation: under investigation.
• Resistance breeding: under investigation.
• Restocking with resistant species: not useful.
• Blocking agents: unknown.
• General husbandry practices: introducing pathogen free seed. Implementation of hygiene practices at farms. Avoid practices that can decrease water quality and/or increase stress such as overcrowding and overfeeding.

3. DIAGNOSTIC METHODS

a) Field diagnostic methods
• Clinical signs: affected fish become lethargic, exhibit severe anaemia, petechiae of the gill, and enlargement of the spleen.

b) Clinical methods
• Gross pathology: pale gills and enlarged spleen.
• Microscopic pathology:
  • Tissue smears: confirm presence of abnormally enlarged cells in Giemsa-stained stamp-smear of the spleen.
  • Fixed sections: confirm presence of abnormally enlarged cells in tissues such as spleen, heart or intestine.
• Electron microscopy/cytopathology: confirm presence of virions (200–240 nm in diameter) in the enlarged cells by electron microscopy.

c) Agent detection and identification methods
• Direct detection methods
  i) Microscopic methods
Examination of histological sections from diseased fish may reveal abnormally enlarged cells from spleen, heart kidney, liver or intestine.
  ii) Agent isolation and identification
Isolation of RSIV and ISKNV is undertaken using the GF cell line. Spleen and/or kidney tissues from diseased fish are suitable samples. Cells should be grown at 25°C in a temperature-controlled incubator to ensure subsequent success in the isolation of RSIV. Viruses to be used as positive controls can be obtained from the OIE Reference Laboratory. Use uninfected cells as negative controls.
Following development of viral cytopathic effect (CPE), virus identification would be undertaken using either antibody-based antigen detection (IFAT) and/or nucleic acid-based (PCR) methods.

- **Isolation of RSIV and ISKNV in cell culture**
  a) **Inoculation of cell monolayers**
     i) Following the virus extraction procedure described in Chapter I.1 (Section B.3.2), make an additional tenfold dilution of the 1/10 spleen homogenate supernatants and transfer an appropriate volume of each of the two dilutions onto 24-hour-old cell monolayers. Inoculate at least 2 cm² of drained cell monolayer with 100 µl of each dilution.
     ii) Allow to adsorb for 0.5–1 hour at 25°C and, without withdrawing the inoculum, add cell culture medium buffered at pH 7.6 and supplemented with 2% fetal calf serum (FCS) (1 ml/well for 24-well cell culture plates), and incubate at 25°C using a temperature-controlled incubator.
  
  b) **Monitoring incubation**
     i) Follow the course of infection in positive controls and other inoculated cell cultures by daily microscopic examination at ×40–100 magnification for 10 days. The use of a phase-contrast microscope is recommended.
     ii) If a CPE appears in those cell cultures inoculated with dilutions of the test homogenates, identification procedures have to be undertaken immediately (see below).
     If a fish health surveillance/control programme is being implemented, provisions may 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 RSIV or ISKNV.
     iii) If no CPE develops in the inoculated cultures (despite normal progression of CPE in the virus controls) after 10 days incubation, the inoculated cultures should be subcultured for a further 7 days. Should the virus control fail to develop CPE, the process should be repeated with fresh susceptible cells and new batches of samples.
  
  c) **Subcultivation procedures**
     i) Collect aliquots of cell culture medium from all monolayers inoculated with dilutions of each supernatant of the test homogenates.
     ii) Inoculate cell monolayers as described in a) above.
     iii) Incubate and monitor as described in b) above.
     iv) If no CPE occurs, the test may be declared negative.

- **Identification of RSIV and ISKNV**

RSIV (probably ISKNV also) cannot be identified by neutralisation tests as the antisera generated by the immunisation of rabbits have few neutralising antibodies.

a) **Indirect fluorescent antibody test**

The IFAT is to be conducted directly after virus isolation in cell culture.
Chapter 2.1.15. - Red sea bream iridoviral disease

Manual of Diagnostic Tests for Aquatic Animals 2006 255

i) Prepare monolayers of cells on cover-slips in order to reach around 80% confluency, which is usually achieved within 24 hours of incubation at 25°C. The FCS content of cell culture medium can be reduced to 2–4%.

ii) When the cell monolayers are ready for infection inoculate the virus suspension to be identified by making tenfold dilution steps directly in the cell culture wells or flasks.

iii) Incubate at 25°C for 24–72 hours.

iv) When CPE appears, remove the cell culture medium, rinse three times with phosphate buffered saline (PBS). Air-dry the infected cells, then fix with cold acetone (stored at –20°C) for 10 minutes.

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

vi) Prepare a solution of MAb (M10) to RSIV or ISKNV at the appropriate dilution (determined previously).

vii) Treat the cell monolayers with the solution of antibody to RSIV or ISKNV for 30 minutes at 37°C in a humid chamber.

viii) Rinse the cells three times for 5 minutes with PBS.

ix) Incubate with a specific anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody (prepared according to the supplier’s instructions) for 30 minutes at 37°C in darkness in a humid chamber.

x) Rinse three times for 5 minutes with PBS.

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

xii) Examine under incident UV light using a microscope with ×10 eye pieces and ×20-40 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. Positive results are indicated by diffuse fluorescence throughout the cytoplasm.

Levels of validation:

• Specificity and sensitivity: MAb M10 can detect both RSIV and ISKNV (31); it does not detect ranaviruses. The reactivity of the MAb against TRBIV has not yet been confirmed.

• ‘Gold’ standard: the CPE is characterised by many enlarged cells and virus is confirmed in the CPE by IFAT.

Interpretation of results:

• The virus isolated is RSIV or ISKNV and the disease is RSIVD (except in the case of freshwater ornamental fish).

Availability of test: Reagents and biologicals are available from the OIE Reference Laboratory or commercial sources. MAb M10 can be supplied from the OIE Reference Laboratory.

• Antibody-based antigen detection method (IFAT)

Samples to be taken: spleen.
Positive controls (air-dried and fixed spleen imprints from infected fish) can be obtained from the OIE Reference Laboratory. Use imprints of healthy fish spleen as negative controls.

- **Indirect fluorescent antibody test**
  
  i) Bleed the fish thoroughly.
  
  ii) Make spleen imprints on cleaned glass microscope slides.
  
  iii) Store at 4°C the spleen pieces together with the other organs required for virus isolation in case this becomes necessary later.
  
  iv) Allow the imprints to air-dry for 20 minutes.
  
  v) Fix with cold acetone.
  
  vi) Treat the imprints with the solution of MAb (M10) to RSIV for 30 minutes at 37°C.
  
  vii) Rinse three times with PBS.
  
  viii) Treat the imprints for 30 minutes at 37°C with a solution of a specific anti-mouse FITC-conjugated antibody prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.
  
  ix) Rinse three times with PBS.
  
  x) Mount the microscope slides with cover-slips using glycerol saline prior to microscopic observation.
  
  xi) Examine under indirect UV light using a microscope with ×10 eye pieces and ×20–40 objective lens having high numeral aperture. Positive and negative controls must be found to give the expected results prior to any other observation.

Levels of validation:

- Specificity and sensitivity: MAb M10 can detect both RSIV and ISKNV (31); it does not detect ranaviruses. The reactivity of the MAb against TRBIV has not yet been confirmed.

- 'Gold' standard: abnormal enlarged cell with strong fluorescence is confirmed by IFAT.

Interpretation of results:

- If the test is positive, the fish from which the samples were obtained is considered infected with RSIV or ISKNV and the disease is RSIVD (except in the case of freshwater ornamental fish). If the test is negative, process the organ samples stored at 4°C for virus isolation in cell cultures as described above.

Availability of test: Reagents are available from the OIE Reference Laboratory or commercial sources. MAb M10 can be supplied from the OIE Reference Laboratory.

- **Molecular technique (PCR)**

Samples to be tested include spleen from affected fish or supernatants from cell cultures that had developed CPE.

  1. DNA extraction from organ sample or cell culture supernatant of isolated virus

Fish samples or cell culture supernatants are prepared as described in Kurita et al. (17) for DNA extraction. Use pre-confirmed RSIV (or ISKNV)-affected organ or supernatant from RSIV (or ISKNV)-infected cell cultures as positive control. Use
organs from healthy fish or supernatants from non-infected cell cultures as negative control.

b) **Polymerase chain reaction amplification**

RSIV and ISKNV have large double-stranded DNA genomes. Two primers, a forward primer 1-F (5’-CTC-AAA-CAC-TCT-GGC-TCA-TC-3’) and reverse primer 1-R (5’-GCA-CCA-ACA-CAT-CTC-CTA-TC-3’) are used for the amplification of the gene sequence (570 bases) of both RSIV DNA and ISKNV DNA by polymerase chain reaction (PCR). Please note that the previous RSIV-specific OIE primers 4-F (5’-CGG-GGG-CAA-TGA-CGA-CTA-CA-3’) and 4-R (5’-CCG-CCT-GTG-CCT-TTT-CTG-GA-3’) (expected product size is 568 bp) do not amplify ISKNV DNA (31). Both primer sets have been described by Kurita et al. (17)

Extracted nucleic acid (1 µl) is added to Taq polymerase buffer containing 1 mM of each primer, 200 mM of deoxynucleotide triphosphate, 1.25 U of ExTaq DNA polymerase in 20 mM Mg²⁺ PCR buffer. The mixture is incubated in an automatic thermal cycler programmed for 30 cycles at 94°C for 30 seconds, 58°C for 60 seconds, and 72°C for 60 seconds, and finally held at 72°C for 5 minutes. Amplified DNA (570 bp) is analysed by agarose gel electrophoresis.

Levels of validation:
- Specificity and sensitivity: primer set 1-F and 1-R can amplify both RSIV and ISKNV DNA with adequate sensitivity. The previous primer set 4-F and 4-R also has adequate sensitivity for RSIV, but it cannot be used to amplify ISKNV DNA. The reactivity of these primer sets against TRBIV has not yet been confirmed.
- ‘Gold’ standard: PCR product of expected size is clearly confirmed by electrophoresis when primer set 1-F and 1-R are used.

Interpretation of results:
- A positive result by PCR using the primer set 1-F and 1-R, and confirmed specificity by sequencing, indicates the presence of RSIV or ISKNV and that the disease is RSIVD (except in the case of freshwater ornamental fish). A positive result by the optional PCR using the previous primer set 4-F and 4-R and run in conjunction with the previous PCR indicates that the virus is RSIV and that the disease is RSIVD by RSIV. A negative result with this optional, secondary PCR indicates that the virus is ISKNV and the disease is RSIVD by ISKNV.

Availability of test: Reagents are available from the OIE Reference Laboratory or commercial sources.

- **Indirect detection methods**

Serological methods using serum of affected fish have not yet been developed.

4. **RATING OF TESTS AGAINST PURPOSE OF USE**

The methods currently available for surveillance, detection, and diagnosis of RSIV-ISKNV are listed in Table 1. The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic sensitivity and specificity; C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and D = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
### Table 1. RSIV-ISKNV surveillance, detection and diagnostic methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance</th>
<th>Presumptive</th>
<th>Confirmatory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Juveniles</td>
<td>Adults</td>
<td></td>
</tr>
<tr>
<td>Gross signs</td>
<td>D</td>
<td>D</td>
<td>B</td>
</tr>
<tr>
<td>Direct LM of stamp-smear</td>
<td>D</td>
<td>D</td>
<td>B</td>
</tr>
<tr>
<td>Histopathology</td>
<td>D</td>
<td>D</td>
<td>B</td>
</tr>
<tr>
<td>Transmission EM</td>
<td>D</td>
<td>D</td>
<td>B</td>
</tr>
<tr>
<td>Virus isolation and identification by one of the following methods:</td>
<td>C</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>Antibody-based assays (IFAT) of isolated virus</td>
<td>C</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>Antibody-based assays (IFAT) of stamp-smear</td>
<td>C</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>DNA Probes – <em>in situ</em></td>
<td>D</td>
<td>D</td>
<td>C</td>
</tr>
<tr>
<td>PCR</td>
<td>C</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>Sequence</td>
<td>D</td>
<td>D</td>
<td>A</td>
</tr>
</tbody>
</table>

LM = light microscopy; EM = electron microscopy; PCR = polymerase chain reaction.

### 5. CORROBORATIVE DIAGNOSTIC CRITERIA

#### a) Definition of suspect case

The presence of RSIV-ISKNV shall be suspected if at least one of the following criteria is met:

1) Presence of typical clinical signs and gross pathology and confirmation of abnormally enlarged cells on stamp-smear or tissue section.

2) Presence of typical clinical signs and gross pathology and confirmation of the presence of virions in abnormally enlarged cells by electron microscopy.

3) Virus isolation with specific CPE.

4) Presence of IFAT positive cells on stamp-smear.

#### b) Definition of confirmed case

The presence of RSIV-ISKNV shall be considered as confirmed if, in addition to criteria in 5a), one or more of the following criteria is met:

1) Virus isolation with specific CPE and positive result of IFAT using infected cell cultures.

2) Virus isolation with specific CPE and positive PCR using extracted DNA from isolated virus as template.

3) Positive PCR using extracted DNA from affected organ as template.
4) Presence of typical abnormally enlarged cells showing IFAT positive on stamp-smear.

6. **DIAGNOSTIC/DETECTION METHODS TO DECLARE FREEDOM**

There are no established detection methods for surveillance, because the carrier state of the agents has not yet been investigated. A tentative method would be virus isolation followed by IFAT. The nested PCR is also suitable for the purpose (1).

**REFERENCES**


* * *

**NB:** There is an OIE Reference Laboratory for red sea bream iridoviral disease (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.1.16.

WHITE STURGEON IRIDO VIRAL DISEASE

SUMMARY

The white sturgeon iridoviral disease (WSIVD) is a significant cause of mortality among farm-raised juvenile white sturgeon (Acipenser transmontanus) in North America and among Russian sturgeon (A. guldenstadi) in Europe (1, 2, 4). White sturgeon are the host from which the causative agent, the white sturgeon iridovirus (WSIV) was first isolated. Lake sturgeon (A. fluvagens) have been experimentally infected with the WSIV, but the susceptibility of other sturgeon is currently unknown (3).

The original description of white sturgeon iridovirus was among the first hatchery-raised white sturgeon in North America. The source of the virus was assumed to originate from captive wild sturgeon adults collected from the Sacramento River in California, United States of America (USA) for use as broodstock (2). The WSIV has also been detected in cultured white sturgeon from the lower Columbia River in Oregon and Washington states of the USA, the Snake River in the southern region of the State of Idaho and the Kootenai River in northern Idaho, USA (4, 5). These cultured white sturgeon were all progeny originating from captured wild adult white sturgeon. The virus has been detected among wild juvenile white sturgeon collected from the lower Columbia River and the virus is potentially enzootic in wild white sturgeon populations throughout the Pacific Northwest of North America (4). An iridovirus similar to WSIV has been identified in Russian sturgeon from Northern Europe where it may be enzootic among cultured populations of several species of sturgeon (1).

The WSIV is an epitheliotropic virus infecting the skin, gills, and upper alimentary tract. Infections of the oral mucosa and olfactory organ epithelium are presumed causes of the cessation of feeding that leads to a progressive emaciation or starvation of the fish – the principal external sign of the disease (6). Cumulative mortality of up to 95% has been reported among groups of infected fish in the hatchery and secondary infections with external protozoa or bacteria often contribute to the overall mortality (2). Infected fish with moderate to severe emaciation began dying 2–3 weeks following exposure to the virus at water temperatures of 17–19°C (7). Haemorrhages on the abdomen and the ventral scuta may be present, but these are not specific for WSIVD. There are no specific internal signs of infection as the virus does not invade systemically. Viral infection is evident on microscopic observation of stained tissue sections from infected fish. Areas of the integument and particularly the skin may show a focal to diffuse hyperplasia with characteristic amphophilic to basophilic enlarged Malpighian cells (6). These cells are filled with virus particles as demonstrated by electron microscopy. The virus can be isolated, but with some difficulty, from infected fish using sturgeon cell lines.

The modes of transmission of WSIV are not completely understood but horizontal transmission via the water has been demonstrated in the hatchery and experimentally in the laboratory (3). There is strong circumstantial evidence from epidemiological investigations at the hatchery that the virus is transmitted vertically from adult broodstock, but the virus has never been isolated or observed in adult fish.

There appears to be little antigenic relationship of WSIV to the systemic iridoviral agents represented by epizootic haematopoietic necrosis virus or the red sea bream iridovirus. The larger size and inner membrane structure of WSIV virion, host cell-line specificity, type of cytopathic effect, and location of target host cells (epitheliotropic and not systemic) distinguish the agent from other groups of fish iridoviruses. Although lymphocystivirus (LCDV) has a similar virion morphology, LCDV infects fibroblasts and not Malpighian cells as in WSIV infections, and the cell line specificity and types of cytopathic effects are clearly different between the two agents.
Chapter 2.1.16. - White sturgeon iridoviral disease

The principal diagnostic methods for WSIV include microscopic observation of characteristic infected cells in stained tissue sections of the oral mucosa, gills and skin, or isolation of the virus in sturgeon cell lines (3). Neutralising polyclonal antibodies and binding monoclonal antibodies recognise WSIV, but not the systemic iridoviral agents. These antibodies can also be used in indirect immunofluorescence tests or for immunohistochemical staining of infected cells in tissue culture and sections of infected tissues.

Control methods currently rely on avoidance of the agent where possible. Because there are currently no methods for detecting the virus in adult broodstocks, quarantine and investigation of juveniles suffering mortality are the principal means to detect WSIV in young fish. Methods to detect the virus in broodstock are currently under development.

DIAGNOSTIC PROCEDURES

The diagnosis of white sturgeon iridovirus (WSIV) is based on the observation of pathognomonic infected cells in stained tissue sections from infected fish and isolation of the virus using sturgeon cell lines. Confirmation of WSIV infection relies on neutralisation of the isolated virus with polyclonal antibodies. The specific binding of monoclonal antibodies (MAbs) to viral antigens in infected cell cultures or impression smears from infected fish tissues are under investigation as alternatives to cell culture isolation and virus neutralisation for identification of WSIV.

Infected fish material suitable for virological examination is:

- **Clinically affected fish:** Fish <6 cm: 1) for virus isolation – gill arches and skin from the fleshy portions of the mouth (oral flap) and fins; 2) for histology – a sagittal section to include gills and the epidermis of the head region, also include larger fins. For larger fish – portions of the gill and fleshy part of the oral flap and punches or portions from fleshy part of larger fins. These tissues can be used for both virus isolation and histological examinations.

- **Asymptomatic fish** (apparently healthy fish): As above.

1. **STANDARD SCREENING METHOD FOR WSIV**

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

   **Cell lines to be used:** WSS-2 (white sturgeon spleen) or WSSK-1 (white sturgeon skin) cells

   Cells should be grown at 20°C in a temperature-controlled incubator using standard minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) that can be reduced to 5% after virus inoculation.

   a) **Inoculation of cell monolayers**

   i) Remove a portion of the gill, oral flap, and abdomen and prepare a supernatant from a homogenate to yield a final tissue dilution of 1/50. Prepare a second dilution representing 1/100 (w/v). Transfer an appropriate volume of each of the two dilutions on to 24-hour-old cell monolayers. Inoculate at least 2 cm² of drained cell monolayer with 100 µl of each dilution.

   ii) Allow to adsorb for 0.5–1 hour at 20°C and, without withdrawing the inoculate, add the cell culture medium buffered at pH 7.6 and supplemented with 5% FCS (2 ml/well for 12-well cell culture plates), and incubate at 20°C using a temperature-controlled refrigerated incubator to ensure successful isolation.

   b) **Monitoring incubation**

   i) Follow the course of infection in inoculated and control cell cultures by daily microscopic examination at \(\times 40\) to \(\times 100\) magnification for 30 days.
ii) If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of tested homogenate supernatants, identification procedures have to be undertaken immediately (see Section 1.2. below).

If a fish health surveillance/control programme is being implemented, provisions may 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 WSIV.

iii) If no CPE develops in the inoculated cultures (despite normal progression of CPE in the virus controls), the inoculated cultures should be subcultured for a further 15 days. Should the virus control fail to develop CPE, the process should be repeated with fresh susceptible cells and new batches of samples.

c) Subcultivation procedures

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

ii) Inoculate cell monolayers as described above in Section 1.1.a.

iii) Incubate and monitor as described above in Section 1.1.b.

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

1.2. Identification of WSIV

a) Neutralisation test

i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge at 2000 g for 15 minutes at 4°C to remove cell debris.

ii) Dilute the virus-containing medium from 10–1 to 10–3.

iii) Mix aliquots (for example 200 µl) of each virus dilution with equal volumes of rabbit anti-WSIV serum diluted 1/100, and similarly treat aliquots of each virus dilution with cell culture medium.

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

v) Transfer aliquots of each of the above mixtures on to cell monolayers (inoculate two cell cultures per dilution) and allow adsorption to occur for 0.5–1 hour at 20°C; 24- or 12-well culture plates are suitable for this purpose, using a 50 µl inoculum.

vi) When adsorption is completed, add cell culture medium supplemented with 5% FCS and buffered to pH 7.4–7.6 into each well and incubate at 20°C.

b) Indirect fluorescent antibody test

This indirect fluorescent antibody test (IFAT) is to be conducted directly after virus isolation in cell culture.

i) Prepare monolayers of WSS-2 cells in 2 cm² wells of cell culture plastic plates or on cover-slips in order to reach around 80% confluency. The FCS content of the cell culture medium can be reduced to 5%.

ii) When the cell monolayers are ready for infection, inoculate the virus suspensions to be identified by making tenfold dilution steps directly in the cell culture wells or flasks.

iii) Dilute the control virus suspension of WSIV in a similar way, in order to obtain a virus titre of about 1000 TCID₅₀ (50% tissue culture infective dose) per ml in the cell culture medium.

iv) Incubate at 20°C for 7 days
v) Remove the cell culture medium, rinse once with 0.01 M phosphate buffered saline (PBS), pH 7.2, then three times briefly with cold fixative. This fixative will be acetone (stored at -20°C) for cover-slips or a mixture of acetone 30%/ethanol 70% (v/v) for plastic, also stored at -20°C.

vi) Let the fixative act for 15 minutes. 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 minutes and process immediately or freeze at -20°C.

viii) Prepare a solution of MAbs to WSIV in 0.01 M PBS, pH 7.2, containing 2% skim milk, at the appropriate dilution.

ix) Rehydrate the dried cell monolayers by four rinsing steps with PBS and remove this buffer completely after the last rinsing.

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

xi) Rinse four times with PBS as above.

xii) Incubate the cell monolayers in appropriately diluted biotinylated anti-mouse antibody (in 2% skim milk in PBS) at 37°C for 1 hour.

xiii) Rinse four times with PBS as above.

xiv) Treat the cell monolayers for 1 hour at 37°C with a solution of FITC conjugate (FITC = fluorescein isothiocyanate).

xv) Rinse four times with PBS.

xvi) Examine the treated cell monolayers on plastic plates immediately, or mount with cover-slips using glycerol saline at pH 8.5 prior to microscopic observation.

xvii) Examine under incident UV light using a microscope with ×10 eye pieces and ×20–40 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. Positive results are indicated by diffuse fluorescence throughout the cytoplasm.

2. Diagnostic Methods for WSIV

Confirmation of WSIV outbreaks depends on the observation of clinical signs of disease among affected sturgeon, presence of pathognomonic infected cells in stained tissue sections or isolation of the virus in cell culture followed by its identification in a neutralisation test. Immunostaining of cells in target tissues with antibodies to WSIV can replace the need to conduct electron microscopy to view the virions of WSIV.

2.1. Virus isolation and subsequent identification

As in Sections 1.1. and 1.2.

2.2. Observation of infected cells in stained tissue sections

a) For fish <6 cm in length, preserve the entire fish in histological fixative

i) Prepare sagittal sections (off midline to include gills) of entire fish for hematoxylin and eosin staining.

ii) Observe the epithelium of the gills, fins (pectoral and caudal) and body including the mouth and esophagus, for evidence of enlarged cells with an amphophilic to basophilic staining cytoplasm and a hypertrophic irregular nucleus.
b) For fish >6 cm in length, remove one gill arch and a 1 cm² portion of the fleshy section of mouth (oral flap), and a punch sample and a similar sized piece of skin from the abdomen.

Fix tissues and prepare stained tissue sections for observation of characteristic infected cells as described above.

2.3. Immunohistochemical test

Begin with tissues fixed in 10% neutral buffered formalin used for histological examinations above.

i) Deparaffinise sections and rehydrate
   a. Xylene-1 for 5 minutes
   b. Xylene-2 for 5 minutes
   c. 100% EtOH for 3 minutes. Ring tissue area with a PAP pen
   d. 95% EtOH for 3 minutes
   e. 70% EtOH for 3 minutes
   f. Rinse in deionised water for 3 minutes

ii) Inactivate endogenous peroxidase by soaking slides in 0.3% H₂O₂ in methanol for 30 minutes at room temperature.

iii) Rinse slide with water then gently wash with PBS.

iv) Block for 30 minutes in PBS with 10% goat serum.

v) Shake off block solution and add 20 µl of biotinylated IIIA11 or IIC7 mouse anti-WSIV MAb in PBS (3 µg/ml) to the section and incubate for 60 minutes at room temperature.

vi) Wash three times.

vii) Add peroxidase-streptavidin (1/1000) in PBS for 10 minutes.

viii) Wash three times.

ix) Stain with AEC (aminoethyl carbazole) for 5–15 minutes at 25°C. Monitor the level of development by viewing the slide on a microscope. Stop development by immersing in PBS.

x) Wash three times.

xi) Counterstain with Mayer’s haematoxylin for 5 minutes at room temperature.

xii) Wash three times.

xiii) Mount slide while wet with Crystal/Mount™ aqueous/dry mounting medium. (AEC substrate is soluble with toluene or organic based mounting media.) After drying overnight, a cover-slip may be mounted on the section using an organic solvent-based mounting media such as Krystalon Mounting Media.

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1 At the end of a staining session, discard the first deparaffinisation xylene. For the following session use xylene 2 as the initial xylene and use fresh xylene in the second step.
2 DAKO protocol calls for 3 minutes × 2 changes for each alcohol bath. Tech service indicates that this is excessive. Other protocols call for 1–3 changes for 1–3 minutes each.
3 PAP Pen order from The Binding Site Inc., San Diego, CA. 1-800-633-4484.
4 AEC Substrate. Zymed AEC Substrate Kit.
5 Mayer’s haematoxylin solution. 0.1% soln. Sigma diagnostics, Cat#MHS-1.
6 Crystal/Mount™ aqueous/dry mounting medium. Biomeca Corp. Cat # MO3.
7 Krystalon Mounting Media, EM Science, 64969/95.
PBS – 2 litres of × 10
1. 160 g. NaCl (0.137 M)
2. 4.0 g. KCl (0.003 M)
3. 28.8 g. Na₂HPO₄ (0.01 M)
4. 4.8 g. KH₂PO₄ (0.002 M)
5. pH to 7.2–7.4 and Q.S. to 2 litres with d.d. H₂O.


Tris buffered saline (TBS)

<table>
<thead>
<tr>
<th>Component</th>
<th>1 litre × 1</th>
<th>2 litres × 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Tris base</td>
<td>6.07 g</td>
<td>121.1 g</td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td>0.409 g</td>
<td>8.18 g</td>
</tr>
<tr>
<td>150 mM NaCl</td>
<td>8.7 g</td>
<td>174 g</td>
</tr>
<tr>
<td>pH to 8.0 with HCl</td>
<td>~2.5 ml conc.</td>
<td>~50 ml conc.</td>
</tr>
</tbody>
</table>

TTBS
To make TTBS add 0.1% Tween-20 (1.0 ml/litre).

REFERENCES


CHAPTER 2.1.17.

KOI HERPESVIRUS DISEASE

CHAPTER IN PREPARATION

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SECTION 2.2.
DISEASES OF MOLLUSCS

CHAPTER 1.2.
GENERAL INFORMATION

1. DISEASES OF MOLLUSCS IN THE OIE AQUATIC MANUAL

Over the past few decades, world mollusc production has been adversely affected by a number of diseases and, given their severe impact on economic and socio-economic development in many countries; some of these diseases have become a primary constraint to the development and sustainability of mollusc aquaculture. Disease agent transfer via transfers of live molluscs has been a major cause of disease outbreaks and epizootics.

For the OIE list of diseases of molluscs, please consult the current edition of the Aquatic Code. For all of these diseases, the OIE Reference Laboratory may provide technical assistance on the practical implementation of the standards included in the Aquatic Code and Aquatic Manual.

2. SAMPLING PROCEDURES

There are at least three purposes for which mollusc stocks may be sampled. These are: 1) surveillance; 2) disease outbreak or suspicion; and 3) confirmatory diagnosis. The number and type of samples to be taken for analysis varies greatly according to which of these purposes applies.

2.1. Collection of specimens

A general approach to surveillance and sampling is given in Chapter 1.1.4 of this Aquatic Manual. The sampling should be designed to enable detection, at a 95% confidence level, of infected animals. The following section gives information relevant to sampling molluscs. For those diagnostic tests where the sensitivity and specificity have been established, sample size may be determined using methods such as FreeCalc (www.ausvet.com.au) or similar programs as outlined in Chapter 1.1.4 Requirements for surveillance for international recognition of freedom from infection. However, for those diagnostic tests where the sensitivity and specificity have not been established, the default sample size should be determined from Table 1 in the present chapter.
Chapter I.2. - Diseases of molluscs: General information

**Table 1. Random sample size based on assumed pathogen prevalence in lot and assuming 100% sensitivity and specificity of the technique**

<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>
<td>23</td>
</tr>
<tr>
<td>250</td>
<td>110</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>500</td>
<td>130</td>
<td>55</td>
<td>26</td>
</tr>
<tr>
<td>1000</td>
<td>140</td>
<td>55</td>
<td>27</td>
</tr>
<tr>
<td>1500</td>
<td>140</td>
<td>55</td>
<td>27</td>
</tr>
<tr>
<td>2000</td>
<td>145</td>
<td>60</td>
<td>27</td>
</tr>
<tr>
<td>4000</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>


### 2.2. Specific recommendations for sampling molluscs

The timing and frequency of sampling should be determined by the cycle of infection by the disease agent and the pre-patent period. An adequate time period should be allocated when sampling for seasonal diseases, for example infection with *Marteilia refringens* or *Haplosporidium nelsoni*, to ensure optimal detection. As disease agents may increase in intensity of infection associated with loss of host condition following spawning, post-spawning sampling is also recommended. Sampling periods should also take account of the transfer of juveniles and spat into outgrowing areas, and the transfer of adults for further fattening or relaying.

Samples should also cover a range of size groups, or target the most susceptible age group when this is known. During sampling, any molluscs showing abnormalities (abnormal growth, gaping valves, elevated or high mortality rates) should be selected. Diseases have sub-clinical stages of development that can escape detection using routine screening methodology. However, the probability of detection of infection may be increased by holding the bivalves in quarantine for a long period and subjecting them to stress (crowding, handling, temperature and salinity changes, etc.). To detect infection, species of molluscs that are more susceptible to infection should be examined histologically. For example, members of the Arcidae (*Arca, Barbatia*), Malleidae (*Malleus*), Isognomonidae (*Isognomon*), Chamidae (*Chama*), Tridacnidae (*Tridacna*) and Veneridae tolerate high prevalence of *Perkinsus olseni* infection and are good indicators of the presence of *P. olseni*.

Prevalence of infection is an important factor affecting the chance of detection. When molluscs are to be moved from natural beds into a farm site or between natural beds in different zones, large numbers of bivalves may be required to detect low prevalence of infection. For example, in Western Australia, *Marteilia sydneyi* and *Perkinsus* sp. occur at 0.1% prevalence in isolated beds untouched by humans. This level of sampling should be commensurate with the level of risk to the receiving waters and the aquatic resources they support.

For each zone, a number of sampling sites should be selected in the most practical way so as to maximise the chances of detecting disease agents. The number of sites should be increased for large zones that contain several discrete areas of cultivation of the susceptible species. Account should be taken of parameters that have an effect on the development of the disease agents, such as stocking density, water flow, and the developmental cycle of the molluscs.
Chapter I.2. - Diseases of molluscs: General information

It is an important requirement that the screening techniques used be the optimum methods available for detection of the disease agent in question, and that when the infection is present, it can be detected. For screening methods, sensitivity and specificity should have been assessed. This information has a strong influence on sample size (see Section 2.1).

2.3. Shipment of samples

All sampled molluscs should be delivered to the approved diagnostic laboratory and should arrive live. The laboratory should be informed of the estimated time of arrival of the sample so the required materials to process the molluscs can be prepared before reception of samples.

Mollusc samples should be packed in accordance with current standards in order to keep them alive. If the sampling site is a long distance from the laboratory, moribund animals or those with foul-smelling tissues may be of little use for subsequent examination. Required samples should be shipped as soon as possible after collection from the water, in order to reduce air storage and possible mortality during transportation, especially for moribund diseased molluscs.

For samples that cannot be delivered live to the diagnostic laboratory, due to advanced stages of disease, long distance or slow transportation connections, etc., specimens should be fixed on site as recommended in the following sections of this chapter or the individual disease chapters of this Aquatic Manual. While this is suitable for, for example, subsequent histology or transmission electron microscopy examination, other techniques, such as fresh smears, tissue imprints, routine bacteriology, mycology or Ray’s fluid thioglycollate culture of Perkinsus spp., cannot be performed. Diagnostic needs and sample requirements should be discussed with the diagnostic laboratory prior to collection of the sample.

Samples should be accompanied with background information, including the reason for submitting the sample (surveillance, abnormal mortality, abnormal growth, etc.), gross observations and associated environmental parameters, approximate prevalence and patterns of mortality, origin and nature of the molluscs (species, age, whether or not the samples are from local mollusc populations or stocks transferred from another site, date of transfer and source location, etc.). This information should identify possible changes in handling or environmental conditions that could be a factor in mortality in association, or not, with the presence of infectious agents.

2.4. Macroscopic examination

The gross observation of molluscs should target, as far as possible, animal behaviour, shell surface, inner shell and soft tissues.

It is often difficult to observe the behaviour of molluscs in open waters. However, observation of molluscs in certain rearing facilities such as brood-stock in tanks and larvae in hatcheries can provide useful indications of disease-related behavioural changes. If signs are noted (e.g. pre-settlement of larvae on the bottom, food accumulation in tanks, signs of weakening, etc.), samples may be examined for gross signs, including observation under a dissecting microscope for abnormalities and deformities, fouling organisms, and fixed for further processing as recommended below. For adults and juveniles, signs of weakening may include gaping, accumulation of sand, mud and debris in the mantle and on the gills, mantle retraction away from the edge of the shell, decreased activity (scallop’ swimming, clam’ burrowing, abalone’ grazing), etc. The righting reflex of abalone after being inverted is not possible in weakened animals, and it is a good indicator of weakness. Open-water mortality should be monitored for patterns of losses and samples collected for further analysis. Environmental factors pre- and post-mortality should be recorded.
Even under culture conditions, the shells of molluscs may not be clean and fouling organisms are normal colonists of mollusc shell surfaces. Organisms such as barnacles, limpets, sponges, polychaete worms, bivalve larvae, tunicates, bryozoans, etc., do not normally threaten health of molluscs. Culture systems, such as suspension and shallow water culture, can even increase exposure to fouling organisms and shells may become covered by other animals and plants. This can affect the health directly by impeding shell opening and closing or indirectly through competition for food resources. Signs of weakening associated with heavy fouling should be a cause for concern rather than fouling itself. Shell damages by boring organisms such as sponges and polychaete worms are usually benign, but under certain conditions may reach proportions that make the shell brittle or pierce through to the soft-tissues. This degree of shell damage can weaken the mollusc and render it susceptible to inter-current pathogen infections. Shell deformities (shape, holes in the surface), fragility, breakage or repair should be noted, but are not usually indicative of a disease concern. Abnormal coloration and smell, however, may indicate a possible soft-tissue infection that may need to be examined at a laboratory.

The molluscs should be opened carefully so as not to damage the soft tissues, in particular the mantle, gills, heart and digestive gland. The presence of fouling organisms on the inner shell surface is a clear indication of weakness. The inner surface of the shell is usually smooth and clean due to mantle and gill action. Perforation of the inner surface may occur, but can be sealed off by the deposition of additional conchiolin and nacre. This may result in formation of mud- or water-filled blisters. Blisters may also form over superficial irritants such as foreign bodies. The degree of shell perforation can be determined by holding the shell up to a strong light. Where abnormalities occurring within the matrix of the shell warrant further investigation, freshly collected specimens can be brought intact to the laboratory or fixed for subsequent decalcification, as required. The appearance of the soft-tissues is frequently indicative of the physiological condition of the animal. Soft tissues should be examined for the presence of abscess lesions, pustules, tissue discoloration, pearls, oedema, overall transparency or wateriness, gill deformities, etc., and, when found in association with weak or dying animals, these abnormalities should be a cause for concern.

Abnormalities and lesions of the tissues should be noted and recorded, as well as any shell deformities, shell-boring organisms and conspicuous mantle inhabitants. Levels of tissue damage should be recorded and samples of affected and unaffected animals collected for laboratory examination as soon as possible.

2.5. Examination of stocks where abnormal mortality occurs

Abnormal mortality of molluscs is usually recognised as a sudden sizeable mortality that occurs in a short time between two observations or inspections of the stocks (for example, about 15 days in the case of facilities located in inter-tidal zone). In a hatchery, abnormal mortality is the failure of successive production of larvae coming from different brood-stock. Given the broad spectrum of species, environments and culture conditions, these definitions should be adapted when and where necessary.

Whenever abnormal mortality occurs in stocks of molluscs, an urgent investigation should be carried out to determine the aetiology.

The samples taken should be collected, preserved or fixed and stored in accordance with the procedures described in this *Aquatic Manual*.

Where and when available, unaffected or control molluscs should also be fixed for histological comparison with abnormal tissues. Whatever the fixative, it is essential that the shell be removed to allow easy ingress of the fixative. Bivalves and operculated gastropods can keep the shell shut against fixative until autolysis begins.
3. Diagnostic Methods

Techniques applicable to molluscan disease agents are limited to direct detection of the causative agent. Classic serological methods cannot be used for diagnostic purposes because molluscs do not produce antibodies. In addition to histology and cytology, immunoassays using monoclonal antibodies or nucleic acid probes can be used for the detection of listed disease agents. From this point of view, the development of DNA-based diagnostic techniques for mollusce disease agents has certainly been the most significant advancement in recent years. Given the development and potential for widespread application of these diagnostic techniques and the inherent problems currently associated with their use, the issue of validation is of the utmost importance.

Three levels of examination procedures are proposed in the following sections. Histology is recommended as a standard screening method because it provides a large amount of information. It is particularly important because macroscopic examination usually gives no pathognomonic signs or solid indicative information. Also, mortality may be due to several disease agents or physiological problems, such as loss of condition following spawning, and this can only be determined using histology. Screening (surveillance) is routinely performed by histology. According to each epidemiological situation, and when it is justified, targeted surveillance may rely on other techniques.

When abnormal mortality outbreaks occur, histology is also recommended. It is particularly important because macroscopic examination usually gives no pathognomonic signs or solid indicative information. Also, mortality may be due to several disease agents or physiological problems, such as loss of condition following spawning, and this can only be determined using histology. Various presumptive diagnostic methods can be used in addition to histology, among which, tissue imprints, Ray’s fluid thioglycollate medium (RFTM) culture or polymerase chain reaction (PCR) may be used, as recommended in the individual disease chapters. Such methods may provide advantages of quick and/or cheap procedures as an answer to suspicion of infection with a given disease agent.

When a disease agent is encountered during screening or mortality outbreaks, molecular methods are increasingly being used, in addition to electron microscopy, for specific identification. Some of the OIE listed diseases for molluscs are caused by disease agents belonging to genera encompassing closely related species. Specific protocols designed to detect certain listed agents are recommended in the following chapters, to be used to confirm histological examination results and/or to give a species-specific diagnosis.

3.1. Histological techniques

Because of the generic use of histology in diagnostic procedures for diseases of molluscs, a detailed technical guideline is provided in this chapter.

Histology is a technique that is used to study the structure of cells and tissues under light microscopy. Tissue preparation involves different steps, including tissue fixation, dehydration, impregnation and embedding of samples, preparation of sections, staining and mounting of slides.

Live moribund animals or freshly dead (within minutes) animals provide the optimum conditions under which to collect tissues. A standard section should be taken through the digestive gland, to include the gills, mantle and palps, where possible. Alternatively for large specimens, several sections should be taken to include all the important tissues.

- 3.1.1. Tissue fixation

The role of the fixative is to maintain the morphology of the tissues as close to in-vivo morphology as possible and to prevent post-sampling necrosis. Recommended fixatives used for the study of marine molluscs are Davidson’s solution and Carson’s solution for large
specimens. For smaller specimens, glutaraldehyde fixatives may be used and are compatible for electron microscopy use. The ratio of fixative to tissue volume should be at least 10:1 to ensure good fixation.

**Davidson’s solution:**

- 1200 ml Sea water
- 1200 ml 95% Alcohol
- 800 ml 35–40% Formaldehyde
- 400 ml Glycerol
- 10% Glacial acetic acid

**Carson’s solution:**

- 23.8 g NaH$_2$PO$_4$·2H$_2$O
- 5.2 g NaOH
- 900 ml Distilled water
- 100 ml 40% Formaldehyde

Adjust the pH to 7.2–7.4

There is no universal fixative and choice should be made taking into account later use of fixed material as well as practical aspects of fixative use (price, component availability, etc.). Davidson’s solution is an excellent choice for preserving the structure of the tissues. In addition, tissue sections fixed in Davidson’s solution can be stained later by different histochemical methods, as well as *in-situ* hybridisation with DNA probes. For this purpose, over-fixation (over 24–48 hours) should be avoided. Carson’s solution may not be as good as Davidson’s solution for histological analysis. Nevertheless it does allow good preservation of the ultrastructure and may be used to preserve samples for later study by electron microscopy. Because electron microscopy may be a valuable adjunct in diagnosing or confirming infections in molluscs, fixation of some samples (especially smaller samples) using glutaraldehyde, as described in Section 6.2 of this chapter, may be considered. Otherwise, material fixed in Carson’s solution, and shown to contain adequate levels of targeted disease agents or abnormalities, can be refixed in glutaraldehyde. It is recommended that part of the mollusc be fixed in Davidson’s solution while the other part be fixed in Carson’s solution for further investigation. If neither are available, 10% formalin buffered with filtered seawater is adequate.

Within each country, the molluscian aquaculture industry should agree on the most effective way of ensuring adequate fixation.

- **3.1.2. Dehydration, impregnation and embedding of the samples**

The embedding of the samples in paraffin requires several steps during which the water contained in the tissues is progressively replaced, first by alcohol, then by xylene or equivalent less toxic clearing solution, and lastly by paraffin.

After having fixed the samples in Carson’s or Davidson’s solution, they are transferred through graded alcohols (70–95 [v/v]) before final dehydration in absolute ethanol. The alcohol contained in the tissues is next eliminated by immersing them in xylene. The tissues are then impregnated with paraffin, which is soluble in xylene, at 60°C. These steps may be all carried out automatically using a machine.

Blocks are produced by letting the tissues cool in moulds filled with paraffin on a cooling table; cooling and moisturising are essential to section cutting.

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1 A saturated 37–39% aqueous solution of formaldehyde gas.
3.1.3. Preparation of the sections

After the blocks have been cooled on a cold plate, which allows the paraffin to solidify, histological sections of about 2–3 µm are cut using a microtome. The sections are recovered on histological slides, drained and dried overnight at 60°C. Drying the samples at this temperature allows the excess moisture to be eliminated and thus the sections adhere to the slides.

3.1.4. Staining and mounting the slides

Before staining, the paraffin is removed from the sections by immersing them in xylene or equivalent less toxic clearing solution for 10–20 minutes. This is repeated once and then the solvent is eliminated by immersion in two successive absolute ethanol baths for 10-minute periods each and rehydrated by immersion in a bath of tap water for 10 minutes. Different topographical or histochemical staining techniques can then be performed.

When staining with haematoxylin–eosin (H&E) is used, (haematoxylin or equivalent) nuclear and basophilic structures stain a blue to dark purple colour, the endoplasmic reticulum stains blue, while the cytoplasm takes on a grey colour. The acid dye eosin stains the other structures pink. This staining technique is simple and reproducible and, although it only allows a limited differentiation of cell structures, it is possible to detect any abnormalities in tissue and cellular structure. Other techniques may be applied to demonstrate particular structures or features as required (e.g. trichrome for connective tissue and cytoplasmic granules).

3.2. Transmission electron microscopy methods

Because of the very frequent use of transmission electron microscopy in confirmatory identification of disease agents in diagnostic procedures for diseases of molluscs, detailed technical guidelines are provided in this chapter for indication.

Fixation for electron microscopy should be done immediately after the animal has been killed, before fixation for histology. Only samples taken rapidly from live animals will be of any use. The preparation of samples for electron microscopy involves the following steps: tissue fixation, decalcification of the samples (when necessary), dehydration, impregnation and embedding of the samples, preparation and counterstaining of the sections.

3.2.1. Tissue fixation

For tissues that are to be examined by electron microscopy, it is important that the fixation be performed correctly in order to cause as little damage as possible to the ultrastructure. The specimens are cut such that their dimensions do not exceed 1–2 mm. This small size allows the various solutions to penetrate rapidly into the sample.

Fixation of the samples is carried out directly in 3% glutaraldehyde for 1 hour. Fixation for longer periods leads to membranous artefacts. The samples are washed in buffer three times, then fixed in 1% osmic acid and washed twice again in buffer. Various formulations of glutaraldehyde fixative and buffers work equally well.

In order to cause as little damage as possible to the ultrastructure, the samples are treated with solutions that have an osmolarity close to that of the tissues. Thus, mollusc tissues are treated with solutions with an osmolarity of around 1000 mOsm. The osmolarity of the solutions is adjusted with NaCl. As mollusc tissues are nearly iso-osmotic with seawater, it is possible to make the glutaraldehyde up with 0.22 µm filtered seawater, and use the filtered seawater for subsequent washes.
Chapter I.2. - Diseases of molluscs: General information

Sodium cacodylate 0.4 M: 8.6 g in 100 ml of distilled water
Sodium chloride 10% in distilled water

_Cacodylate buffer, pH 7.4:
1000 mOsm
Sodium cacodylate 50 ml from 0.4 M stock solution
NaCl 20 ml from 10% stock solution
Distilled water 30 ml
Adjust the pH to 7.4

_3% Glutaraldehyde:
1000 mOsm
25% gluteraldehyde 2.5 ml
0.4 M sodium cacodylate 5 ml
10% NaCl 3.5 ml
Distilled water 9 ml

_1% Osmic acid:
1000 mOsm
4% Osmic acid 1 volume
0.4 M sodium cacodylate 1 volume
NaCl 1 volume from 10% stock solution
Distilled water 1 volume

_5% EDTA:
Disodium EDTA 5 g
Cacodylate buffer 100 ml
EDTA dissolves when the pH is above 8. When the solution becomes clear adjust the pH to 7.4 by adding concentrated HCl.

If the samples have been previously fixed and stored in Carson’s solution, they should be washed several times in a bath of buffer before fixative with 3% glutaraldehyde.

- **3.2.2. Dehydration, impregnation and embedding of the samples**

The samples are dehydrated in successive baths of ethanol: 70% ethanol once, 95% ethanol twice, absolute ethanol three times. The dehydration is completed by two baths of propylene oxide, which allows the subsequent impregnation with Epon or other resin.

The samples are impregnated progressively. After a first bath in a mixture of polypropylene oxide–Epon (50/50), the samples are placed in a bath of Epon. The longer the incubation, the better is the impregnation of the tissues.

Embedding is carried out by placing the samples in moulds filled with Epon resin. A label identifying the sample is included in each block and the blocks are then placed at 60°C (the temperature at which Epon resin polymerises) for 48 hours.

- **3.2.3. Preparation of the sections and the counterstaining**

The blocks are cut to appropriate sizes with a razor blade and the sections are then cut using an ultra microtome. Semi-thin sections (0.5–1 µm) are cut and placed on glass slides. These will be used to control the quality of the samples by light microscopy and to find the areas of interest on the section.
The semi-thin sections are stained at 90–100°C with 1% toluidine blue solution. After drying, the slides are mounted under cover-slips with a drop of synthetic resin and observed under the light microscope.

Ultra-thin sections 80–100 nm thick are placed on mesh copper grids for electron microscopy analysis. Uranyl acetate and lead citrate are used to counterstain the ultra-thin sections.

3.3. Molecular methods

Molecular techniques usually offer an advantage in sensitivity that is frequently offset by technical problems. PCR is particularly dependent on the conditions under which it is run, yielding false-positive or false-negative results. Whenever molecular techniques are used, they should be performed with caution and with special attention to the inclusion of adequate positive and negative controls in order to overcome the possible lack of robustness and to maintain adequate accuracy.

PCR, PCR-RFLP (restriction fragment length polymorphism), sequencing, *in-situ* hybridisation and immuno-histochemistry are increasingly used in confirmatory identification of disease agents. For these techniques, samples should be prepared to preserve the DNA of the pathogen. Likewise, samples intended for testing with antibody-based methods should be preserved to retain the reactive antigenic sites for the antibodies used.

- **3.3.1. Sample preparation**

Samples selected for DNA-based or antibody-based diagnostic tests should be handled and packaged with the greatest care to minimise the potential for cross-contamination among the samples or target degradation before the assay can be performed. To prevent contamination, new containers (plastic sample bags or bottles) should be used. A water-resistant label, with the appropriate data filled out, should be placed within each package or container for each sample set.

Some suitable methods for preservation and transport of samples taken for molecular or antibody-based tests are:

- **Live iced specimens or chilled specimens:** for specimens that can be rapidly transported to the laboratory for testing within 24 hours, pack samples in sample bags surrounded by an adequate quantity of wet ice around the bagged samples in an insulated box and ship to the laboratory.

- **Frozen whole specimens:** select live specimens according to the purpose of sampling, quick freeze in the field using crushed dry-ice, or freeze in a field laboratory using a mechanical freezer at −20°C or lower temperature. Prepare and insert the label into the container with the samples, pack samples with an adequate quantity of dry-ice in an insulated box, and ship to the laboratory.

- **Alcohol-preserved samples:** in regions where the storage and shipment of frozen samples is problematic, 90–95% ethanol may be used to preserve, store, and transport certain types of samples. Whole molluscs (when the specimen is small), excised tissues from larger molluscs. Pack for shipment according to the methods described above.

- **Fixed tissues for *in-situ* hybridisation and immuno-histochemistry:** for this purpose, classic methods for preservation of the tissues are adequate. Davidson’s solution is usually a good choice for later use of molecular probes. For DNA, specifically, over-fixation (over 24–48 hours) should be avoided.
3.3.2. DNA extraction

For DNA extraction, grind the preserved tissues to powder. Around 10 volumes of extraction buffer (NaCl [100 mM], ethylene diamine tetra-acetic acid [EDTA, 25 mM], pH 8, sodium dodecyl sulfate [SDS, 0.5%]) are added with proteinase K (100 µg/ml). Following overnight incubation at 50°C, DNA is extracted using a standard phenol/chloroform protocol, and precipitated with ethanol.

Considering time constraints and risks for laboratory staff, commercially available kits may provide satisfactory technical alternative. Use of commercial kits should be validated by comparison with standard phenol/chloroform protocol prior to its routine use in diagnostic laboratories.

3.3.3. Preparation of slides for in-situ hybridisation

For in-situ hybridisation, molluscs are fixed in Davidon’s fixative for approximately 24 hours and then embedded in paraffin according to methods described above for histology. Sections are cut at 5 µm thick and placed on aminoalkylsilane-coated slides, which are then baked overnight in an oven at 40°C. The sections are dewaxed by immersing in xylene for 10 minutes. This step is repeated once and then the solvent is eliminated by immersion in two successive absolute ethanol baths for 10 minutes each. The sections are then rehydrated by immersion in an ethanol series. The protocol may require a step of membrane permeabilisation enabling access to the target DNA. For this purpose, sections are treated with proteinase K (100 µg/ml) in TE buffer (Tris [50 mM], EDTA [10 mM]), at 37°C for 30 minutes.

KEY REFERENCES


* * *
CHAPTER 2.2.1.

INFECTION WITH BONAMIA OSTREAE

1. CASE DEFINITION

For the purpose of this chapter, infection with *Bonamia ostreae* is considered to be infection with *Bonamia ostreae*.

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

a) Agent factors

- Aetiological agent, agent strains: *Bonamia ostreae*, no strain identified.
- Survival outside the host: currently unknown.
- Stability of the agent: peracetic acid (0.001% and 0.005%) has been shown to reduce contamination of oysters by *B. ostreae* (32).
- Life cycle: life cycle outside the host is unknown but transmission of the parasite directly from host to host by cohabitation or by inoculation of purified parasites is possible (1, 35, 51).

b) Host factors

- Susceptible host species:
  - Natural host: European flat oysters *Ostrea edulis*.
  - Oyster species infected when moved into endemic zones: *Ostrea puelchana*, *O. angasi*, *O. chilensis* (= *Tiostrea chilensis*, *T. lutaria*) (7, 10, 33, 49).
  - *Ostrea conchaphila* (= *O. lurida*), *Crassostrea angulata* and *C. ariakensis* (= *C. rivularis*) have been speculated to be infected with *B. ostreae* (19, 29, 37), but confirmatory diagnosis has not been achieved.
  - Susceptible stages of the host: spat and particularly adult; individuals older than 2 years appear to be more susceptible to the disease (3, 26, 32, 52).
  - Species or sub-population predilection: *Ostrea edulis* is the only natural known susceptible species, infection intensity increases concurrently to mortality with age and/or size of the oysters (12, 26, 32).
  - Target organs and infected tissue: *Bonamia ostreae* is an intrahaemocytic protozoan (21, 50) but can be observed extracellularly between epithelial or interstitial cells in gills and stomach or in necrotic connective tissue areas (3). Intraepithelial localisation has also been reported in gills (46). A controversial description mentioned the presence of the parasite in ovarian tissue (56). Advanced infections become systemic (20).
  - Persistent infection with lifelong carriers: infection is often fatal depending on host and environmental conditions.
  - Vectors: none required
c) Disease pattern

- Transmission mechanisms: direct transmission from host to host is possible (1, 51). The infective form and ways of entry and release remain undetermined. A lag time of at least 3 months is generally observed before detecting the parasite in free batches moved in infected areas (45, 54).

- Prevalence: variable (0% to 80%). Prevalence is higher in individuals older than 2 years. The disease occurs and can be transmitted throughout the year, but there is a seasonal variation in infection with Bonamia ostreae; prevalence and intensity of infection tends to increase during the warm season presenting peaks in autumn (26, 32, 44, 57).

- Geographical distribution: Europe (France, Ireland, Italy, Netherlands, Spain and United Kingdom excluding Scotland) (11, 21, 41, 44, 50, 55), Canada (British Columbia) and United States of America (California, Maine and Washington States) (4, 28, 30, 31, 37).

- Mortality and morbidity: infection is often lethal, death usually occurs concurrently to highest intensity infection level (9).

- Economic and/or production impact of the disease: Bonamia ostreae, in conjunction with earlier epizootics caused by Marteilia refringens, caused a drastic drop in the French production of Ostrea edulis from 20,000 tonnes per year in the 1970s to 1,800 tonnes in 1995 (33, 42). Bonamia ostreae has also had a significant negative impact on O. edulis production throughout its distribution range in Europe.

d) Control and prevention

- Vaccination: none.

- Chemotherapy: none.

- Immunostimulation: none.

- Resistance breeding: selective breeding has been shown to be effective in reducing susceptibility and mortality caused by Bonamia ostreae (5, 6, 23, 25, 40, 47, 48).

- Restocking with resistant species: none.

- Blocking agents: none.

- General husbandry practices: mortalities due to bonamiasis can be reduced using suspension culture (38), lower stocking densities (34) or by culturing Ostrea edulis with Crassostrea gigas, which are not naturally susceptible to infection (39). Oyster seed from hatcheries are preferred to seed from natural settlement, which appears to be significantly more parasitised (22).

3. Diagnostic methods

a) Field diagnostic methods

- Clinical signs: dead or gaping oysters. These clinical signs are not pathognomonic to infection with Bonamia ostreae.

b) Clinical methods

- Gross pathology: sometimes yellow discoloration, extensive lesions including perforated ulcers in the connective tissues of the gills, mantle and digestive gland (21, 53). These gross signs are not pathognomonic to infection with Bonamia ostreae and most of the infected oysters appear normal.

- Clinical chemistry: none.
• Microscopic pathology
  • Wet mounts: none.
  • Imprints: no information.
  • Fixed sections: dense infiltrations of haemocytes, some of them containing parasites, in the connective tissue of the gill and mantle, and in the vascular sinuses around the stomach and intestine (21).
  • Electron microscopy/cytopathology: no information.

C) Agent detection and identification methods

• Direct detection methods

  i) Microscopic methods

    • Tissue imprints

      Samples to be taken: oyster spat or heart ventricle or gills from live adult hosts.

      Technical procedure: after drying tissues on absorbent paper, several imprints are made on a glass slide. Slides are air-dried, fixed in methanol or in absolute ethanol and stained using a commercially available blood-staining kit, in accordance with the manufacturer's instructions. After rinsing in tap water and drying, the slides are mounted with a coverslip using an appropriate synthetic resin. Slides are observed first at ×200 magnification and then under oil immersion at ×1000 magnification.

      Positive controls: recommended and available from the OIE Reference Laboratory.

      Levels of validation:

      • Specificity and sensitivity: low specificity, sensitivity better than histological examination (2, 27), it would appear however that the heart imprint technique is not reliable for detecting latent infections (24).

      • Gold standard: sensitivity higher than histology, which is the gold standard although it is not species specific.

      Interpretation of results:

      • A positive result is the presence of small spherical or ovoid organisms (2–5 µm wide) within haemocytes. However, the parasite might also occur extracellularly. These organisms show a basophilic cytoplasm and an eosinophilic nucleus (colours may vary with used stain); because they spread on the slide, they can appear wider than on histological examination. Multinucleated cells can be observed (9). The technique is not species specific.

      Availability of tests: quick staining kits are commercially available (e.g. Hemacolor®).

    • Histopathology

      Samples to be taken: live or freshly dead oysters.

      Technical procedure: sections of tissue that include gills, digestive gland, mantle, and gonad should be fixed for 24 hours in Davidson’s fixative followed by normal processing for paraffin histology and staining, for example with haematoxylin and eosin. Observations are made at increasing magnifications to ×1000.

      Positive controls: recommended and available from the OIE Reference Laboratory.

      Levels of validation:

      • Specificity and sensitivity: low specificity, sensitivity is good for moderate to high intensity infections, but low for low intensity infections.
• Gold standard: histology is the gold standard and is the recommended surveillance method.

Interpretation of results:
• A positive result is the presence of parasites as very small cells of 2–5 µm wide within the haemocytes or freely in connective tissue or sinuses of gill, gut and mantle epithelium, often associated with intense inflammatory reaction. To avoid any doubt the parasite has to be observed inside the haemocyte for a positive diagnosis. The technique is not species specific.

Availability of tests: no commercially available tests.

• Transmission electron microscopy
Samples to be taken: live or freshly dead oysters.
Technical procedure: described in chapter I.2 of this Aquatic Manual.
Positive controls: no.
Levels of validation:
• Specificity and sensitivity: better specificity than imprints and histology. Transmission electron microscopy may help to differentiate *B. ostreae* from other closely related microcells like *B. exitiosa*.

Interpretation of results:
• A positive result is the presence of parasites within haemocytes. Different stages including uninucleate, diplocaryotic and plasmodial stages have been reported (9, 46, 50). Intracellular structures include mitochondria, haplosporosomes, Golgi apparatus and persistent intranuclear microtubules.
• Dense forms of *B. ostreae* are more dense, slightly smaller in size (2.4 ± 0.5 µm mean diameter \( \bar{n} = 64 \) in comparison with *B. exitiosa* with a mean diameter of 3 ± 0.3 µm, \( \bar{n} = 64 \)), have fewer haplosporosomes, mitochondrial profiles and lipid bodies per ultrastructure section, and have larger tubulo-vesicular mitochondria than *B. exitiosa*. In addition, dense forms of *B. ostreae* lack nuclear membrane-bound Golgi/nuclear cup complexes and a vacuolated stage (36).

Availability of tests: no commercially available tests.

ii) Agent isolation and identification

• Cell culture/artificial media: none.

• Antibody-based antigen detection methods
An immunofluorescent technique based on monoclonal antibodies was developed and had sensitivity similar to tissue imprint (8). However, this technique gave unclear results when tested extensively on oysters from Maine, USA (58). Although direct monoclonal antibody sandwich immunoassay for the detection of *B. ostreae* in haemolymph samples of *O. edulis* was developed (16) and marketed commercially for a few years in the mid-1990s, it is no longer available on the market. The specificity and sensitivity of this last technique against histology were 76.7% and 106%, respectively (16).

• Molecular techniques: polymerase chain reaction (PCR)
Samples to be taken: live or freshly dead oysters.
Technical procedure: tissue samples are placed in 95% ethanol or frozen at –80°C until DNA is extracted. DNA extraction is accomplished by proteinase K digestion overnight at 50–55°C and phenol-chloroform extraction and ethanol precipitation (13, 17) or spin-column methodology using commercially available kits (e.g. QIAGEN) (13).
Two PCR protocols with two different primer pairs targeting the SSU rDNA have been developed for *Bonamia ostreae*.

The first primer pair is 5'-CAT-TTA-ATT-GGT-CGG-GCC-GC-3' and 5'-GGG-GGA-TCG-AAG-ACG-ATC-AG-3', designated Bo and Boas respectively, and amplifies a 300 bp product (17). PCR mixtures contain buffer (500 mM KCl, 100 mM Tris/HCl [pH 9.0 at 25°C] and 1% Triton® X-100), 2.5 mM MgCl₂, 0.2 mM dNTP mix, 1 µM forward and reverse primers, 0.02 units/µl Taq DNA polymerase, and 0.2 ng/µl of the DNA template in a total volume of 50 µl. Samples are denatured in a thermocycler for 5 minutes at 94°C before being submitted to 30 cycles (94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute) followed by a final extension of 10 minutes at 72°C.

The second primer pair is 5'-CGG-GGG-CAT-AAT-TCA-GGA-AC-3' and 5'-CCA-TCT-GCT-GGA-GAC-ACA-G-3', designated CF and CR respectively, and amplifies a 760 bp product (13). PCR reaction mixtures contain buffer (200 mM Tris/HCl [pH 8.4], 500 mM KCl), 1.5 mM MgCl₂, 0.2 mM dNTP mix, 0.05 µM forward and reverse primers, 0.05 units/µl Taq DNA polymerase, and 1 ng/µl of the DNA template in a total volume of 50 µl. Samples are submitted to 35 cycles (94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute) followed by a final extension of 10 minutes at 72°C.

Positive/negative controls: compulsory. Positive controls are DNA from purified *B. ostreae* cells, or genomic DNA from heavily infected hosts. Negative controls are genomic DNA from no infected hosts.

Levels of validation:

- Specificity and sensitivity: based on target DNA sequence similarity, the first assay should amplify all microcell haplosporidians and the second one at least *B. ostreae* and *B. exitiosa* (15). Sensitivity of both assays is higher than histocytological methods.

- Gold standard: neither PCR assay has yet been formally validated against histology for the detection of *B. ostreae*.

Interpretation of results:

- A positive result is an amplicon of the appropriate size, with all negative controls negative and all positive controls positive.

- Neither assay is species specific. The sequence of the SSU rDNA gene of *B. ostreae* shows polymorphism with that of *B. exitiosa* or *B. roughleyi* by restriction fragment length polymorphism (RFLP) analysis, by digesting PCR product Bo-Boas with *Hae* II and *Bgl* I. The obtained profiles vary according to the parasite species. *Bonamia ostreae* and *B. exitiosa* present the same profile (two products of 115 and 189 bp) when digested with *Hae* II while *B. roughleyi* is not digested. The *B. ostreae* profile consists of two bands of 120 and 180 bp when digested with *Bgl* I while *B. exitiosa* and *B. roughleyi* are not digested (18, 36).

Availability of tests: no commercially available tests.

- **Molecular techniques: in-situ hybridisation (ISH)**

Samples to be taken: live or freshly dead oysters.

Technical procedure: two ISH protocols have been developed. The first one (17) uses a 300 bp digoxigenin-labelled probe and the second one (14) uses three fluorescein-labelled oligonucleotide probes. All these probes target the SSU rDNA gene. Tissue samples are placed in Davidson’s fixative for 24 hours and then embedded in paraffin. Sections are cut 5 µm thick, placed on silane-coated slides and then baked overnight in an oven at 50–60°C. After de-waxing, slides are treated with proteinase K (100 µg/ml) in TE buffer (50 mM Tris, 10 mM EDTA [ethylene diamine tetra-acetic acid]) at 37°C for 30 minutes in the first protocol or in PBS (phosphate buffered saline) buffer (150 mM NaCl, 12.5 mM Na₂HPO₄, 3 mM KH₂PO₄, pH 7.2) for 15 minutes at 37°C in the second protocol.
• In the first protocol, slides are dehydrated by immersion in an ethanol series and air dried. The slides are then covered with hybridisation buffer (4 × SSC [standard saline citrate; 60 mM NaCl, 0.01 M NaCl, pH 7], 50% formamide, 1 × Denhardt's solution, 250 ng/ml yeast tRNA, 10% dextran sulfate) containing 20 ng of the digoxigenin-labelled probe. After denaturation for 5 minutes at 95°C, hybridisation is performed by incubating slides in a humid chamber overnight at 42°C. The probe is produced by PCR using the previously described primer pair Bo Boas with digoxigenin incorporation. The PCR is performed as described in the section on PCR except that DIG dUTP 25 mM is added to the reaction mixture. The detection steps are performed according to the manufacturer's instructions.

• In the second protocol, after proteinase K treatment, slides are washed in several baths including PBS plus 0.2% glycine for 5 minutes, acetylated using acetic anhydride 5% in 0.1 M triethanolamine/HCl (pH 8), for 10 minutes at room temperature, washed again in PBS for 10 minutes and lastly equilibrated in 5 × SET (750 mM NaCl, 0.4 mM EDTA, 100 mM Tris Base) for 10 minutes at room temperature. Slides are then covered with 200 µl of prehybridisation buffer (5 × SET, 0.02% bovine serum albumin, 0.025% sodium dodecyl sulphate [SDS]) for 30 minutes at 45°C. Prehybridisation buffer is replaced with 10 to 12 µl of the prehybridisation buffer containing 2–10 ng/µl of the oligonucleotides and slides are incubated overnight in humid chamber at 45°C. Slides are then washed three times in 0.2 × SET for 5 minutes at 42°C, air dried and mounted before being examined using an epifluorescent microscope at ∼600–1000. Probes consist of a cocktail of oligo-fluorescein-labelled probes specific for *B. ostreae*: UME-BO-1 (5'-CGA-GGC-AGG-GTT-TGT-3'), UME-BO-2 (5'-GGG-TCA-AAC-TCG-TTG-AAC-3') and UME-BO-3 (5'-GGC-TCT-TAT-CCA-CCT-AAT-3').

Positive/negative controls: compulsory. Positive controls are histological sections from infected hosts. Negative controls are histological sections from uninfected hosts. In the second protocol, an additional positive control consists in using an oligonucleotide which binds both *O. edulis* and *B. ostreae*: UME-OE-385 (5'-TCA-TGC-TCC-CTC-TCC-GG-3').

Levels of validation:
• Specificity and sensitivity: specificity and sensitivity are higher than histological examination. However, the probe Bo-Boas is able to detect *Haplosporidium nelsoni* in *Crassostrea virginica*, *Bonamia exitiosa* in *Ostrea chilensis*, but not *Mikrocytos mackini* in *C. gigas* (17). The specificity of the oligoprobe cocktail UME-BO-1, 2 and 3 has been tested and proved against *H. nelsoni* (14) but this ISH assay probably detects other microcells including *B. exitiosa* (15).
• Gold standard: ISH has not yet been validated against histology.

Interpretation of results:
• A positive result corresponds to labelled parasites inside haemocytes, with all negative controls negative and all positive controls positive. In the first described protocol, it appears like dark spots whereas in the second protocol, it corresponds to small green rings, as green fluorescence surrounded an eccentric dark region.

Availability of tests: DIG nucleic acid detection kit, Boehringer Mannheim for the first protocol.

• **Agent purification**: *Bonamia ostreae* can be purified from highly infected oysters (43).

**Indirect detection methods**

• Serological methods: none applicable.
4. RATING OF TESTS AGAINST PURPOSE OF USE

The methods currently available for surveillance, detection, and diagnosis of B. ostreae are listed in Table 1. The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic sensitivity and specificity; C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and D = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility.

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<tr>
<th>Method</th>
<th>Surveillance</th>
<th>Presumptive</th>
<th>Confirmatory</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>In known host and geographical range</td>
<td>Outside known host and geographical range</td>
<td>In known host and geographical range</td>
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<tr>
<td>Gross signs</td>
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<td>Tissue imprints</td>
<td>A</td>
<td>B</td>
<td>A</td>
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<tr>
<td>Histopathology</td>
<td>B</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>PCR</td>
<td>B</td>
<td>B</td>
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<tr>
<td>PCR-RFLP</td>
<td>D</td>
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<td>Transmission EM</td>
<td>D</td>
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<td>In-situ hybridisation</td>
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EM = electron microscopy; PCR = polymerase chain reaction, RFLP = Restriction Fragment Length Polymorphism

5. CORROBORATIVE DIAGNOSTIC CRITERIA

a) Definition of suspect case

In known susceptible species within the known geographical range of B. ostreae, a suspect case of infection with B. ostreae is a positive result by one of the following methods: histopathology, tissue imprints, or PCR.

In other host species or outside the known range of B. ostreae, a suspect case is a positive result by histopathology, tissue imprints, PCR or in-situ hybridisation.

b) Definition of confirmed case

A confirmed case of B. ostreae is a positive result by tissue imprints, histology or in-situ hybridisation combined with a positive result by PCR-RFLP.

6. PRESCRIBED DIAGNOSTIC/DETECTION METHODS TO DECLARE FREEDOM

Prescribed methods for targeted surveillance to declare freedom from infection as outlined in the Aquatic Code are: histology, tissue imprints (heart or gills) or PCR.

REFERENCES


*  *  *

NB: There is an OIE Reference Laboratory for Infection with Bonamia ostreae (see Table at the end of this Aquatic Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.2.2.

INFECTION WITH *BONAMIA EXITIOSA*

1. CASE DEFINITION

For the purpose of this chapter, infection with *Bonamia exitiosa* is considered to be infection with *Bonamia exitiosa* previously named *B. exitiosus* (1, 13).

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

a) Agent factors

- Aetiological agent, agent strains: *Bonamia exitiosa*, no strain identified.
- Survival outside the host: infective particles can survive for 48 hours in sea water at 18°C (4, 6).
- Stability of the agent: currently unknown.
- Life cycle: transmission of the parasite directly from host to host is possible and transmission by infective stages carried passively on currents between oyster beds is suspected (4, 11).

b) Host factors

- Susceptible host species (natural hosts): New Zealand dredge oyster *Ostrea chilensis* (=*Tiostrea chilensis*, =*T. lutaria*) (7) and the Australian mud oyster, *O. angasi* (11, 15).
- Susceptible stages of the host: in *O. chilensis*, recruit-sized oysters (oysters greater than or equal to 58 mm in length) are known to be susceptible (7). There are no data concerning the other oyster stages, including spat.
- Species or sub-population predilection: *Ostrea chilensis* and *O. angasi* (7, 15) greater than or equal to 58 mm in length. In *O. chilensis*, female and spent oysters appear significantly more infected than male and hermaphrodite oysters (14).
- Target organs and infected tissue: *Bonamia exitiosa* is an intrahaemocytic protozoan but it can be observed extracellularly (7). This intrahaemocytic protozoan quickly becomes systemic and can be found in different organs, especially in the connective tissues of gills and mantle (9). In *Ostrea angasi*, the parasite is epitheliotropic; apparently very light infections may cause a massive focal haemocyte infiltration and necrotic foci.
- Persistent infection with lifelong carriers: infection often fatal depending on host and environmental conditions.
- Vectors: none required.

c) Disease pattern

- Transmission mechanisms: in *O. chilensis* transmission of the parasite directly from host to host is possible. Released infective particles are ingested by oysters and enter the haemolymph from the gut (9, 10). Infective particles are phagocytosed by agranular haemocytes, but are able to resist lysis within the haemocyte (16).
• Prevalence: variable in *O. chilensis* (0% to nearly 80%) (4). Infection with *Bonamia exitiosa* shows the highest prevalence from January to April, with the parasite barely detectable in September and October (9). Stressors such as exposure to extreme temperatures (7 or 26°C) and salinity (40%), starvation (prolonged holding in filtered sea water), handling (vigorous stirring four times per day), or heavy infection with an apicomplexan (12), can affect the disease dynamics of *B. exitiosa* in *Ostrea chilensis* (14).

• Geographical distribution: *O. chilensis*, Foveaux Strait and other locations around South Island, New Zealand (7, 8) and in *O. angasi*, in Australia (Port Philip Bay, Victoria; Georges Bay, Tasmania; and Albany, Western Australia) (11, 15).

• Mortality and morbidity: infection is often lethal. In *O. chilensis*, death usually occurs concurrently to highest intensity infection level, particularly in association with high intensity apicomplexan infections (12, 16).

• Economic and/or production impact of the disease: in *O. chilensis*, large-scale mortalities of native dredge oyster (91% between 1975 and 1992 in Foveaux Strait, New Zealand [8]) have been attributed to this parasite. This fishery was closed in 1993 with severe economic impact on local communities.

d) Control and prevention

• Vaccination: none.
• Chemotherapy: none.
• Immunostimulation: none.
• Resistance breeding: none.
• Restocking with resistant species: none.
• Blocking agents: none.
• General husbandry practices: development of lighter dredges and less damaging fishing strategies should reduce the chance of disease outbreaks by lowering disturbance (4). Avoiding stressors such as exposure to extreme temperatures (7 or 26°C) and salinity (40%), starvation, handling, or heavy infection with other parasites, as well as decreasing density, should help to reduce the impact of the disease (4, 14).

3. DIAGNOSTIC METHODS

a) Field diagnostic methods

• Clinical signs: dead or gaping oysters. These clinical signs are not pathognomonic to infection with *Bonamia exitiosa*.

b) Clinical methods

• Gross pathology: most of the live infected oysters appear normal but sometimes, gills can appear eroded (7).
• Clinical chemistry: none.
• Microscopic pathology
  • Wet mounts: none.
  • Imprints: no information.
  • Fixed sections: in *O. chilensis*, lesions occur in the connective tissue of the gill and mantle, and in the vascular sinuses around the stomach and intestine. The sub-epithelial layer of mantle connective tissue, which is infiltrated by groups of parasitised granular haemocytes, presents a dissociated appearance (7). In *O. angasi*, apparently light infections of gill and
digestive diverticular epithelia by faintly staining pale basophilic intracellular *B. exitiosa*, results in massive epithelial hyperplasia.

- Electron microscopy/cytology: no information.

c) Agent detection and identification methods

- Direct detection methods

i) Microscopic methods

- **Tissue imprints**

  Samples to be taken: oyster spat or heart ventricule or gills from live adult hosts.

  Technical procedure: after drying tissues on absorbent paper, several imprints are made on a glass slide. Slides are air-dried, fixed in methanol or in absolute ethanol and stained using a commercially available blood-staining kit, in accordance with the manufacturer's instructions. After rinsing in tap water and drying, the slides are mounted with a coverslip using an appropriate synthetic resin. Slides are observed first at ×200 magnification and then under oil immersion at ×1000 magnification. Infections in *O. angasi* are usually very light, even at the point of death, and therefore infection can be very hard to detect using tissue imprints.

  Positive controls: recommended and available from the OIE Reference Laboratory.

  Levels of validation:

  - Specificity and sensitivity: low specificity, sensitivity better than histological examination (5). When compared with the polymerase chain reaction (PCR) and the *in-situ* hybridisation (ISH) combined, heart imprints show a sensitivity of 59.3% and a specificity of 100% (5).
  - Gold standard: tissue imprint sensitivity higher than histology, which is the gold standard although it is not species specific.

  Interpretation of results:

  - A positive result is the presence of small spherical or ovoid organisms (2–5 µm wide) within haemocytes. However, the parasite might also occur extracellularly. These organisms show a basophilic cytoplasm and an eosinophilic nucleus (colours may vary with used stain); because they spread on the slide, they can appear larger than on histological examination.

  Availability of tests: quick staining kits are commercially available (e.g. Hemacolor®).

- **Histopathology**

  Samples to be taken: live or freshly dead oysters.

  Technical procedure: sections of tissue that include gills, digestive gland, mantle, gonad should be fixed for 24 hours in Davidson's fixative followed by normal processing for paraffin histology and staining, for example with haematoxylin and eosin. Observations are made at increasing magnifications to ×1000.

  Positive controls: recommended and available from the OIE Reference Laboratory.

  Levels of validation:

  - Specificity and sensitivity: in *O. doliensis*, low specificity, sensitivity is good for moderate to high intensity infections, but low for low intensity infections. When compared with the PCR and the ISH combined, histology shows a sensitivity of 44% (lower than heart imprints) and a specificity of 100% (5).
  - Gold standard: histology is the gold standard and is the recommended surveillance method.
Interpretation of results:
- A positive result is the presence of parasites as very small cells of 2–5 µm wide within the haemocytes or freely in connective tissue or sinuses of gill, gut and mantle epithelium in *O. chilensis*, often associated with an intense disseminated haemocyte infiltration in *O. chilensis*, but intense focal haemocyte infiltration in *O. angasi*. To avoid any doubt the parasite has to be observed inside the haemocyte for a positive diagnosis. The technique is not species specific.

Availability of tests: no commercially available tests.

- **Transmission electron microscopy**
  Samples to be taken: live or freshly dead oysters.
  Technical procedure: described in chapter I.2 of this *Aquatic Manual*.
  Positive controls: no.
  Levels of validation:
  - Specificity and sensitivity: better specificity than imprints and histology. Transmission electron microscopy permits the differentiation of *B. exitiosa* from other closely related microcells like *B. ostreae*.

Interpretation of results:
- Positive result is the presence of parasites within haemocytes. In *O. chilensis*, four parasite development stages have been described in infected oysters corresponding to dense forms (Stage 1), intermediate forms (Stage 2), plasmodial form (Stage 3) and vacuolated form (Stage 4) (10, 13). Intracellular structures include mitochondria, haplosporosomes, Golgi apparatus and persistent intranuclear microtubules.
- Unlike other haplosporidians including *B. ostreae*, a stage containing a large vacuole derived from enlargement of one or more mitochondria has been observed in *B. exitiosa* (10, 13). Dense forms of *B. exitiosa* are less dense, slightly larger in size (3 ± 0.3 µm mean diameter *n* = 61 in comparison with *B. ostreae*, with a mean diameter of 2.4 ± 0.5 µm, *n* = 64), have more haplosporosomes, mitochondrial profiles and lipid bodies per ultrastructure section, and have smaller tubulo-vesicular mitochondria than *B. ostreae*. In addition, dense, but not light, forms of *B. ostreae* lack nuclear membrane-bound Golgi/nuclear cup complexes and a vacuolated stage (12).

Availability of tests: no commercially available tests.

**ii) Agent isolation and identification**

- **Cell culture/artificial media:** none.
- **Antibody-based antigen detection methods:** not currently available, but one of the two monoclonal antibodies raised against the cell membrane of *B. ostreae* reacted with *B. exitiosa* (16).
- **Molecular techniques: polymerase chain reaction (PCR)**
  Samples to be taken: live or freshly dead oysters.
  Technical procedure: tissue samples are placed in 95% ethanol or frozen at −80°C until DNA is extracted. DNA extraction is accomplished by proteinase K digestion overnight at 50°C and phenol-chloroform extraction and ethanol precipitation (3).
  A PCR protocol developed for the detection of *Bonamia ostreae* has been shown to allow *B. exitiosa* detection (2, 5, 13). The primer pair Bo-Boas (5'-CAT-TTA-ATT-GGT-CGG-GCC-GC-3' and 5'-GGG-GGA-TCG-AAG-ACG-ATC-AG-3', respectively) amplifies a 304 bp product from the SSU rDNA region (3). PCR mixtures contain buffer (500 mM KCl, 100 mM Tris/HCl (pH 9.0 at 25°C) and 1% Triton® X-100), 2.5 mM MgCl₂, 0.2 mM dNTP mix, 1 µM forward and reverse primers, 0.02 units/µl Taq DNA polymerase, and 0.2 ng/µl of the DNA template in a total volume of 50 µl.
Samples are denaturated in a thermocycler for 5 minutes at 94°C before being submitted to 30 cycles (94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute) followed by a final extension of 10 minutes at 72°C.

A second primer pair C₅ and C₆ (5'-CGG-GGG-CAT-AAT-TCA-GGA-AC-3' and 5'-CCA-TCT-GCT-GGA-GAC-ACA-G-3', respectively) also designated to amplify a 760 bp product from the SSU rDNA of *B. ostreae* should amplify *B. exitiosa* (2).

Positive/negative controls: compulsory. Positive controls are genomic DNA from heavily infected hosts. Negative controls are genomic DNA from uninfected hosts.

Levels of validation:

- **Specificity and sensitivity:** based on target DNA sequence similarity, the primer pair Bo-Boas should amplify all microcell haplosporidians (2). Sensitivity of the assay is higher than histocytological methods but lower than *in-situ* hybridisation (5).
- **Gold standard:** when compared with histology and heart imprints combined, and assuming that these latter techniques present 100% sensitivity and specificity, PCR shows a sensitivity of 88.2% (lower than ISH) and a specificity of 36.4% (5).

Interpretation of results:

- A positive result is an amplicon of the appropriate size, with all negative controls negative and all positive controls positive.
- The PCR assay is not species specific. The sequence of the SSU rDNA gene of *Bonamia exitiosa* shows polymorphism with that of *B. ostreae* and *B. roughleyi* by restriction fragment length polymorphism (RFLP) analysis by digesting Bo-Boas PCR products by *Bgl* I and *Hae* II. *Bonamia ostreae* and *B. exitiosa* present a same profile (two products of 115 and 189 bp) when digested with *Hae* II while *B. roughleyi* PCR product is not digested. The *B. ostreae* profile consists of two bands of 120 and 180 bp when digested with *Bgl* I while *B. exitiosa* and *B. roughleyi* PCR products are not digested (2, 13).

Availability of tests: no commercially available tests.

- **Molecular techniques:** *in-situ* hybridisation (ISH)

Samples to be taken: live or freshly dead oysters.

Technical procedure: an ISH protocol developed for the detection of *Bonamia ostreae* has been shown to allow *B. exitiosa* detection (3, 5). This assay uses a 300 bp digoxigenin-labelled probe targeting the SSU rDNA gene. Tissue samples are placed in Davidson’s fixative for 24 hours and then embedded in paraffin. Sections are cut 5 µm thick, placed on silane-coated slides and then baked overnight in an oven at 60°C. After dewaxing, slides are treated with proteinase K (100 µg/ml) in TE buffer (50 mM Tris, 10 mM EDTA [ethylene diamine tetra-acetic acid]) at 37°C for 30 minutes. Slides are dehydrated by immersion in an ethanol series and air dried. The slides are then covered with hybridisation buffer (4 × SSC [standard saline citrate; 60 mM NaCl, 600 mM NaCl, pH 7], 50% formamide, 1 × Denhardt’s solution, 250 µg/ml yeast tRNA, 10% dextran sulfate) containing 20 ng of the digoxigenin-labelled probe. After denaturation 5 minutes at 95°C, hybridisation is performed by incubating slides in a humid chamber overnight at 42°C. The probe is produced by PCR using the previously described primer pair Bo Boas with digoxigenin incorporation. The PCR is performed as described in the section on PCR except that DIG dUTP 25 mM is added to the reaction mixture. The detection steps are performed according to the manufacturer’s instructions.

Positive/negative controls: compulsory. Positive controls are histological sections from infected hosts. Negative controls are histological sections from uninfected hosts.

Levels of validation:

- **Specificity and sensitivity:** specificity is higher than histocytological methods.
- However, the probe Bo-Boas is also able to detect *Haplosporidium nelsoni* in
Crasostrea virginica, Bonamia ostreae in Ostrea edulis but not Mikrocytos mackini in C. gigas (2, 3). Sensitivity of the assay is higher than histocytological methods and PCR (5).

- Gold standard: when compared with histology and heart imprints combined, and assuming that these latter techniques present 100% sensitivity and specificity, ISH shows a sensitivity of 100% (higher than PCR) and a specificity of 27.3% (5).

**Interpretation of results:**
- A positive result corresponds to dark labelled parasites inside haemocytes, with all negative controls negative and all positive controls positive.

**Availability of tests:** DIG nucleic acid detection kit, Boehringer Mannheim.

- Agent purification: no.

**Indirect detection methods**
- Serological methods: none applicable.

### 4. RATING OF TESTS AGAINST PURPOSE OF USE

The methods currently available for surveillance, detection, and diagnosis of Bonamia ostreae are listed in Table 1. The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic sensitivity and specificity; C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and D = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility.

**Table 1.** Bonamia exitiosa surveillance, detection and diagnostic methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance</th>
<th>Presumptive</th>
<th>Confirmatory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In known host and geographical range</td>
<td>Outside known host and geographical range</td>
<td>In known host and geographical range</td>
</tr>
<tr>
<td>Gross signs</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Tissue imprints</td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>Histopathology</td>
<td>B</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>PCR</td>
<td>B</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>PCR-RFLP</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Transmission EM</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>In situ hybridisation</td>
<td>C</td>
<td>C</td>
<td>D</td>
</tr>
</tbody>
</table>

EM = electron microscopy; PCR = polymerase chain reaction, RFLP = Restriction Fragment Length Polymorphism

### 5. CORROBORATIVE DIAGNOSTIC CRITERIA

**a) Definition of suspect case**

In known susceptible species within the known geographical range of B. exitiosa, a suspect case of infection with B. exitiosa is a positive result by one of the following methods: histopathology, tissue imprint, PCR or in-situ hybridisation.
In other host species or outside the known range of *B. exitiosa*, a suspect case is a positive result by histopathology, tissue imprint, PCR or *in situ* hybridisation.

**b) Definition of confirmed case**

A confirmed case of *B. exitiosa* is a positive result by tissue imprints, histology or *in situ* hybridisation combined with a positive result by PCR-RFLP.

### 6. PRESCRIBED DIAGNOSTIC/DETECTION METHODS TO DECLARE FREEDOM

Prescribed methods for targeted surveillance to declare freedom from infection as outlined in the *Aquatic Code* are: tissue imprints (heart or gills), PCR or histology.

**REFERENCES**


* * *

\textbf{NB}: There is an OIE Reference Laboratory for Infection with \textit{Bonamia exitiosa} (see Table at the end of this Aquatic Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.2.3.

INFECTION WITH *HAPLOSPORIDIUM NELSONI*

1. CASE DEFINITION

For the purpose of this chapter, infection with *Haplosporidium nelsoni* is considered to be infection with *Haplosporidium nelsoni* or MSX disease.

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

   a) Agent factors

   - Aetiological agent, agent strains: the aetiological agent is *H. nelsoni*; there are no data on whether more than one strain exists.
   - Survival outside the host: maximum time unknown, but spore stage can likely survive weeks to months.
   - Stability of the agent: no data available.
   - Life cycle: unknown.

   b) Host factors

   - Susceptible stages of the host: all stages after settlement.
   - Species or subpopulation predilection: *Crassostrea virginica* is the most susceptible species; infections in *C. gigas* are rare (3).
   - Target organs and infected tissue: epithelium and connective tissue of all organs; sporulation restricted to epithelium of digestive tubules (1, 4, 7).
   - Persistent infection with lifelong carriers: infection usually fatal at salinities greater than 15 parts per thousand (ppt); lifelong carriers may occur at salinities between 10 and 15 ppt or in tolerant stocks.
   - Vectors: unknown.

   c) Disease pattern

   - Transmission mechanisms: mechanisms unknown, but infection ‘window’ is from May to October (8).
   - Prevalence: highly variable depending on salinity, but prevalences of 50–90% are not uncommon at salinities above 15 ppt (1, 4).
   - Geographical distribution: east coast of North America from the east coast of Florida, USA to Nova Scotia, Canada in *C. virginica* (2, 8, 12). *Haplosporidium nelsoni* is also present in *C. gigas* in California and Washington, USA (3) and in *C. gigas* in France, Japan and Korea (3, 11).
   - Mortality and morbidity: infection is usually rapidly fatal; death occurs within 1–3 months of infection. Infections acquired in late summer may overwinter and cause mortality the following April/May (1, 4).
• Economic and/or production impact of the disease: *Haplosporidium nelsoni* has caused epizootic mortality of *C. virginica* in Chesapeake Bay and Delaware Bay since the late 1950s (1, 4), and more recently in Long Island Sound in the USA (16), and in Bras d'Or Lakes, Nova Scotia, Canada (12). The pathogen is a continuing problem mainly in Chesapeake Bay, with only occasional epizootics in other areas. Oysters in Delaware Bay have acquired some natural resistance to mortality from *H. nelsoni* (2).

d) Control and prevention

• Vaccination: none.
• Chemotherapy: none.
• Immunostimulation: none.
• Resistance breeding: selective breeding has demonstrated effectiveness for reducing mortality caused by *H. nelsoni* (5, 10).
• Restocking with resistant species: disease tolerant strains of *C. virginica* are used in restoration efforts in Chesapeake Bay and in oyster aquaculture in Long Island Sound. Consideration is also being given to introduction of a non-susceptible host, *C. ariakensis*, in Chesapeake Bay.
• Blocking agents: none.
• General husbandry practices: farming in areas where salinity is less than 12 ppt, and use of fast-growing, disease-tolerant strains has shown some benefit.

3. DIAGNOSTIC METHODS

a) Field diagnostic methods

• Clinical signs: dead or gaping oysters. These clinical signs are not specific to infection with *H. nelsoni*.

b) Clinical methods

• Gross signs: mantle recession; thin, watery tissue; pale digestive gland (7). These gross signs are not specific to infection with *H. nelsoni*.
• Clinical chemistry: none.
• Microscopic examination
  • Wet mounts: none.
  • Smears: none.
  • Histology: extensive haemocyte infiltration at sites of infection. Mechanical damage and tissue lysis caused by proliferating parasites; sporulation causes metaplasia and disruption of the digestive tubule epithelium (7). Phagocytosis of plasmodia is rare.
  • Electron microscopy: no information.

c) Agent detection and identification methods

• Direct detection methods
  
  i) Microscopic methods
    
    • *Histology*
      
      Samples to be taken: live or freshly dead oysters.
Technical procedure: sections of tissue no more than 5 µm thick, that include digestive gland, gill and mantle should be fixed for 24 hours in Davidson’s AFA (10% glycerin, 20% formaldehyde, 30% ethanol [95%], 30% distilled water, 10% glacial acetic acid), then transferred to 70% ethanol until processed for paraffin histology and staining with haematoxylin and eosin. Observations are made at increasing magnifications to ×400.

Positive controls: recommended and available from the OIE Reference Laboratory.

Levels of validation:
- Specificity and sensitivity: species specificity is low unless spores are present; sensitivity is good for light to high intensity systemic infections, but poor for very light, focal infections. Histology is the gold standard for diagnosis of *H. nelsoni* infections.

Interpretation of results:
- A positive result is the occurrence of spherical to oval multinucleate plasmodia ranging in diameter from 4 to 50 µm, and staining eosinophilic. Spores occur only in the epithelium of the digestive tubules; they are 6–8 µm in length.
- In susceptible host species within the known range of *H. nelsoni*, the presence of spores of the proper size in digestive tubule epithelium is strong evidence of *H. nelsoni* infection. The presence of multinucleate plasmodia in estuarine areas where salinity is less than 25 ppt is also presumptive evidence of *H. nelsoni*. On the north-east coast of North America, *H. costale* can be present in areas where salinity is greater than 25 ppt and plasmodia of *H. costale* are not distinguishable from *H. nelsoni* plasmodia (14), however, *H. costale* sporulates in the vesicular connective tissue, not the digestive tubule epithelium. In all cases, species identification should be confirmed by species-specific polymerase chain reaction (PCR) and/or in-situ hybridisation (ISH) with species-specific DNA probes.

Availability of tests: no commercially available tests.

ii) Agent isolation and identification

- *Cell culture/artificial media:* Plasmodia have been cultured *in vitro* for up to 5 weeks in a medium consisting of 40% sterile sea water, 35% sterile oyster haemolymph and 25% 1/1 DME/Ham’s F-12 medium, with antibiotics, at a pH of 7.4 (9). Culture medium is inoculated with haemolymph drawn from the adductor muscle sinus. Continuous culture of *H. nelsoni* has not been successful.
- *Antibody-based antigen detection methods:* none developed.
- *Molecular techniques:* PCR (11, 15).

Samples to be taken: live or freshly dead oysters.

Technical procedure: gill and digestive gland tissue samples are placed in 95% ethanol or frozen at −70°C until DNA is extracted. DNA extraction is accomplished by proteinase K digestion overnight at 56°C and spin-column methodology using commercially available kits (e.g. Qiagen Dneasy tissue kit).

Primer pairs that target the SSU rRNA gene have been developed for *H. nelsoni*. Primers are MSX-A’ (5’-CGA-CTT-TGG-CAT-TAG-GTT-TCA-GAC-C-3’) and MSX-B (5’-ATG-TGT-TGG-TGA-CGC-TAA-CCG-3’) and amplify 573 base pair product. PCR reaction mixtures contain 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 10 µg/ml gelatin; 400 µg/ml bovine serum albumin; 200 µM each of dATP, dCTP, dGTP, dTTP; 0.6 units AmpliTaq DNA polymerase; 25 pmoles of each primer (MSX-A’ and MSX-B); 200–400 ng template DNA in a total volume of 25 µl. Cycling parameters are an initial denaturation of 4 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 59°C, 1.5 minutes at 72°C with a final extension of 5 minutes at 72°C.
Positive/negative controls: compulsory. Positive controls are genomic DNA from heavily infected hosts (available from the OIE Reference Laboratory). Negative controls are no target DNA reactions.

Levels of validation:
- Specificity and sensitivity: SSU region primers were tested for specificity against a wide variety of other haplosporidians and other oyster parasites (12). Sensitivity is high, but subsampling error in very light, localised infections may lead to false negatives.
- Gold standard: unpublished data from the OIE Reference Laboratory indicate that the PCR assay for H. nelsoni is much more sensitive than paraffin histology; however some light, localised infections detected by histology are missed by PCR, leading to false negatives by PCR.

Interpretation of results:
- A positive result is a PCR amplicon of the appropriate size, with all negative controls negative and all positive controls positive.

Availability of tests: not commercially available.

Molecular techniques: In-situ hybridisation (13, 14).

Samples to be taken: live or freshly dead oysters.

Technical procedure: a specific DNA probe has been developed for H. nelsoni (MSX1347: 5'-ATG-TGT-TGG-TGA-CGC-TAA-CCG-3'); it should be 5’ end-labelled with digoxigenin. Follow procedure for ‘fixed sections’ above, except that tissue sections must be placed on positively charged glass slides or slides coated with 3-aminopropyl-triethoxylane, without staining. Deparaffinise sections in xylene for 10 minutes and then rehydrate in an alcohol series. Wash sections twice for 5 minutes in phosphate-buffered saline (PBS).

The sections are treated with proteinase K, 50 µg/ml in PBS, at 37°C for 18 minutes. The reaction is then stopped by washing the sections in PBS with 0.2% glycine for 5 minutes. The sections are then placed in 2 × SSC (standard saline citrate; 20 × SSC = 3 M NaCl; 0.3 M Na-citrate; pH 7.0) for 10 minutes.

The sections are prehybridised for 1 hour at 42°C in prehybridisation solution (4 × SSC, 50% formamide, 5 × Denhardt’s solution, 0.5 mg/ml yeast tRNA, and 0.5 mg/ml heat-denatured salmon sperm DNA).

The prehybridisation solution is then replaced with prehybridisation buffer containing 2 ng/µl of the digoxigenin-labelled H. nelsoni probe or 3 ng/µl each of the digoxigenin-labelled H. costale probes. The sections are covered with in-situ hybridisation plastic cover-slips and placed on a heating block at 90°C for 12 minutes. The slides are then cooled on ice for 1 minute before hybridisation overnight at 42°C in a humid chamber.

The sections are washed twice for 5 minutes each in 2 × SSC at room temperature, twice for 5 minutes each in 1 × SSC at room temperature, and twice for 10 minutes each in 0.5 × SSC at 42°C. The sections are then placed in Buffer 1 (100 mM Tris, pH 7.5, 150 mM NaCl) for 1–2 minutes.

The sections are placed in Buffer 1 supplemented with 0.3% Triton X-100 and 2% sheep serum for 30 minutes. Anti-digoxigenin alkaline phosphatase antibody conjugate is diluted 1/500 (or according to the manufacturer’s recommendations) in Buffer 1 supplemented with 0.3% Triton X-100 and 1% sheep serum and applied to the tissue sections. The sections are covered with in-situ hybridisation cover-slips and incubated for 3 hours at room temperature in the humid chamber.

The slides are washed twice in Buffer 1 for 5 minutes each and twice in Buffer 2 (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM MgCl₂) for 5 minutes each. The slides are then placed in colour development solution (337.5 µg/ml nitroblue tetrazolium,
175 µg/ml 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, 240 µg/ml levamisole in Buffer 2) for 2 hours in the dark. The colour reaction is stopped by washing in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA [ethylene diamine tetra-acetic acid]).

The slides are then rinsed in dH₂O. The sections are counterstained with Bismarck Brown Y, rinsed in dH₂O, and cover-slips are applied using an aqueous mounting medium.

Positive/negative controls: compulsory. Positive controls are tissue sections from *H. nelsoni*-infected oysters. Negative controls are either no-probe assays or assays with uninfected oysters.

Levels of validation:
- Specificity and sensitivity: the *H. nelsoni* DNA probe has been tested for specificity against a variety of other haplosporidians and other oyster parasites (13). Sensitivity is equal to the gold standard, paraffin histology.

Interpretation of results
- A positive result is the presence of purple-black labelling of the parasite cells, with all negative controls negative and all positive controls positive.
- *Agent purification:* plasmodia of *H. nelsoni* in oyster haemolymph can be partially purified from oyster haemocytes by the technique of panning (6).

**Indirect detection methods**

- Serological methods: none available.

4. RATING OF TESTS AGAINST PURPOSE OF USE

The methods currently available for surveillance, detection, and diagnosis of *H. nelsoni* are listed in Table 1. The designations used in the table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic specificity and sensitivity; C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and D = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity, and utility.

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance</th>
<th>Presumptive</th>
<th>Confirmatory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross signs</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Histology plasmodia only spores present</td>
<td>A</td>
<td>D&lt;sup&gt;1&lt;/sup&gt;</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Polymerase chain reaction</td>
<td>C</td>
<td>B</td>
<td>B&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>In-situ</em> hybridisation</td>
<td>D</td>
<td>D</td>
<td>A</td>
</tr>
</tbody>
</table>

<sup>1</sup>Histology can establish that an infection is present, but cannot reliably distinguish species of *Haplosporidium*.

<sup>2</sup>Useful as a confirmatory diagnosis only if plasmodia have been visualised by histology.

5. CORROBORATIVE DIAGNOSTIC CRITERIA

a) Definition of suspect case

In known susceptible species within the known geographical range of *H. nelsoni*, a suspect case of infection with *H. nelsoni* is a finding of plasmodia in histological sections, or a positive result by
PCR alone. In other host species or outside the known range of H. nelsoni, a suspect case is a positive result by PCR. Such cases should be referred to the OIE Reference Laboratory.

b) Definition of confirmed case

A confirmed case of H. nelsoni is either 1) the finding of spores of the appropriate size in the epithelium of digestive tubules; 2) a combination of plasmodia in histological sections combined with a positive result by PCR; or 3) a positive result with in-situ hybridisation.

6. Diagnostic/detection methods to declare freedom

Methods for targeted surveillance to declare freedom from infection as outlined in the Aquatic Code are: PCR with subsequent histology of positive cases.

REFERENCES


*  
*  *

**NB:** There is an OIE Reference Laboratory for Infection with *Haplosporidium nelsoni* (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: [www.oie.int](http://www.oie.int)).
CHAPTER 2.2.4.

INFECTION WITH MARTEILIA REFRINGENS

1. CASE DEFINITION

For the purpose of this chapter, infection with *Marteilia refringens* covers infection with *Marteilia refringens* as defined by Le Roux *et al.* (21). This definition excludes infections with *Marteilia sydneyi* (30), *M. lenghei* (13) and *M. christenseni* (14). *Marteilia* spp. that are not identified to the species level (10, 15, 25–27) should be referred to the appropriate OIE Reference Laboratory.

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

a) Agent factors

- Aetiological agent, agent strains: *Marteilia refringens* (16, 18) as defined by Le Roux *et al.* (21).
- Survival outside the host: depending on the environmental conditions from several days up to 2–3 weeks (17).
- Stability of the agent: no data available.
- Life cycle: indirect – includes *Paracartia grani* (2–4). Other species have not been shown to be involved in the life cycle (for detailed information see Table 1 in Ref. 2).

b) Host factors

- Susceptible host species: *Ostrea* species including *O. edulis* (18), *O. chilensis* (17, 19), *O. puelchana* (28), *O. angasi* (17) and *Mytilus* species including *M. edulis* (5, 21) and *M. galloprovincialis* (23, 28, 32, 37, 38).
- Susceptible stages of the host: juveniles and older life stages (17).
- Species or sub-population predilection (probability of detection): usually causes clinical infection in *Ostrea edulis* (5, 18) and *Ostrea* spp. (10, 17, and 19). In wild populations, molluscs aged more than 2 years usually have higher levels of infection (2).
- Target organs and infected tissue: digestive tract. Young plasmodia are mainly found in the epithelium of labial palps and the stomach (18). Sporulation takes place in the digestive gland tubules and ducts. Propagules are released into the lumen of the digestive tract and shed into the environment in faeces (3, 9, 10).
- Persistent infection with lifelong carriers: infection with *Marteilia refringens* is a lethal disease of oysters (1, 3, 9, 18). Death occurs during the second year after initial infection (1, 6, 9, 17). Therefore infection may persist for more than 1 year and may be lifelong (6). Mussels are usually not adversely affected by *M. refringens* (7, 10), but whether sporulation of *M. refringens* occurs and whether mussels can be carriers of *M. refringens* is not known (10, 21).
- Vectors: unknown.
c) **Disease pattern**

- Transmission mechanisms: via intermediate host (3, 9). Transmission from *P. grani* to *O. edulis* has not been demonstrated experimentally (3). In oysters, early stages of disease occur in the stomach, palps and even gill epithelia. It is thought that initial infection occurs via feeding currents.

- Prevalence: highly variable – up to 98%. Higher prevalence is expected according to farming practices and more than 1 year of exposure to infection (10, 17).

- Geographical distribution: reported in Croatia, France, Greece, Italy, Morocco, Portugal and Spain.

- Mortality and morbidity: infection is lethal for oysters – death usually occurs during summer and autumn, and is associated with sporulation of the parasite (9, 17, 18). Similarly, morbidity is higher during warmer periods. Mussels are less affected by infection (7, 10).

- Economic and/or ecological impact of the disease: between 1980 and 1983 in France *Marteilia refringens* caused serious losses evaluated at 440 million euros (17).

- Environmental factors (e.g. temperature, salinity, season, etc.): 17°C is regarded as the threshold for parasite sporulation and transmission – common in estuaries where prevalence is usually higher in the upper parts (2, 4, 10, 17).

d) **Control and prevention**

- Vaccination: none.

- Chemotherapy: none.

- Immunostimulation: none.

- Resistance breeding: none.

- Restocking with resistant species: none. Attempts have been made in Europe with different species of genus *Ostrea* that all have demonstrated their susceptibility (17, 19, 29). Naive stocks of *Ostrea edulis* and *Mytilus edulis* are highly susceptible to the infection.

- Blocking agents: none.

- General husbandry practices: stocking at low density or in association with resistant mollusc species such as *Crassostrea gigas* has been shown to be effective (11).

### 3. **Diagnostic methods**

a) **Field diagnostic methods**

- Clinical signs: dead or gaping oysters; weak animals particularly susceptible to any additional stress (17, 18). These clinical signs are not specific to infection with *M. refringens*.

b) **Clinical methods**

- Gross signs: pale digestive gland, thin watery flesh, mantle retraction and reduced growth rate (10, 17, 18). These gross signs are not specific to infection with *M. refringens*.

- Clinical chemistry: none.
• Microscopic examination:
  • Wet mounts: none.
  • Smears: none.

• Histology: the digestive gland, in which *M. refringens* and other *Marteilia* species occur, is a site of intracellular food digestion and one of the main sites for storage of metabolic reserves (10). In heavy infections, *M. refringens* significantly reduces absorption of organic matter (12, 34). Severe infections may also cause loss of condition as a consequence of reduced energy acquisition. Furthermore, the parasite may interfere directly with host feeding and absorption simply by its physical presence. Development of adipo-granular storage cells in the mantle of *Mytilus galloprovincialis* was shown to be inhibited in the presence of *M. refringens* (36). Apparently, *M. refringens* also interferes with glycogen storage in *Ostrea edulis* (31).

• Electron microscopy/cytopathology: none.

c) Agent detection and identification methods

• Direct detection methods

  i) Microscopic methods

  • *Wet mounts*: in advanced infection.

    Samples to be taken: gaping oysters or freshly dead oysters.

    Technical procedure: squash a piece of digestive gland or faeces on a glass slide; observations are made at 400× and can potentially show refringent granules in sporangia

    Positive/negative controls: no.

    Levels of validation:
    • Specificity and sensitivity: unknown, presumably low;
    • ‘Gold’ standard: not validated against histology.

    Interpretation of results:
    • A positive result is the presence of large (20–30 µm) spherical bodies containing thick wall structures;
    • In susceptible species within the known geographical range of infection with *M. refringens*, a positive result is indicative of infection with *M. refringens*;
    • In other species or outside the known geographical range of infection with *M. refringens*, a positive result is indicative of infection with a *Marteilia* species that needs to be confirmed.

    Availability of test: no kits available commercially.

  • *Smears*: in advanced infection, digestive gland imprints.

    Samples to be taken: gaping oysters or freshly dead oysters.

    Technical procedure: blot a piece of digestive gland on a glass slide; observations are made at 400× after Giemsa staining.
Chapter 2.2.4. - Infection with *Marteilia refringens*

Positive/negative controls: recommended. Positive and negative controls are available on request from the OIE Reference Laboratory.

Levels of validation:
- Specificity and sensitivity: unknown, presumably low;
- ‘Gold’ standard: not validated against histology.

Interpretation of results:
- A positive result is the observation of cells ranging in size up to 30–40 µm. Cytoplasm stains basophilic; nucleus stains eosinophilic. Pale halo around large, strongly stained (refringent) granules and in larger cells, cell within cell arrangements are observed (8, 10, 16, 18);
- In susceptible species within the known geographical range of infection with *M. refringens*, a positive result is strongly indicative of infection with *M. refringens*;
- In other species or outside the known geographical range of infection with *M. refringens*, a positive result is indicative of infection with a *Marteilia* species that needs to be confirmed.

Availability of test: commercially available quick-staining kits include Difquick®/Hemacolor®.

- **Fixed sections**

Samples to be taken: live oysters.

Technical procedure: for histology, sections should be cut through the digestive gland including the gills and palps. Observations are made at increasing magnification to 400× after conventional histological staining, i.e. H&E (haematoxylin–eosin).

Positive/negative controls: recommended. Positive and negative controls are available on request from the OIE Reference Laboratory.

Levels of validation:
- Specificity and sensitivity: Values of sensitivity and specificity for histology were estimated at 0.7 and 0.99, respectively (35);
- ‘Gold’ standard: histology is the gold standard; *in-situ* hybridisation co-validated with histology.

Interpretation of results:
- A positive result is the observation of cells ranging in size from 4 up to 40 µm. Young plasmodia (uninucleate) are mainly found in the epithelium of labial palps and stomach. Sporulation involves divisions of cells within cells and takes place in the digestive gland tubules and ducts. Refringent granules appear in the course of sporulation, but are not observed in early stages. In late phases of infection, sporangia are observed free in the lumen of the digestive tract. Cytoplasm stains basophilic; nucleus stains eosinophilic; granules can range from deep orange to deep red.
- In susceptible species within the known geographical range of infection with *M. refringens*, a positive result is conclusive of infection with *M. refringens*;
- In other species or outside the known geographical range of infection with *M. refringens*, a positive result, even with refringent granules being observed, is indicative of infection with a *Marteilia* species that needs to be confirmed.

Availability of test: no kits available commercially.
ii) Agent isolation and identification

- **Cell culture/artificial media:** not available.

- **Antibody-based antigen detection methods (IFAT, ELISA, etc.):** not currently available or used for diagnostic purpose but monoclonal antibodies have been developed and published (10).

- **Agent purification:** currently not used for diagnostic purpose but purification protocols have been developed and published (24, 33).

- **Molecular techniques:** Polymerase chain reaction (PCR) protocols have been developed and published (8, 21, 22).

Samples to be taken: live oysters or freshly dead oysters.

Technical procedure: For DNA extraction, animals are frozen at –80°C and tissues ground to powder. Around 10 volumes of extraction buffer (NaCl [100 mM], ethylene diamine tetra-acetic acid [EDTA, 25 mM], pH 8, sodium dodecyl sulfate [SDS, 0.5%]) are added with proteinase K (100 µg/ml). Following an overnight incubation at 50°C, DNA is extracted using a standard phenol/chloroform protocol, and precipitated with ethanol. PCR is carried out in 50 µl volume. After denaturation of DNA at 94°C for 5 minutes, 30 cycles are run as follows: denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and elongation at 72°C for 1 minute per kilo-base pair. A final elongation step of 10 minutes at 72°C is performed. For the detection of *M. refringens*, PCR is performed with primers that target the ITS1 region (5'-CCG-CAC-ACG-TTC-TTC-ACT-CC-3' and 5'-CTC-GCG-AGT-TTC-GAC-AGA-CG-3') (21).

Positive/negative controls: compulsory. Positive controls are: 1) PCR with specific primers on genomic DNA from infected host or DNA from purified parasite; 2) nonspecific amplification (actin, Small Sub-Unit, etc.) of samples. Negative controls are: 3) no target DNA reactions; 4) PCR with specific primers on genomic DNA from non-infected hosts. Positive controls (22) are available on request from the OIE Reference Laboratory.

Levels of validation:

- **Specificity and sensitivity:** unknown values. No cross-reaction has occurred with tested samples and specificity is considered very high (20, 22). This PCR is expected to detect *M. refringens*. Because infection may be focal and also because infection targets different tissues in the early and late stages, the sensitivity of PCR detection may be lower than expected theoretical PCR performances;

- **‘Gold’ standard:** not validated against histology.

Interpretation of results:

- A positive result is positive PCR amplification at the expected size; and all negative controls being negative and all positive controls being positive;

- In susceptible species within the known geographical range of infection with *M. refringens*, a positive PCR result, associated with a positive result by means of histology or smears, is confirmatory of infection with *M. refringens*;

- In other species or outside the known geographical range of infection with *M. refringens*, a positive PCR result, associated with a positive result by means of histology or smears, is strongly indicative of infection with *M. refringens*, but PCR product sequencing is necessary before confirmatory diagnosis.

Availability of test: no kits available commercially.
• **In-situ hybridisation (ISH):** ISH protocols have been developed and published (8, 21).

Samples to be taken: live oysters or gaping oysters.

Technical procedure: For ISH, molluscs are fixed in Davidson’s fixative for approximately 24 hours and then embedded in paraffin. Sections are cut 5 µm thick and placed on aminoalkylsilane-coated slides, which are then baked overnight in an oven at 40°C. The sections are dewaxed by immersing in xylene for 10 minutes. This step is repeated once and then the solvent is eliminated by immersion in two successive absolute ethanol baths for 10 minutes each. The sections are then rehydrated by immersion in an ethanol series. The sections are treated with proteinase K (100 µg/ml) in TE buffer (Tris [50 mM], EDTA [10 mM]), at 37°C for 30 minutes. Slides are dehydrated by immersion in an ethanol series and then air dried. Sections are then incubated with 100 µl of hybridisation buffer (4 × SSC [standard saline citrate], 50% formamide, 1 × Denhardt’s solution, 250 µg/ml yeast tRNA, 10% dextran sulfate) containing 10 ng (1 µl of the PCR reaction) of the digoxigenin-labelled probe. Sections are covered with in-situ plastic cover-slips and placed on a heating block at 95°C for 5 minutes. Slides are then cooled on ice for 1 minute before overnight hybridisation at 42°C in a humid chamber. Sections are washed twice for 5 minutes in 2 × SSC at room temperature, and once for 10 minutes in 0.4 × SSC at 42°C. The detection steps are performed according to the manufacturer’s instructions. The slides are then rinsed in dH2O. The sections are counter-stained with Bismarck Brown Yellow, rinsed in dH2O, and cover-slips are applied using an aqueous mounting medium.

Positive/negative controls: compulsory. Positive controls are: 1) ISH on infected host; 2) nonspecific ISH (SSU rDNA) on samples. Negative controls are: 3) no probe ISH reactions; 4) ISH on non-infected hosts. Positive controls (21) are available on request from the OIE Reference Laboratory.

Levels of validation:
• Specificity and sensitivity: 0.9 and 0.99 respectively in the case of in-situ hybridisation (34). This ISH protocol is expected to detect *M. refringens*;
• ‘Gold’ standard: co-validated with histology.

Interpretation of results:
• A positive result is demonstrated by the purple-black labelling of *M. refringens* cells within known target tissues; and all negative controls being negative and all positive controls being positive;
• In susceptible species within the known geographical range of infection with *M. refringens*, a positive ISH result is confirmatory of infection with *M. refringens*;
• In other species or outside the known geographical range of infection with *M. refringens*, a positive ISH result is still confirmatory of infection with *M. refringens*. Such a case should be referred to the appropriate OIE Reference Laboratory.

Availability of test: probe can be obtained from the appropriate OIE Reference Laboratory.

• **Sequencing:** as stated above, sequencing is recommended as one of the final steps for confirmatory diagnostic. Targeted regions are SSU rDNA and ITS1. Although sequences are available in gene banks, it is recommended to refer such cases to the appropriate OIE Reference Laboratory.

• **Indirect detection methods**

• Serological methods: none applicable.
4. **RATING OF TESTS AGAINST PURPOSE OF USE**

Here ‘fitness for purpose’ is a combination of applicability and performance.

The methods currently available for surveillance, detection and diagnosis of infection with *M. refringens* are listed in the Tables below.

The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic specificity and sensitivity; C = the method has some applications in some situations, but cost, accuracy, or other factors severely limits its application; and D = the method is presently not recommended for this purpose. These designations (A, B, C and D) are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests in category A or B have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, make them acceptable.

**Table 1.** *Marteilia refringens* surveillance, detection and diagnostic methods

<table>
<thead>
<tr>
<th>Susceptible species in infected area</th>
<th>Surveillance</th>
<th>Presumptive diagnostic</th>
<th>Confirmatory diagnostic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field diagnostic methods</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Clinical methods</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Squash</td>
<td>C</td>
<td>B</td>
<td>D</td>
</tr>
<tr>
<td>Smears</td>
<td>B</td>
<td>A</td>
<td>C</td>
</tr>
<tr>
<td>Histology</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>PCR</td>
<td>B</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>ISH</td>
<td>C</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>Sequence</td>
<td>D</td>
<td>D</td>
<td>A</td>
</tr>
</tbody>
</table>

**Table 1 (cont).** *Marteilia refringens* surveillance, detection and diagnostic methods

<table>
<thead>
<tr>
<th>Other species or an area of unknown status</th>
<th>Surveillance</th>
<th>Presumptive diagnostic</th>
<th>Confirmatory diagnostic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field diagnostic methods</td>
<td>D</td>
<td>D</td>
<td>D</td>
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<tr>
<td>Clinical methods</td>
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<tr>
<td>Squash</td>
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<td>B</td>
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<tr>
<td>Sequence</td>
<td>D</td>
<td>D</td>
<td>A</td>
</tr>
</tbody>
</table>

5. **CORROBORATIVE DIAGNOSTIC CRITERIA**

a) **Definition of suspect case**

- In susceptible species within the known geographical range of infection with *M. refringens*, a suspect case of infection with *M. refringens* is a positive result by one of the following methods: squash, smears, or PCR.

- In other species or outside the known geographical range of infection with *M. refringens*, a suspect case of infection with *M. refringens* is a positive result by histology or PCR.
b) Definition of confirmed case

- In susceptible species within the known geographical range of infection with *M. refringens*, a confirmed case of infection with *M. refringens* is:
  - a positive result by histology or ISH; or
  - a suspected case with PCR alone combined with positive histology or ISH; or
  - a suspected case with squash or smears alone combined with positive PCR.

- In other species and in susceptible species outside the known geographical range, a confirmed case of infection with *M. refringens* is:
  - a case suspected by PCR combined with a positive result with histology and ISH; or
  - a case suspected by histology combined with a positive result with PCR and ISH.

All such cases should be referred immediately to the OIE Reference Laboratory for *M. refringens* whether or not clinical signs are associated with the case.

6. Diagnostic/Detection Methods to Declare Freedom

Methods for targeted surveillance to declare freedom from infection as outlined in the *Aquatic Code*, and based on the information provided above are:

- Histology; or
- ISH.

REFERENCES


Chapter 2.2.4. - Infection with Marteilia refringens


* * *

**NB:** There is an OIE Reference Laboratory for Infection with *Marteilia refringens* (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.2.5.

INFECTION WITH MIKROCYTOS MACKINI

1. CASE DEFINITION

For the purpose of this chapter, infection with Mikrocytos mackini is considered to be infection with Mikrocytos mackini.

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

a) Agent factors

- Aetiological agent, agent strains: Mikrocytos mackini, no known strains (13).
- Survival outside the host (i.e. in the natural environment) is unknown. However, direct transmission between oysters occurs via the water column (1, 14, 18).
- Stability of the agent unknown. No evidence of a cell wall has been observed and the apparent reliance on the organelles of its host for nutrients (15) suggests that this parasite lacks stability when outside its host.
- Life cycle: direct from host to host.

b) Host factors

- Susceptible host species: oysters – Crassostrea gigas, C. virginica, Ostrea edulis and O. conchaphila (= O. lurida) (7); the clam Panope abrupta is resistant to infection (4).
- Susceptible stages of the host: all stages after settlement (4). The susceptibility of larvae is not known.
- Species or sub-population predilection (probability of detection): all susceptible species held at less than 10°C for at least 3 months are vulnerable to the disease (7, 14). Crassostrea virginica, Ostrea edulis and Ostrea conchaphila appear to be more susceptible to infection and disease than Crassostrea gigas (7).
- Target organs and infected tissue: connective tissue of all organs, adductor muscle fibres, and haemocytes (15). Recently, M. mackini was observed in the epithelium of the digestive gland (17).
- Persistent infection with lifelong carriers: infection can be fatal depending on host and environmental conditions (1, 2, 9). Subclinical infections occur but the persistence of infection over several years and occurrence of lifelong carriers is not known (6).
- Vectors: none required.

c) Disease pattern

- Transmission mechanisms: transmission is direct from host to host (14). Viable cells released upon death of the host and possibly with the host faeces, and diapedesis through the gills are probably acquired through feeding mechanisms.
- Prevalence: historically during the spring (April and May), mortalities up to 40% have been reported at low intertidal levels among old (3+ years) C. gigas grown on the beach substrate.
About 10% of the oysters from a suspended culture site that could not be harvested on schedule died with heavy *M. mackini* infections the following spring. Up to 45% of the oysters from this infected population had symptomatic lesions (yellowy green pustules). However, this disease does not usually have an impact on oysters under intensive culture (harvest within 3 years). Laboratory exposure experiments indicated that spat are susceptible to infection and resulting high mortality (4). The impact of *M. mackini* on spat during commercial culture is not known but expected to be negligible if spat are deployed after the end of the natural transmission period that occurs in the spring (18).

- Geographical distribution: Canadian west coast, probably ubiquitous throughout the Strait of Georgia and confined to other specific localities around Vancouver Island, and adjacent areas of the State of Washington, USA.
- Mortality and morbidity: infection can be lethal if environmental temperatures are <10°C for 3–4 months (9, 14). However, about half of the exposed oysters seem to be resistant to infection.
- Economic and/or production impact of the disease: management techniques can be employed by the aquaculture industry to circumvent the impact of *M. mackini* (1). Susceptible species other than *Ostrea conchaphila* are not native to areas where *M. mackini* is known to occur. Populations of *O. conchaphila* are low and this species has been identified as a species of special concern by the Committee on the Status of Endangered Wildlife in Canada (12). The impact of *M. mackini* on *O. conchaphila* populations is not known.

**d) Control and prevention**
- Vaccination: none
- Chemotherapy: none
- Immunostimulation: none
- Resistance breeding: none
- Restocking with resistant species: none
- Blocking agents: none
- General husbandry practices: harvest market-sized oysters within 3 years of planting and prior to February of the third year of grow-out. Oyster spat should not be deployed at lower tide levels or adjacent to infected stock in suspended culture before June (1, 18).

### 3. DIAGNOSTIC METHODS

**a) Field diagnostic methods**
- Clinical signs: dead or gaping oysters during the spring. Focal yellowy green pustules up to 5 mm in diameter observed in the soft tissues and often with a brown scar on the shell, adjacent to the abscess on the mantle surface. Apart from the pustules, infected oysters are usually in good condition up to the time of death. These clinical signs are not specific to infection with *M. mackini*.

**b) Clinical methods**
- Gross signs: focal yellowy green pustules up to 5 mm in diameter, within the body wall, adductor muscle or on the surfaces of the labial palps or mantle (3). These gross signs are not specific to infection with *M. mackini*.
- Clinical chemistry: none.
• Microscopic examination
• Wet mounts: none
• Tissue imprints: see description below
• Fixed sections: foci of haemocyte infiltration in the mantle, labial palps and adductor muscle. Tissue necrosis may occur at the centre of the lesion (8). In high intensity infections induced in the laboratory, haemocyte infiltration may not be present.
• Electron microscopy/cytopathology: see description below.

c) Agent detection and identification methods

• Direct detection methods
  i) Microscopic methods

    • Tissue imprints: in advanced infection only.

    Samples to be taken: live hosts with pustules.

    Technical procedure: excise pustule and cut in half with scalpel. Blot pustule side of tissue on absorbent paper to remove excess fluid. Touch tissue to several areas of a clean glass slide and air dry. Observations are made at ×1000 (oil emersion) after staining with Wright–Giemsa (Romanovsky) stains or with a commercially available staining kit for blood cells in accordance with the manufacturer’s instructions.

    Positive/negative controls: no.

    Levels of validation:
    • Specificity and sensitivity: very low specificity because *M. mackini* resembles other microcells in tissue imprints; sensitivity is better than routine histology but only when pustules are present (14).

    Interpretation of results:
    • Presence of small microcells (can be distorted to about 4 µm in diameter) that are usually observed outside of the host cells. The parasite, usually 2–3 µm in diameter has blue (basophilic) cytoplasm and a small red (eosinophilic) nucleus (colours may vary with stain used). The technique is not species specific.

    Availability of tests: quick staining kits are commercially available (e.g. Hemacolor®, Merck; Diff-Quick®, Baxter).

    • Histology

    Samples to be taken: live or freshly dead oysters.

    Technical procedure: sections of tissue that include pustules and the adductor muscle should be fixed for 24 hours in Davidson’s solution or 10% buffered formalin followed by normal processing for paraffin histology and staining with haematoxylin and eosin stain. Observations are made at increasing magnifications to ×1000.

    Positive controls: recommended and available from the OIE Reference Laboratory.

    Levels of validation:
    • Specificity and sensitivity: species specificity is very low for microcells observed in haemocytes but high when microcells are found within the cytoplasm of vesicular connective tissue cells; sensitivity is good for moderate to high intensity infections especially when the connective tissue immediately around pustules is examined, but low for tissues distant from pustules and for low intensity infections.
Interpretation of results:
- A positive result is the occurrence of spherical microcells about 2–3 µm in diameter within the cytoplasm of vesicular connective tissue cells and/or myocytes, usually intracellular in host cells immediately adjacent to focal intense haemocyte infiltration.
- In susceptible host species within the known range of *M. mackini*, a positive result is presumptive evidence of *M. mackini* infection. Outside the known range, a positive result must be confirmed by DNA sequencing of the small-subunit ribosomal RNA gene (SSU rDNA) region and comparison of the sequence with the 1457 bp fragment from *M. mackini* published in GenBank (Accession number AF477623, URL: http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=30515676).

Availability of tests: no commercially available tests.

Transmission electron microscopy

Samples to be taken: live or freshly dead oysters.

Technical procedure: described in chapter I.2 of this *Aquatic Manual*.

Positive controls: no.

Levels of validation:
- Specificity and sensitivity: better specificity than histology. Transmission electron microscopy can be used to differentiate *M. mackini* from other microcells such as all species of *Bonamia*. Sensitivity is very low.

Interpretation of results:
- Positive result is the presence of parasites within vesicular connective tissue cells, myocytes and haemocytes. Three morphological forms have been described (15). The quiescent cells (QC) has a central round to ovoid nucleus, less than seven cisternae of inactive nuclear membrane-bound Golgi apparatus, few vesicles and lyosome-like bodies. They occur in the vesicular connective tissue cells, haemocytes (hyalinocytes), adductor and heart myocytes, and extracellularly. The vesicular cells (VC) contain many small coated and uncoated vesicles, lack nuclear membrane-bound Golgi-like arrays and the nuclear membrane is sometimes dilated to form a cisternal chamber. They are rarely extracellular and usually occur in adductor and heart myocytes, in close association with host cell mitochondria. The endosomal cells (EC) have a dilated nuclear membrane, a well developed anastomosing endoplasmic reticulum connecting the nuclear and plasma membranes and endosomes are present in the cytoplasm. They occur in the vesicular connective tissue cells, haemocytes (hyalinocytes), and extracellularly.
- Unlike all other microcells, *M. mackini* has no mitochondria or their equivalents, few cytoplasmic organelles, and with the nucleolus located towards the centre of the nucleus. The few organelles in all forms of *M. mackini* may be due to obligate parasitism and the use of host cell organelles, thus reducing the need for parasite organelles (15).

Availability of tests: no commercially available tests.

ii) Agent isolation and identification

- **Cell culture/artificial media:** none.
- **Antibody-based antigen detection methods:** monoclonal and polyclonal antibodies have been developed for *M. mackini*, (14), but their specificity and sensitivity have not been fully assessed.
- **Molecular techniques:** polymerase chain reaction (PCR).

Samples to be taken: live or freshly dead molluscs.
Technical procedure: tissue samples are placed in 95% ethanol until DNA extraction using a commercially available kit (e.g. DNeasy Kit; QIAGEN).

SSU rDNA region assay (5, 11): primer pairs that target the SSU region have been developed for *M. mackini*. Primers are 5'-AGA-TGG-TTA-ATG-AGC-CTC-C-3' and 5'-GCG-AGG-TGC-CAC-AAG-GC-3' and they amplify a 546 bp product (11). Samples should be diluted with either sterile ddH₂O or sterile Tris/EDTA (TE) buffer (pH 7.0–7.2) to a concentration between 10 and 40 ng/µl before being assayed. PCR reaction mixture contains the following ingredients at final concentrations: 20 mM Tris/HCl (pH 8.4), 50 mM KCl, 1.25 mM MgCl₂, 200 µM each dATP, dCTP, dGTP, and dTTP, 0.05 µM of each primer and 0.05 units/µl and 1.5 µl DNA template in a total volume of 15 µl. Cycling parameters begin with an initial denaturation at 94°C for 10 minutes, followed by 40 cycles of 94°C for 1 minute, 60.5°C for 1 minute, and 72°C for 1 minute, and end with a final extension at 72°C for 10 minutes. Products can be electrophoresed on 1.5% agarose gels containing 2.0 µl 5000X SybrGreen or 0.1 µg/ml ethidium bromide and visualised by exposure to UV light.

Positive/negative controls: compulsory and available from the OIE Reference Laboratory. Positive controls are genomic DNA from heavily infected hosts. Negative controls are no template DNA reactions.

Levels of validation:
- Specificity and sensitivity: The specificity of this PCR assay has not been fully assessed. However, the PCR assay detected three to four times more *M. mackini* infections in 1056 wild oysters from Denman Island, British Columbia than did standard histopathology. *Miknytos mackini* prevalence estimates based on both methods increased (PCR from 4.4 to 7.4%, histopathology from 1.2 to 2.1%) when gross lesions were processed in addition to standard samples (i.e. transverse sections for histopathology, left outer palp DNA for PCR). The use of histopathology and tissue imprints plus PCR, and standard samples plus observed gross lesions, represented a total evidence approach to *M. mackini* diagnosis that provided the most realistic estimates that could practically be obtained of the prevalence of *M. mackini* in an oyster sample (11).

Interpretation of results:
- A positive result is a PCR amplicon of the appropriate size, with all negative controls negative and all positive controls positive.

Availability of tests: not commercially available.

Molecular techniques: in-situ hybridisation (ISH).

Samples to be taken: live or freshly dead molluscs.

Technical procedure: sections of tissue that include pustules and the adductor muscle should be fixed for 24 hours in Davidson’s solution followed by normal processing for paraffin histology and hybridised with labelled oligonucleotide probes. Probes labelled with 5’ Oregon Green hybridise strongly to *M. mackini* (11) however, host tissue orientation is difficult. Hybridisation with the probe MACKINI-1 (5’-AGC-CCA-CAG-CCT-TCA-C-3’) with a 3’end digoxigenin label (QIAGEN Inc., Canada) and a counter stain of 0.5% Bismark Brown Y in 30% ethanol (17) depicts the parasite location within the host tissues.

Positive controls: recommended and available from the OIE Reference Laboratory.

Levels of validation:
- Specificity and sensitivity: the MACKINI-1 probe hybridised strongly to *M. mackini*, but did not hybridise to oyster tissues or with the other shellfish parasites and a bacterium tested: *Bonamia ostreae* in *O. edulis*, *Perkinsus gugwadi* in *Patinopecten yessoensis*, *Trichodina* sp. in *C. gigas*, an amoeba-like protistan in *Protobasina staminata*, *Hematodinium* sp in *Chionoecetes tanneri*; SPP (a protistan parasite of uncertain
taxonomic affiliation (10) in *Pandalus platyceros*; and *Nocardia crassostreae* in *C. gigas* (17). This probe with a digoxigenin label was considerably more sensitive for detecting infections when compared with standard histological sections stained with haematoxylin and eosin stain. Infection could be detected at lower magnification (×100 in comparison with ×1000) and in basophilic-staining tissues such as the digestive gland, gut epithelium and gonad (17).

Interpretation of results:
- A positive result is a staining reaction of the appropriate size, with all negative controls negative and all positive controls positive.

Availability of tests: not commercially available.
- Agent purification: isolates of *M. mackini* free of contamination host cell nuclei have not been obtained. However, a filtration technique that concentrates this parasite has been described (16).

**4. RATING OF TESTS AGAINST PURPOSE OF USE**

The methods currently available for surveillance, detection, and diagnosis of *M. mackini* are listed in Table 1. The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic sensitivity and specificity; C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and D = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility.

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance</th>
<th>Presumptive</th>
<th>Confirmatory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross signs*</td>
<td>C</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Tissue imprints*</td>
<td>C</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>Histology</td>
<td>B</td>
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<td>Transmission electron microscopy</td>
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<td>Polymerase Chain Reaction</td>
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<td>Sequence</td>
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<td>A</td>
</tr>
<tr>
<td>DNA Probes – <em>in-situ</em> hybridisation</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
</tbody>
</table>

*The technique is not species specific, but can be used in hosts, areas and during the spring of the year when the disease caused by *M. mackini* is evident.

**5. CORROBORATIVE DIAGNOSTIC CRITERIA**

**a) Definition of suspect case**

In known susceptible species within the known geographical range of *M. mackini* during the spring of the year, a suspect case of infection with *M. mackini* is a positive result by one of the following methods: gross signs in combination with tissue imprints of pustules, histology, or PCR.
In other host species or outside the known range of *M. mackini*, a suspect case is a positive result by histology, PCR or *in-situ* hybridisation.

**b) Definition of confirmed case**

A confirmed case of *Mikrocytos mackini* is a positive result by tissue imprints of pustules, histology, PCR or *in-situ* hybridisation combined with a positive result with transmission electron microscopy. Sequencing of the SSU region is recommended as a final step for a confirmatory diagnosis of *M. mackini*.

**6. Diagnostic/detection methods to declare freedom**

Methods for targeted surveillance to declare freedom from infection as outlined in the *Aquatic Code* are: histology screening of 3+ year old oysters at low intertidal levels or suspended culture during the spring of the year.

**REFERENCES**


*  

**NB:** There is an OIE Reference Laboratory for Infection with *Mikrocytos mackini* (see Table at the end of this Aquatic Manual or consult the OIE Web site for the most up-to-date list: [www.oie.int](http://www.oie.int)).
CHAPTER 2.2.6.

INFECTION WITH PERKINSUS MARINUS

1. CASE DEFINITION

For the purpose of this chapter, infection with *Perkinsus marinus* is considered to be infection with *Perkinsus marinus*.

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

a) **Agent factors**

- Aetiological agent, agent strains: *P. marinus*, all strains (26).
- Survival outside the host: maximum time unknown.
- Stability of the agent: *P. marinus* is relatively stable because of its thick cell wall. Dessication, chlorination (>300 ppm), and UV light (>28,000 µWs/cm²) have all been shown to inactivate *P. marinus* cells (7, 8). UV irradiation from 4,000 to 14,000 µWs/cm² will inhibit proliferation of *P. marinus* (7).
- Life cycle: direct from host to host; all life stages infective (1).

b) **Host factors**

- Susceptible host species: oysters – *Crassostrea virginica*, *C. gigas*, *C. ariakensis*, *C. rhizophorae* (1, 10, 11, 29).
- Susceptible stages of the host: all stages after settlement.
- Species or sub-population predilection (probability of detection): *Crassostrea virginica* is the most susceptible species; *C. gigas* and *C. ariakensis* can be infected but do not develop the disease (10, 11). Infection intensity increases with age of host in *C. virginica* (1).
- Target organs and infected tissue: gut epithelium, connective tissue of all organs, and haemocytes (22).
- Persistent infection with lifelong carriers: infection often fatal depending on host and environmental conditions (1, 3). Persistent infection with lifelong carriers can occur.
- Vectors: none required; life cycle is direct.

c) **Disease pattern**

- Transmission mechanisms: transmission is direct from host to host. All life stages are infective (29, 30). Viable cells released in host faeces or upon death of the host and probably acquired through feeding mechanisms (7).
- Prevalence: highly variable, but often 100% in *C. virginica*. Prevalence is expected to be higher in individuals with more than 1 year of exposure to the pathogen (1, 3).
- Geographical distribution: east coast of North America from Maine, USA, to Tabasco, Mexico.
• Mortality and morbidity: infection is often lethal for *C. virginica*, death usually occurs 1 or 2 years after infection. Death usually occurs during or shortly after the warmest annual water temperatures (3).

• Economic and/or production impact of the disease: *Perkinsus marinus* has caused serious losses of *C. virginica* in Chesapeake Bay, Delaware Bay, the coastal bays of North Carolina and in the Gulf of Mexico (1, 22). The pathogen is still a serious problem in all these areas during drought years. A decline in growth and condition precedes death and has an impact on marketability.

**d) Control and prevention**

• Vaccination: none.

• Chemotherapy: Bacitracin and cycloheximide have been shown to reduce, but not eliminate *P. marinus* in infected oyster hosts (9, 17). Their use may be relevant for aquaculture, but is not practical in the natural environment.

• Immunostimulation: none.

• Resistance breeding: selective breeding has demonstrated effectiveness for reducing mortality caused by *P. marinus* (24).

• Restocking with resistant species: disease-tolerant strains of *C. virginica* are used in restoration efforts in Chesapeake Bay (24), and consideration is being given to introduction of *C. ariakensis* in Chesapeake Bay (11).

• Blocking agents: none.

• General husbandry practices: farming in areas where salinity is less than 12 ppt and use of fast-growing, disease-tolerant strains has shown some benefit (1, 3).

### 3. DIAGNOSTIC METHODS

**a) Field diagnostic methods**

• Clinical signs: dead or gaping oysters. These clinical signs are not specific to infection with *P. marinus*.

**b) Clinical methods**

• Gross signs: thin, watery tissue, pale digestive gland. These gross signs are not specific to infection with *P. marinus*.

• Clinical chemistry: none

• Microscopic examination
  • Wet mounts: none
  • Smears: none
  • Histology: large multifocal lesions in gut epithelium or connective tissue of any organ, containing *P. marinus* cells (22). Haemocyte infiltration and phagocytosis in most infections. In high intensity infections, the gut epithelium may be almost completely destroyed.

• Electron microscopy: no information.
c) Agent detection and identification methods

i) Microscopic methods

- **Smears:** in advanced infection only.

Samples to be taken: live hosts

Technical procedure: bleed host with a needle and syringe inserted into the adductor muscle. Place a drop of haemolymph on a glass slide and smear. Observations are made at ×100–400 after Giemsa staining.

Positive/negative controls: No

Levels of validation:
- Specificity and sensitivity: very low specificity; sensitivity unknown.
- ‘Gold’ standard: sensitivity not validated against fluid thioglycollate culture (whole body burden assay [18]).

Interpretation of results:
- presence of spherical cells 2-15 µm in diameter with a large vacuole and eccentric nucleus indicates the presence of *Perkinsus* sp. The technique is not species specific.

Availability of tests: quick staining kits are commercially available (e.g. Difquick®)

- **Histology**

Samples to be taken: live or freshly dead oysters.

Technical procedure: sections of tissue that include digestive gland and gills should be fixed for 24 hour in Davidson’s AFA or other suitable fixative followed by normal processing for paraffin histology and staining with haematoxylin and eosin. Observations are made at increasing magnifications to 400×.

Positive controls: recommended and are available from the OIE Reference Laboratory.

Levels of validation:
- Specificity and sensitivity: species specificity is very low; sensitivity is good for moderate to high intensity infections, but low for low intensity infections.
- ‘Gold’ standard: fluid thioglycollate culture (whole body burden assay) is the gold standard although it is not species specific. Histology is less sensitive, but not formally validated against fluid thioglycollate culture.

Interpretation of results:
- Positive result is the occurrence of spherical cells ranging from about 2–10 µm in diameter with a large vacuole and an eccentrically displaced nucleus. Cells are often phagocytosed by host haemocytes. *Perkinsus marinus* cells stain basophilic.
- In susceptible host species within the known range of *P. marinus*, a positive result is presumptive evidence of *P. marinus* infection, but should be confirmed by species-specific polymerase chain reaction (PCR) and/or DNA sequencing of the ITS region because of the possible presence of *Perkinsus chesapeakei* or undescribed *Perkinsus* species.

Availability of tests: no commercially available tests.
• Ray's fluid thiglycollate culture

Samples to be taken: live or freshly dead molluses.

Technical procedure:

Tissue assay (25): Tissue samples measuring approximately 5–10 mm are excised giving preference to rectal, gill and mantle tissue from oysters, and placed in fluid thiglycollate medium (Difco) containing antibiotics. Recommended antifungal/antibiotics are 200 units of mycostatin (Nystatin), 500 units penicillin G and 500 mg dihydro-streptomycin per ml of media. Chloromycetin can be used in place of penicillin/streptomycin. Incubation is at 22–25°C for between 4 and 7 days, in the dark. After incubation, the fragments of tissue are collected and macerated with a scalpel blade on a glass slide, a drop of Lugol's iodine 1/5 solution is added, and the preparation is covered with a cover-slip and allowed to sit for 10 minutes. The preparations are examined in the fresh state.

Whole body burden assay (13, 18): The entire host, cut into 2–5 mm pieces, is placed into fluid thiglycollate culture medium and incubated as in the tissue assay above. Centrifuge at 1500 \( g \) for 10 minutes and discard supernatant. Add 2 M NaOH (20 ml/g tissue) and incubate at 60°C for 2–6 hours until tissue is digested. Centrifuge at 1500 \( g \) for 10 minutes and discard supernatant. Wash three times in deionised water, resuspend pellet in 1 ml Lugol's iodine solution, and count cells.

Levels of validation:

• Specificity and sensitivity: specificity is low; the technique does not distinguish species of *Perkinsus*. Sensitivity is high, especially of the whole body burden assay (5).

• ‘Gold’ standard: fluid thiglycollate culture (whole body burden assay) is the gold standard. The fluid thiglycollate culture tissue assay is the recommended surveillance method; it has been validated against the whole body burden assay (5) and shown to be less sensitive.

Interpretation of results:

• Cultured parasites enlarge from 2–10 to 20–70 \( \mu m \) during incubation. *Perkinsus* spp. cells are spherical and the walls stain blue or bluish-black with Lugol's iodine solution (5, 25).

Availability of tests: no commercial kits available

ii) Agent isolation and identification

• Cell culture/artificial media: *Perkinsus marinus* cells are easily cultured in a variety of media (14, 19–21). Culture medium is usually inoculated with heart, haemolymph, or gill tissue. Comparisons of commercially available media have been made (14); growth was supported in all media, but was at a maximum in 1/1 DME/Ham’s F-12 medium.

• Antibody-based antigen detection methods: monoclonal and polyclonal antibodies have been developed for *P. marinus*, (15, 29), but they are not specific to *P. marinus* and the polyclonal antibody has been shown to cross react with some dinoflagellate species (4).

• Molecular techniques: polymerase chain reaction (PCR).

Samples to be taken: live or freshly dead molluses.

Technical procedure: Tissue samples are placed in 95% ethanol or frozen at –70°C until DNA is extracted. DNA extraction is accomplished by proteinase K digestion.
overnight at 56°C and spin-column methodology using commercially available kits (e.g., QIAGEN).

NTS region assay (23, 27): primer pairs that target the NTS region have been developed for \textit{P. marinus} (23). Primers are 5' CAC TTG TAT GAA GCA CCC 3' and 5'-TTG-GTG-ACA-TCT-CCA-AAT-GAC-3' and they amplify a 307 bp product. (27). PCR reaction mixtures contain buffer (10 mM Tris, pH 9.2; 1.5 mM MgCl$_2$; 75 mM KCl; 0.02% Tween-20; 10 µM tetrathymammonium chloride [TMAC]; 10 µg/ml bovine serum albumin [BSA]; 2.5% dimethyl sulfoxide [DMSO]; and 5% formamide); 1 µM of each primer; 200 µM each dATP, dCTP, dGTP, and dTTP; 1.5 units \textit{Taq} polymerase; and the DNA template in a total volume of 25 µl. Samples are heated in a thermocycler to 91°C for 3 minutes and then cycled 35 times at 91°C for 1 minute, 58°C for 1 minute (plus 1 second/cycle), and 72°C for 1 minute (plus 2 seconds/cycle) with a final extension at 72°C for 10 minutes. A modification of this assay that incorporates an enzyme-linked immunoassorbent assay has also been developed (16).

ITS region assay (2): primer pairs that target the ITS region have also been developed for \textit{P. marinus}. Primers are 5'-CTT-TTG-YTW-GAG-WGT-TGC-GAG-ATG-3' and 5'-CGA-GTT-TGC-GAG-TAC-CTC-KAG-AG-3' and amplify a 509 bp product. PCR reaction mixtures contain 20 mM Tris/HCl, pH 8.4; 50 mM KCl; 1.5 mM MgCl$_2$; 0.2 mM each dATP, dGTP, dCTP, and dTTP; 25 pmol of each primer; 0.625 units of \textit{Taq} polymerase; and the DNA template in a total volume of 25 µl. Samples are denatured in a thermocycler at 94°C for 4 minutes, followed by 40 cycles of 1 minute at 94°C, 1 minute at 57°C, and 3 minutes at 65°C with a final extension of 10 minutes at 65°C.

Positive/negative controls: compulsory. Positive controls are DNA from purified \textit{P. marinus} cells, or genomic DNA from heavily infected hosts. Negative controls are no target DNA reactions.

Levels of validation:
- Specificity and sensitivity: NTS region primers were tested for specificity against \textit{P. olseni} and \textit{P. chesapeaki} (27). ITS region primers have been tested for specificity against \textit{P. olseni}, \textit{P. chesapeaki}, \textit{P. andrewsi} and \textit{P. mediterraneus}, as well as a number of dinoflagellate species (2). Sensitivity is high in both assays, with the ability to detect one \textit{P. marinus} cell in 30 mg of oyster tissue, but subsampling error in light, localised infections may lead to false negatives. The ITS primers were developed with a more complete understanding of the sequence variability in this region of the \textit{P. marinus} genome and are recommended over the NTS assay because the ITS primers are more likely to amplify all \textit{P. marinus} strains.

- ‘Gold’ standard: the NTS PCR assay has been validated against fluid thioglycollate culture (tissue assay) (27) and shown to be more sensitive. The ITS PCR assay has not been validated against fluid thioglycollate. However, a previous ITS PCR assay (31) was validated against thioglycollate culture of haemolymph, tissue, and whole body burden. The assay was as sensitive as the body burden thioglycollate assay and more sensitive than the haemolymph thioglycollate assay in oysters from Chesapeake Bay. It is now known, however, that the ITS primers developed by Yarnall et al. (31) do not amplify all strains of \textit{P. marinus} and their use is not recommended. The ITS primers developed by Audemard et al. (2) do amplify all known strains of \textit{P. marinus} and should be more sensitive than previous ITS primers.

Interpretation of results:
- A positive result is a PCR amplicon of the appropriate size, with all negative controls negative and all positive controls positive.

Availability of tests: not commercially available.
**Perkinsus** genus PCR assay (12): Primer pairs that target all known species of *Perkinsus* except *P. qugwadi* have been developed (12). The primers are 5'-CCG-CTT-TGT-TTG-GA/CTC-CC-3' and 5'-ACA-TCA-GGC-CTT-CTA-ATG-ATG-3', designated PerkITS85 and PerkITS750, respectively (11). These primers can be used to detect any known, and possibly unknown, species of *Perkinsus* except *P. qugwadi*. DNA sequencing of the ITS region can then be done to identify the species. Cycling parameters are an initial denaturation of 4 minutes at 95°C followed by 40 cycles of 1 minute at 95°C, 1 minute at 53°C, 3 minutes at 65°C and a final extension of 5 minutes at 65°C.

- **Molecular techniques:** *In-situ* hybridisation.

  No *in-situ* hybridisation assay has been developed that is specific for *P. marinus*.

- **Agent purification:** *P. marinus* can be purified by development of clonal cultures.

- **Indirect detection methods**

- Serological methods: none applicable.

### 4. RATING OF TESTS AGAINST PURPOSE OF USE

The methods currently available for surveillance, detection, and diagnosis of *P. marinus* are listed in Table 1. The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic sensitivity and specificity; C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and D = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility.

**Table 1.** *Perkinsus marinus* surveillance, detection and diagnostic methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance</th>
<th>Presumptive</th>
<th>Confirmatory</th>
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<tbody>
<tr>
<td>Gross signs</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Haemolymph smears</td>
<td>C</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>Histology</td>
<td>C</td>
<td>B</td>
<td>D</td>
</tr>
<tr>
<td>Fluid thioglycollate culture: tissue assay*</td>
<td>A</td>
<td>B</td>
<td>D</td>
</tr>
<tr>
<td>Fluid thioglycollate culture: whole body burden assay*</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>Polymerase chain reaction</td>
<td>B</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Sequence</td>
<td>D</td>
<td>D</td>
<td>A</td>
</tr>
</tbody>
</table>

*The technique is not species specific, but can be used reliably in hosts/areas where only one species of *Perkinsus* is known.

### 5. CORROBORATIVE DIAGNOSTIC CRITERIA

a) **Definition of suspect case**

In known susceptible species within the known geographical range of *P. marinus*, a suspect case of infection with *P. marinus* is a positive result by one of the following methods: haemolymph smear, histology, fluid thioglycollate culture or PCR.
In other host species or outside the known range of *P. marinus*, a suspect case is a positive result by PCR.

b) Definition of confirmed case

A confirmed case of *P. marinus* is a positive result by haemolymph smear, histology or fluid thioglycollate culture combined with a positive result with PCR. Sequencing of the ITS region is recommended as a final step for a confirmatory diagnosis.

6. DIAGNOSTIC/DETECTION METHODS TO DECLARE FREEDOM

Methods for targeted surveillance to declare freedom from infection as outlined in the *Aquatic Code* are: fluid thioglycollate culture using the whole body burden assay.

REFERENCES


Chapter 2.2.6. - Infection with *Perkinsus marinus*


* *

**NB:** There is an OIE Reference Laboratory for Infection with *Perkinsus marinus* (see Table at the end of this Aquatic Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.2.7.

INFECTION WITH *PERKINSUS OLSENI*

1. CASE DEFINITION

For the purpose of this chapter, infection with *Perkinsus olseni* is considered to be infection with *Perkinsus olseni*. *Perkinsus atlanticus* is considered to be a synonym (23).

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

a) Agent factors

- Aetiological agent, agent strains: *Perkinsus olseni*.
- Survival outside the host: maximum time unknown, but at least several days up to a few weeks.
- Stability of the agent: *P. olseni* is relatively stable because of its thick cell wall. *Perkinsus olseni* cells were killed by freshwater within 10 minutes at room temperature and by 6 ppm chlorine within 30 minutes (16). *Perkinsus olseni* cells in host tissue were much more resistant to these treatments. UV light (>28,000 µWs/cm²) has been shown to inactivate *P. marinus* cells (4, 5) and this method may be effective for *P. olseni* as well. UV irradiation from 4,000 to 14,000 µWs/cm² will inhibit proliferation of *P. marinus* (5).
- Life cycle: Direct from host to host; all life stages infective (28).

b) Host factors

- Susceptible host species: clams – *Anadara trapezia*, *Austrovenus stutchburyi*, *Tapes decussatus*, *Tapes philippinarum*, *Pitar rostrata*; oysters – *Crassostrea gigas*, *Crassostrea ariakensis*, *Crassostrea sikamea*; pearl oysters – *Pinctada margaritifera*, *Pinctada martensi*; abalone – *Haliotis rubra*, *Haliotis laevigata*, *Haliotis scalaris*, *Haliotis cyclobates* (2, 16, 18, 24, 28). Other bivalve and gastropod species might be susceptible to this parasite especially in the known geographical range. Members of the Arcidae, Malleidae, Isognomonidae, Chamidae and Veneridae are particularly susceptible, and their selective sampling may reveal the presence of *P. olseni*, when only light infections occur in other families in the same habitat.
- Susceptible stages of the host: all stages after settlement.
- Species or sub-population predilection (probability of detection): wide host range; infection intensity increases with age of host.
- Target organs and infected tissue: connective tissue of all organs, haemocytes.
- Persistent infection with lifelong carriers: infection often fatal depending on host and environmental conditions. Persistent infection with lifelong carriers can occur.
- Vectors: none required.

c) Disease pattern

- Transmission mechanisms: transmission is direct from host to host (28). All life stages are infective.
- Prevalence: highly variable, but often 100%. Prevalence is expected to be higher in individuals with more than 1 year of exposure to the pathogen.
Chapter 2.2.7. - Infection with *Perkinsus olseni*

- Geographical distribution: widespread throughout the tropical Pacific Ocean, Australia, north island of New Zealand, Korea, Japan, China (People’s Rep. of), Portugal, Spain, France, Italy, and Uruguay (2, 16, 18, 24, 28). Not known from North America. The *Perkinsus* sp. reported from the undulated surf clam *Paphia undulata* is almost certainly *P. olseni* based on DNA sequence similarity of the ITS-1 (internal transcribed spacer) and ITS-2 locus (21).

- Mortality and morbidity: infection is often lethal; death may occur 1 or 2 years after infection.

- Economic and/or production impact of the disease: *Perkinsus olseni* has caused serious losses of *Haliotis* spp. abalone in Australia, and in *Tapes* spp. clams in Korea, Japan, Spain and Portugal (2, 16, 18, 24, 27). A decline in growth and condition precede death and impact marketability.

**d) Control and prevention**

- Vaccination: none.
- Chemotherapy: cyclohexamide, pyrimethamine, deferoxamine (DFO) and 2, 2-bipyridyl inhibit *P. olseni in vitro*, and DFO inhibits *P. olseni in vivo*. (13). Bacitracin has been shown to reduce, but not eliminate *P. marinus* in infected oyster hosts (13). This compound may be effective for *P. olseni* as well.
- Immunostimulation: none.
- Resistance breeding: none for *P. olseni*, although selective breeding has demonstrated some effectiveness for *P. marinus* (25).
- Restocking with resistant species: none.
- Blocking agents: none.
- General husbandry practices: low density stocking may reduce transmission of the pathogen.

3. DIAGNOSTIC METHODS

**a) Field diagnostic methods**

- Clinical signs: dead or gaping molluscs. These clinical signs are not specific to infection with *P. olseni*.

**b) Clinical methods**

- Gross signs: thin, watery tissue, pale digestive gland, nodules in gills of some hosts (2). These gross signs are not specific to infection with *P. olseni*.
- Clinical chemistry: none
- Microscopic examination
  - Wet mounts: none
  - Smears: none
- Histology: large multifocal lesions in connective tissue containing *P. olseni* cells. Haemocyte infiltration and phagocytosis in most infections. In clam hosts, *P. olseni* cells are often encapsulated by a thick layer of eosinophilic material derived from haemocyte degranulation (28).
- Electron microscopy: no information.
c) Agent detection and identification methods

• Direct detection methods

i) Microscopic methods

• Smears: in advanced infection only.

Samples to be taken: live hosts

Technical procedure: bleed host with a needle and syringe inserted into an adductor muscle of oysters and clams, or the cephalic sinus of abalone. Place a drop of haemolymph on a glass slide and smear. Observations are made at 100-400× after Giemsa staining.

Positive/negative controls: no

Levels of validation:
• Specificity and sensitivity: very low specificity; sensitivity unknown.
• ‘Gold’ standard: sensitivity not validated against fluid thioglycollate culture (whole body burden assay).

Interpretation of results:
• The presence of spherical cells 5-15 µm in diameter with a large vacuole and eccentric nucleus indicates the presence of Perkinsus sp. The technique is not species specific.

Availability of tests: quick staining kits are commercially available (e.g. Difquick®).

• Histology

Samples to be taken: live or freshly dead molluscs.

Technical procedure: sections of tissue that include digestive gland and gills should be fixed for 24 hours in Davidson's AFA followed by normal processing for paraffin histology and staining with haematoxylin and eosin. Observations are made at increasing magnifications to 400×.

Positive controls: recommended and may be available from the OIE reference laboratory, depending on the host.

Levels of validation:
• Specificity and sensitivity: species specificity is very low; sensitivity is good for moderate to heavy infections, but low for low intensity infections.
• ‘Gold’ standard: fluid thioglycollate culture (whole body burden assay) is the gold standard although it is not species specific. Histology not formally validated against fluid thioglycollate culture.

Interpretation of results:
• Positive result is the occurrence of spherical cells ranging from about 5–15 µm in diameter with a large vacuole and an eccentrically displaced nucleus with a prominent nucleolus. Cells are often phagocytosed or encapsulated. Perkinsus olseni cells stain basophilic.
• In susceptible host species in an area where only P. olseni is known to occur, a positive result is presumptive evidence of P. olseni infection, but should be confirmed by DNA sequencing because of the possible presence of undescribed Perkinsus species.
Availability of tests: no commercially available tests.

- **Ray's fluid thioglycollate culture**

Samples to be taken: live or freshly dead molluscs.

Technical procedure:

Tissue assay (26): Tissue samples measuring approximately 5–10 mm are excised giving preference to rectal, gill and mantle tissue from abalone and clams, and adductor or foot muscles or mantle for abalone, and placed in fluid thioglycollate medium (Difco) containing antibiotics. Recommended antifungal/antibiotics are 200 units of mycostatin (Nystatin), 500 units penicillin G and 500 mg dihydro-streptomycin per ml of media (25). Chloromycetin can be used in place of penicillin/streptomycin (26). Incubation is at 22–25°C for between 4 and 7 days, in the dark. After incubation, the fragments of tissue are collected and macerated with a scalpel blade on a glass slide, a drop of Lugol's iodine 1/5 solution is added, and the preparation is covered with a cover-slip and allowed to sit for 10 minutes. The preparations are examined in the fresh state.

Whole body burden assay (1, 8, 14): The entire host, cut into 2–5 mm pieces, is placed into fluid thioglycollate culture medium and incubated as in the tissue assay above. Centrifuge at 1500 g for 10 minutes and discard supernatant. Add 2 M NaOH (20 ml/g tissue) and incubate at 60°C for 2–6 hours. Centrifuge at 1500 g for 10 minutes and discard supernatant. Wash three times in deionised water, resuspend pellet in 1 ml Lugol’s iodine solution, and count cells.

Levels of validation:

- Specificity and sensitivity: Specificity is low; the technique does not distinguish species of *Perkinsus*. Sensitivity is high, especially of the whole body burden assay (3).
- ‘Gold’ standard: Fluid thioglycollate culture (tissue assay) is the recommended surveillance method. The tissue assay has not been validated against the whole body burden assay for *P. olseni*, but for *P. marinus* the tissue assay has been shown to be less sensitive (3).

Interpretation of results:

- Cultured parasites enlarge from 5–15 to 50–70 µm during incubation. *Perkinsus* spp. cells are spherical and the walls stain blue or bluish-black with Lugol’s iodine solution (26).

Availability of tests: no commercial kits available

**ii) Agent isolation and identification**

- **Cell culture/artificial media**: *Perkinsus* spp. cells are easily cultured in a variety of media (6, 11, 15, 19, 20). Culture medium is usually inoculated with heart, haemolymph, or gill tissue. Comparisons of commercially available media for the culture of *P. marinus* have been made (11).

- **Antibody-based antigen detection methods**: Polyclonal antibodies have been developed for a cell wall component of *P. olseni* (22), but they also bind to *P. marinus*. No diagnostic assay has been developed using these antibodies.

- **Molecular techniques**: polymerase chain reaction (PCR).

Samples to be taken: live or freshly dead molluscs.
NTS region assay (10, 27): Tissue samples are placed in 95% ethanol until DNA is extracted. DNA extraction is accomplished by proteinase K digestion overnight at 56°C and spin-column methodology using commercially available kits (e.g. QIAGEN). Two sets of primer pairs that target the NTS region have been developed for *P. olseni*. Primers are 5’-ATG-CTA-TGG-TTG-GTT-GCG-GAC-C-3’ and 5’-GTA-GCA-AGC-CGT-AGA-ACA-GC-3’ and they amplify a 690 bp product (27). PCR reaction mixtures contain buffer (100 mM Tris, pH 9.2; 1.5 mM MgCl₂; 750 mM KCl); 0.6 µM of each primer; 200 µM each dATP, dCTP, dGTP, and dTTP; 1.5 units Taq polymerase; and DNA template in a total volume of 25 µl. Samples are heated in a thermocycler to 94°C for 4 minutes and then cycled 35 times at 92°C for 1 minute, 60°C for 1 minute (plus 1 second/cycle), and 72°C for 1 minute (plus 2 seconds/cycle) with a final extension at 72°C for 7 minutes. A second set of primers (11) is 5’-ACC-AGT-CAC-AGG-GCG-TAA-T-3’ and 5’-GTA-GCG-TGC-TCT-GAT-GAT-CAC-T-3’ and they amplify a 554 bp product.

Positive/negative controls: compulsory. Positive controls are DNA from purified *P. olseni* cells, or genomic DNA from infected hosts. Negative controls are no target DNA reactions.

Levels of validation:
• Specificity and sensitivity: The first set of primers (27) was tested for specificity against *P. marinus* and *P. chesapeaki*, but not other species; the second set of primers (10) has not been tested for specificity. Both sets of primers were developed from *P. olseni* isolates from *Ruditapes decussatus* in Spain. The intraspecific variation in the NTS region of *P. olseni* has not been assessed across its wide host and geographic range and there is a possibility of false negative assays in other hosts or regions.
• ‘Gold’ standard: The PCR assay for *P. olseni* has not been validated against fluid thioglycollate culture.

Interpretation of results:
• A positive result is a PCR amplicon of 690 bp or 554 bp (depending on primers used) and all negative controls negative and all positive controls positive.

Availability of tests: not commercially available.

*Perkinsus* genus specific assay (7): Primer pairs that target all known species of *Perkinsus* except *P. qugwadi* have been developed (7). The primers are 5’-CCG-CTT-TGT-TTG-GA/CTC-CC-3’ and 5’-ACA-TCA-GGC-CTT-CTA-ATG-ATG-3’, designated PerkITS85 and PerkITS750, respectively (7). These primers can be used to detect any known, and possibly unknown, species of *Perkinsus* except *P. qugwadi*. DNA sequencing of the ITS region can then be done to identify the species. Cycling parameters are an initial denaturation of 4 minutes at 95°C followed by 40 cycles of 1 minute at 95°C, 1 minute at 53°C, 3 minutes at 65°C and a final extension of 5 minutes at 65°C.

• **Molecular techniques**: *In-situ* hybridisation.

No *in-situ* hybridisation assay has been developed that is specific for *P. olseni*.

• **Agent purification**: *P. olseni* can be purified by development of clonal cultures.

• **Indirect detection methods**

• Serological methods: none applicable.
4. **RATING OF TESTS AGAINST PURPOSE OF USE**

The methods currently available for surveillance, detection, and diagnosis of *P. olseni* are listed in Table 1. The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic sensitivity and specificity; C = the method has application in some situations, but cost, accuracy, or other factors severely limit its application; and D = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility.

**Table 1.** *Perkinsus olseni* surveillance, detection and diagnostic methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance</th>
<th>Presumptive</th>
<th>Confirmatory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross signs</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Haemolymph smears</td>
<td>C</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>Histology</td>
<td>C</td>
<td>B</td>
<td>D</td>
</tr>
<tr>
<td>Fluid thioglycollate culture: tissue assay*</td>
<td>A</td>
<td>B</td>
<td>D</td>
</tr>
<tr>
<td>Fluid thioglycollate culture: whole body burden assay</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>Polymerase chain reaction</td>
<td>B</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Sequence</td>
<td>D</td>
<td>D</td>
<td>A</td>
</tr>
</tbody>
</table>

*The technique is not species specific, but can be used reliably in hosts/areas where only one species of *Perkinsus* is known. It is also useful for initial surveillance in areas where more than one species of *Perkinsus* is present, with subsequent species identification of positive cases by PCR.

5. **CORROBORATIVE DIAGNOSTIC CRITERIA**

a) **Definition of suspect case**

In known susceptible species within the known geographical range of *P. olseni*, a suspect case of infection with *P. olseni* is a positive result by one of the following methods: haemolymph smear, histology, fluid thioglycollate culture or PCR.

In other host species or outside the known range of *P. olseni*, a suspect case is a positive result by PCR.

b) **Definition of confirmed case**

A confirmed case of *P. olseni* is a positive result by histology or fluid thioglycollate culture combined with a positive result with PCR. Sequencing of the ITS region is recommended as a final step for a confirmatory diagnosis.

6. **DIAGNOSTIC/DETECTION METHODS TO DECLARE FREEDOM**

Methods for targeted surveillance to declare freedom from infection as outlined in the *Aquatic Code* are: 1) fluid thioglycollate culture using the whole body burden assay for oysters and clams; 2) for large organisms such as abalone where whole body burden fluid thioglycollate culture may be impractical, it may be possible to do whole tissue burden for tissues known to be infected.
REFERENCES


* * *

**NB:** There is an OIE Reference Laboratory for Infection with *Perkinsus olseni* (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.2.8.

INFECTION WITH XENOHALIOTIS CALIFORNIENSIS

1. CASE DEFINITION

Infection with *Xenohaliotis californiensis* causes disease (termed withering syndrome [ref. 11]) in wild and farmed abalone, *Haliotis* spp. (Archeogastropoda: Mollusca [refs 11, 13, 16, 17, 19, 20]). While all post-larval life stages have been demonstrated to be susceptible to infection with *X. californiensis*, clinical disease is typically observed in animals >1–2 years of age. Gross signs of the disease include pedal atrophy, mottled digestive gland, anorexia, weakness, and lethargy. The disease is characterised by intracytoplasmic bacterial inclusions within the posterior oesophagus, intestine and absorptive/transport epithelia of the digestive gland; moderate to advanced infections are associated with degenerative or metaplastic changes within the digestive gland followed by pedal muscle atrophy. Clinical disease has only been observed in infected individuals exposed to elevated seawater temperatures (e.g. ~18°C). Associated losses may reach 99% of the population and vary depending on seawater temperatures and host species. Corroborative diagnostic criteria are summarised in Section 5 of this chapter.

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

a) Agent factors

*Xenohaliotis californiensis* is an intracellular bacterium in the family Rickettsiaceae and is closely related to members of the genera *Ehrlichia*, *Anaplasm* and *Cowdria* (5–7). The disease caused by this bacterium is known as withering syndrome (6) and may be more appropriately termed abalone rickettsiosis. No information exists on the presence of varying strains of this bacterium. The dimorphic rod-to-spherical-shaped bacterium measures an average of 332 × 1550 nm in the bacillus form and an average of 1405 nm in the spherical morphotype. The bacterium reproduces within intracytoplasmic vacuoles 14–56 µm in diameter within gastrointestinal epithelia (10). Although *X. californiensis* is thought to be an obligate intracellular organism, the bacterium may survive outside the host for an undetermined period of time as evidenced by water-borne transmission studies (3, 4). Based on the susceptibility of *Escherichia coli* to bleach (0.09 ppm; 18), this bacterium is thought to be readily inactivated by immersion in <10% bleach. In addition, 1% tamed iodine in freshwater (e.g. Prepodyne) for 1 hour is an effective disinfectant based on the use of these disinfection methods at a marine laboratory with flow-through seawater and a lack of detection of this pathogen in adjacent abalone populations (7, Friedman, pers. obs.). The bacterium divides by binary fission (5) and transmission is direct and horizontal (3, 4, 6, 15). Although not typically observed in farmed abalone until they are in grow-out conditions (>2.5 cm in maximum size), polymerase chain reaction (PCR) examination of exposed 6-week-old abalone has shown that 1–2 mm abalone can become infected (Moore *et al.*, unpublished observations).

b) Host factors

*Xenohaliotis californiensis* infects members of the genus *Haliotis* including black abalone (*H. cracherodi*), white abalone (*H. sorenseni*), red abalone (*H. rufescens*), pink abalone (*H. corrugata*), and green abalone (*H. fulgens*) in the wild or culture facilities as well as flat (*H. wallallensis*) and Japanese abalone (*H. discus-hannai*) in laboratory challenges (Friedman, unpublished observations). Other abalone species have not been tested. While all post-larval life stages have been demonstrated susceptible to infection with *X. californiensis*, clinical disease is typically
observed in animals >1 years of age in farmed abalones (Friedman, unpublished observations) and all abalone size classes observed in wild populations surveyed to date (e.g. 3, 4, 8, 11, 20). Probability of detection increases with increasing abalone size. Animals less than 10 mm in size have a reduced probability of detection using histology but equal probability of detection using PCR. *Xenohaliotis californiensis* infects the gastrointestinal epithelial cells of the posterior oesophagus, digestive gland and, to a lesser extent, intestine. Infections may persist for long periods without the development of clinical disease when the host is maintained at cool water temperatures (e.g. ≤15°C for red abalone); exposure to elevated seawater temperatures (e.g. >17°C for red, black and white abalones) typically results in clinical disease (7, 14). Although no alternate, non-haliotid hosts have been identified, it has been suggested that some colonial ascidians may concentrate the bacterium (based on PCR evidence); thus the possibility of such species acting as a vector for the bacterium exists, but further investigation of possible vectors are warranted (J.D. Moore, unpublished observations).

Disease (withering syndrome) occurs at elevated water temperatures (~18°C and above) in abalone with moderate to severe infections (5, 6, 14). The incubation period of withering syndrome is prolonged and ranges between 3 and 7 months (3, 4, 6, 8, 14, 15). Clinical disease is characterised by morphological changes in the digestive gland, which vary between species and may include degeneration (atrophy of tubules, increase in connective tissues and inflammation) and/or metaplasia of the digestive tubules. Metaplasia involves the replacement of terminal secretory/absorptive acini with absorptive/transport ducts similar in appearance to the post-oesophagus. These morphological changes are accompanied by anorexia, depletion of glycogen reserves followed by use of the foot muscle as an energy source and death (3, 4, 8, 10, 12, 14, 15). The foot of affected abalone contains fewer and less organised muscle bundles, abundant connective tissue and may contain more cerous cells than unaffected individuals (10, 14, 20). Surviving abalone appear to remain infected, even in low water temperature environments, such as in northern California (7).

c) Disease pattern

Transmission of *X. californiensis* is horizontal and is postulated to be via a faecal–oral route.

<table>
<thead>
<tr>
<th>Species</th>
<th>PCR prevalence</th>
<th>Histology prevalence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild</td>
<td>Farmed</td>
<td>Wild</td>
</tr>
<tr>
<td><em>Haliotis rufescens</em></td>
<td>N.D.</td>
<td>0–100%</td>
<td>1–75%&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Haliotis cracherodii</em></td>
<td>N.D.</td>
<td>N.A.</td>
<td>74–98%</td>
</tr>
<tr>
<td><em>Haliotis sorenseni</em></td>
<td>0</td>
<td>0–100%</td>
<td>0</td>
</tr>
<tr>
<td><em>Haliotis fulgens</em></td>
<td>N.D.</td>
<td>N.D.</td>
<td>44–100%</td>
</tr>
<tr>
<td><em>Haliotis corrugata</em></td>
<td>N.D.</td>
<td>N.D.</td>
<td>62–63%</td>
</tr>
<tr>
<td><em>Haliotis walallensis</em></td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td><em>Haliotis discus-hannai</em></td>
<td>N.D.</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>1</sup>Prevalences of 1–17% have been observed in northern California and up to 75% in central and southern California. <sup>2</sup>Larger abalone typically have a higher prevalence of infection. ND = no data; NA = not applicable.

*Xenohaliotis californiensis* occurs along the south-west coast of North America in California, USA and Baja California, Mexico. However, as infected abalone have been transported to Chile, Japan, Israel, Iceland and possibly other countries, the geographical range of the aetiological agent is suspected to be broad where California red abalone, *Haliotis rufescens*, are cultured.

Susceptibility varies with species; the bacterium is known to cause disease in black (up to 99% mortality; 16), white (up to 100% mortality; Friedman & McCormick, unpublished observations),
red (up to 35% mortality; 14, 15), pink (also called yellow) and green (also called blue) abalone (20). Unlike the other abalone species studied to date, the magnitude of abalone mortality is not well documented in pink and green abalones. However, in Baja California, Mexico, up to 100% of green (blue) and 63% of pink (yellow) abalone may be infected, with up to 43% of the green and 71% of the pink abalones having microscopic signs of disease (degenerated or metaplastic digestive gland; 19). The incubation period varies with temperature but typically involves a prolonged 3–7-month prepatent period. Mortality typically occurs 1–2.5 months after the onset of visible clinical signs (8).

*Xenohaliotis californiensis* infections have resulted in severe economic impact to abalone culturists along the west coast of North America and in other locations where it is found. Reduced profits have been associated with farm closures as well as contributing to the closure of commercial abalone fisheries in California. Two farms in California reported losses of over USD 1.5 M because off this disease. Recurring disease episodes are also implicated in failure of wild abalones to repopulate historic habitats.

d) Control and prevention

The pathogen and disease (withering syndrome) may occur year round, but losses in farmed red abalone due to the disease occur most often in the summer and autumn, after a 3–4-month period when temperatures are elevated (~18°C or higher). Reducing densities and application of an oxytetracycline-medicated diet may reduce losses (9). Oral administration of 12–19% TM-100 in a medicated diet for 10 or 20 days provides protection against bacterial re-infection for several months. Recent data suggest that a single day *per os* administration of 12% TM-100 can reduce bacterial infections from 80% to 10% prevalence and mean infection intensity from 1.4 to 0.1 on a scale of 0–3 (5). Interest in selecting for resistant abalones, particularly for restoration purposes, is increasing. Wild black abalone are recruiting along the California Channel Islands and some recruits survive suggesting that these individuals may be more resistant to this rickettsial disease (Van Blaricom, pers. obs.).

Husbandry practices to reduce problems caused by *X. californiensis* are typical of those for any bacterial disease and include the purchase of inspected seed (devoid of evidence of infection), maintaining separate families or groups (i.e. avoid high grading and mixing of disparate groups), rinsing hands and equipment in freshwater or iodinated water and drying them in between uses. Isolation of infected groups is recommended if possible. If oxytetracycline treatment is employed, therapeutic application under federal guidelines (e.g. FDA-CVM1 in the USA) prior to a warm water season may reduce losses and infection. Typically, only a single application during the second or third year of growth is required during a typical 3–4 year culture cycle.

3. Diagnostic methods

a) Field diagnostic methods

Abalone with *X. californiensis* infections may be subclinically infected during the prepatent period or at water temperatures ≤ 15°C or severely emaciated (atrophied) under permissive water temperatures. During an epidemic, affected abalone will often cling to horizontal (as opposed to vertical or inverted) substrates and appear weak (easily removed from the substrate by hand) and emaciated (withered) (11). Farmed abalone will also be anorexic. In addition, the presence of an abnormally high number of fresh shells may also indicate disease. If moribund abalone are found, the observation of a mottled digestive gland (dark brown with small foci of tan coloured tissue) indicative of metaplastic changes provides further presumptive evidence of this disease.

1 United States Food and Drug Administration – Center for Veterinary Medicine
b) Clinical methods

Clinical characterisation of *X. californiensis* disease relies on a combination of tissue morphological changes in conjunction with the presence of the agent. Morphological changes include an atrophied foot muscle that is visible at the gross and microscopic level (histology). Affected individuals contain less pedal glycogen and fewer muscle bundles than do unaffected individuals (3, 4, 10). As a direct result of pedal catabolism, infected abalone excrete substantially higher levels of ammonia than do unaffected individuals (12). In some abalones, an increase in cerous cells may be observed in the foot muscle (20). These signs are not pathognomonic for this disease. However, metaplastic changes in the digestive gland that include the transformation of the terminal secretory acini into absorptive/transport epithelia is amplified in abalones infected with *X. californiensis* (3, 4, 6, 14, 15). Although metaplasia has been observed in all affected species examined to date, the response to infection may vary between hosts. Red abalone and white abalone, for example, typically respond with a metaplastic change (3, 4, 14), while black abalone generally respond with a combination of metaplasia, digestive tubule degeneration and inflammation (6, 8).

c) Agent detection and identification methods

• Direct detection methods

  i) Microscopic methods

    a) Stain method: Excise a section of posterior oesophagus and blot on to slide. Fix the smear in methanol and stain with a modified Giemsa (e.g. Hemacolor [Merck] or Diff Quik [Dade Behring]). Dry and observe under oil immersion for rickettsial inclusions or coverslip and examine at ×200–400 magnification.

    b) Fluorescent method: Excise a section of the post-oesophagus, mince and lay on a slide, dry with a hair dryer for ~20 minutes. Stain the slides using a fluorescent stain for nucleic acid such as propidium iodide or Hoechst 33258 (13). Incubate in the dark for 3 minutes and view by epifluorescence at ×200 magnification. Bacterial inclusions are differentiated from host nuclei by size and frequency. However, if the sample slides are to be retained for future examination, they should be thoroughly dried and stored desiccated until staining.

    Inclusions of the parasite, 14–56 µm in diameter, appear interspersed with the smaller host nuclei. An observation time of 5 minutes per slide is sufficient at ×200 magnification (13). This method is less sensitive than histology and bacterial morphology cannot be differentiated. This is best employed as a rapid examination method within the known range of this disease. This test is not commercially available.

  • Histology

  The histological procedure is detailed in Chapter I.2 of this *Aquatic Manual*. Remove the shell and cut several 3–5 mm cross sections that contain posterior oesophagus (post-oesophagus), digestive gland, and foot muscle and placed in Davidson’s or Carson’s solutions (see Chapter I.2 of this *Aquatic Manual*) for 24 hours and process for routine paraffin histology. Cross sections are most easily handled when placed in cassettes prior to fixation. The ratio must be no more than one volume of tissue to ten volumes of fixative.
Deparaffinised 3–5 µm sections should be stained with haematoxylin and eosin and viewed by light microscopy for bacterial inclusions (oblong, basophilic intracytoplasmic vacuoles 14–56 µm in diameter \[5\]) in the post-oesophagus and digestive gland, and morphological changes in the digestive gland and foot. It is recommended that sections should be examined at ×200 or ×400 magnification.

*Xenohaliotis californiensis* may be morphologically similar to other marine rickettsial bacteria. Definitive diagnosis of the bacterium may include molecular tools (e.g., *in-situ* hybridisation). Definitive diagnosis of withering syndrome by histology must include the presence of the bacterium and morphological changes to the digestive gland, metaplasia and or degeneration, and may include those of the foot muscle.

Where losses have been observed within the known geographical range of withering syndrome, visualisation of intracellular bacterial foci within digestive epithelia, by histological examination, may be considered to be a confirmatory method and is considered the gold standard for this disease. However, confirmation by using histology in conjunction with PCR and sequence analysis or *in-situ* hybridisation is recommended to verify the identity of the rickettsial bacteria in abalone species previously not known to be susceptible to the bacterium or in a new geographical location.

This test is not commercially available.

- *Transmission electron microscopy examination*

  Transmission electron microscopy procedures are described in Chapter I.2 of this *Aquatic Manual*. Rod-shaped, ribosome-rich prokaryotes with trilaminar cell walls accumulated into intracellular colonies within membrane-bound vacuoles in the cytoplasm of gastrointestinal epithelial cells are observed. The dimorphic rod-to-spherical-shaped bacterium measures an average of 332 × 1550 nm in the bacillus form and an average of 1405 nm in the spherical morphotype. The bacterium reproduces within intracytoplasmic vacuoles 14–56 µm in diameter \(5\). This test is not commercially available.

- **Agent isolation and identification**

  - *Polymerase chain reaction*

    A positive PCR amplification is only a presumptive diagnosis because it detects DNA and not necessarily a viable pathogen. Other techniques, preferably histology and *in-situ* hybridisation, must be used to visualise the pathogen. When used in conjunction with histology, PCR may be used for confirmation. Examination of the amplified sequence is recommended when examining a new host species or a new geographical area. Sequences must be consistent with the known 16S rDNA sequence of this bacterium (GenBank Accession AF133090; ref. 1).

    Samples for PCR should be excised from the post-oesophagus or digestive gland and processed using the classical phenol-chloroform extraction method or the Qiagen QIAmp DNA mini stool kit (due to the presence of PCR inhibitors in abalone digestive gland). Post-oesophagus tissue is recommended because infections are consistently more intense than in digestive gland tissue. A positive control reaction should always be included in the PCR and should consist of genomic DNA extracted from a known infected individual or the use of a plasmid containing an insert of the amplified product. If a plasmid positive control is to be used, it is recommended that an insert of ~100 bp should be added to the cloned fragment to alleviate concerns over cross contamination of aerosolised plasmid DNA. A negative control consisting of master mix without the addition of template should also be included in each PCR. All reactions should be run in duplicate. Observation of a 160 bp band in tissue samples and positive control reactions as well as no bands in the negative control reactions characterise a successful
Chapter 2.2.8 - Infection with Xenohaliotis californiensis

test. The sensitivity and specificity is of this test are in the process of being formally assessed (Moore & Friedman, unpublished data.). No commercial tests are currently available.

The PCR primers developed for X. californiensis detection specifically amplify a 160 bp segment of the Rickettsia-like pathogen. Primers are currently designated as: RA 5-1 (5’-GTT-GAA-CGT-GGC-TTC-AGT-TTA-C-3’) and RA 3-6 (5’-ACT-TGG-ACT-CAT-TCA-AAA-GCG-GA-3’). They target small subunit ribosomal DNA and have been shown to be sensitive and specific for this pathogen (1). PCR amplification is performed in a standard 25 µl reaction volume containing 1 × PCR buffer, 1.5 mM MgCl₂, 400 ng/ml BSA, 200 µM of dNTPs, 0.5 mM of each primer, 0.8 units of Taq polymerase, and 100 ng template DNA. The reaction mixtures are cycled in a thermal cycler. The programme for amplification reaction is: initial denaturation at 95°C for 5 minutes, 40 cycles at 95°C for 1 minute, 62°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. An aliquot of each PCR reaction is checked for the 160 base pair amplification product by agarose gel electrophoresis and ethidium bromide staining.

This test is not commercially available.

- In-situ hybridisation

*In-situ* hybridisation is the method of choice for confirming identification because it allows visualisation of a specific probe hybridised to the target organism. DNA probes must be thoroughly tested for specificity and validated in comparative studies before they can be used for confirmatory identification.

*In-situ* hybridisation has recently been developed to detect Rickettsiales-like prokaryotes in tissue sections (2). Specific labelled oligonucleotide probes hybridise with the small subunit ribosomal RNA of the bacterium. This hybridisation is detected by an antibody conjugate that recognises the labelled probes. Substrate for the antibody conjugate is added, causing a colorimetric reaction that enables visualisation of probe–parasite DNA hybridisations. Although, this method has not been formally validated, tests for specificity using several bivalve and fish rickettsial organisms suggested that the test is specific for X. californiensis (2).

The procedure of *in-situ* hybridisation is conducted as follows. Positive (known infected tissues) and negative (uninfected or those infected with a different bacterium) controls must be included in the procedure.

1. After removing the shell, a transverse section (3–5 mm) is cut that contains posterior oesophagus (post-oesophagus), digestive gland, and foot muscle and placed in Davidson’s AFA fixative (glycerin [10%], formalin [20%], 95% ethanol [30%], dH₂O [30%], glacial acetic acid [10%]) for 24–48 hours, then transferred to 70% ethanol until processed by histological procedures (step ii). The ratio must be no more than 1 volume of tissue to 10 volumes of fixative.

2. The samples are subsequently embedded in paraffin by conventional histological procedures. Sections are cut at 5–6 µm and placed on positively charged slides or 3-aminopropyl-triethoxylane-coated slides. Histological sections are then dried overnight in an oven at 40°C.

3. The sections are deparaffinised by immersion in xylene or other less toxic clearing agent for 10 minutes. The solvent is eliminated by immersion in two successive absolute ethanol baths for 10 minutes each and rehydrated by immersion in an ethanol series. The sections are then washed twice for 5 minutes in phosphate buffered saline (PBS).
iv) The sections are treated with proteinase K, 50 µg/ml in PBS, at 37°C for 15 minutes. The reaction is then stopped by washing the sections in PBS with 0.2% glycine for 5 minutes. The sections are then placed in 2 × SSC (standard saline citrate) for 10 minutes.

v) The sections are prehybridised for 1 hour at 42°C in prehybridisation buffer (4 × SSC, 50% formamide, 5 × Denhardt’s solution, 0.5 mg/ml yeast tRNA, and 0.5 mg/ml heat-denatured herring sperm DNA).

vi) The prehybridisation solution is then replaced with prehybridisation buffer containing 2 ng/µl of the digoxigenin-labelled oligonucleotide probes. The sequences of the probes designated as RA 5-1, RA 3-6, RA 3-8 and RA 5-6 (2) are, respectively: 5′-GTT-GAA-CGT-GCC-TTC-AGT-TTA-C-3′, 5′-ACT-TGG-AC-T-GAT-TCA-AAA-GCG-GA-3′, 5′-CCA-CTG-TGA-GTG-GTT-ATC-TCC-TG-3′, and 5′-GAA-GCA-ATA-TTG-TGA-GAT-AAA-GCA-3′. The sections are covered with in-situ hybridisation plastic cover-slips and placed on a heating block at 90°C for 12 minutes. The slides are then cooled on ice for 1 minute before hybridisation overnight at 40°C in a humid chamber.

vii) The sections are washed twice for 5 minutes in 2 × SSC at room temperature, twice for 5 minutes in 1 × SSC at room temperature, and twice for 10 minutes in 0.5 × SSC at 40°C. The sections are then placed in Buffer 1 (100 mM Tris, pH 7.5, 150 mM NaCl) for 1–2 minutes.

viii) The sections are placed in Buffer 1 (see step vii) supplemented with 0.3% Triton X-100 and 2% sheep serum for 30 minutes. Anti-digoxigenin alkaline phosphatase antibody conjugate is diluted 1/500 (or according to the manufacturer’s recommendations) in Buffer 1 supplemented with 0.3% Triton X-100 and 1% sheep serum and applied to the tissue sections. The sections are covered with in-situ hybridisation cover slips and incubated for 3 hours at room temperature in the humid chamber.

ix) The slides are washed twice in Buffer 1 for 5 minutes each (see step vii) and twice in Buffer 2 (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM MgCl₂) for 5 minutes each. The slides are then placed in colour development solution (337.5 µg/ml nitroblue tetrazolium, 175 µg/ml 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, 240 µg/ml levamisole in Buffer 2) for 2 hours in the dark. The colour reaction is stopped by washing in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA [ethylene diamine tetra-acetic acid]).

x) The slides are then rinsed in dH₂O. The sections are counterstained with Bismarck Brown Y, rinsed in dH₂O, and cover-slips are applied using an aqueous mounting medium. The presence of the pathogen is demonstrated by the purple-black labelling of the parasitic cells.

This test is not commercially available.

• Indirect detection methods

Serological methods: not applicable.

4. RATING OF TESTS AGAINST PURPOSE OF USE

The methods currently available for surveillance, detection, and diagnosis of X. californiensis are listed in Table 1 below. The designations used in the table are as follows: A = this method is the recommended method due to availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic sensitivity and specificity; C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and D = the method is presently not recommended for this purpose. These are somewhat subjective as
suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

**Table 1. Xenohaliotis californiensis surveillance, detection and diagnostic methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance</th>
<th>Presumptive</th>
<th>Confirmatory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Larvae</td>
<td>Juveniles</td>
<td>Adults</td>
</tr>
<tr>
<td>Gross signs</td>
<td>D</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>Bioassay</td>
<td>D</td>
<td>D</td>
<td>C</td>
</tr>
<tr>
<td>Tissue imprint-Giemsa stain</td>
<td>D</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>Histopathology</td>
<td>D</td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td>Transmission EM</td>
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<td>DNA probes – <em>in situ</em></td>
<td>D</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>PCR</td>
<td>D</td>
<td>A</td>
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</tr>
<tr>
<td>SSU rDNA sequence</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
</tbody>
</table>

1For valuable broodstock, it is possible to use PCR of faeces as a first screen and, if negative, to subsequently use the bioassay method in combination with histology (See Section 6). 2Tissue imprints should be used in combination with PCR and possibly sequencing to confirm the agent. 3In new cases, such as a new geographical location, PCR and sequencing are recommended to confirm identity of the bacterium. 4PCR alone is not confirmatory but when used in combination with histology, it may be considered confirmatory.

5. CORROBORATIVE DIAGNOSTIC CRITERIA

In accordance with the *Aquatic Code*, all cases in other species should be referred immediately to the appropriate OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.

a) Definition of suspect case

A suspect case of *X. californiensis* infection and associated clinical disease (withering syndrome) may include the observation of gross clinical signs (weakness, lethargy, anorexia, pedal atrophy, mottled digestive gland) and mortality in association with warm water conditions, particularly within the known geographic range of this disease. In farmed abalones, anorexia may be a first sign of disease. These clinical signs in combination with microscopic observation of an atrophied foot muscle, inclusion bodies in gastrointestinal epithelia or PCR evidence also represent a suspect case.

b) Definition of confirmed case

Confirmation of *X. californiensis* infection relies on observation of the agent using histology and/or *in-situ* hybridisation. Gross signs and tissue imprints alone cannot be used for confirmatory diagnosis and must be supported by histology, *in-situ* hybridisation or PCR tests.

Confirmation of withering syndrome relies on both presence of the agent and presence of microscopic signs of the disease. At a minimum, digestive gland metaplasia or degeneration must accompany *X. californiensis* infection to diagnose clinical withering syndrome.
6. Diagnostic/detection methods to declare freedom

The method for targeted surveillance to declare freedom from *X. californiensis* is histology in combination with PCR. Given the chronic nature of the disease and influence of temperature it is recommended that animals in the nursery and grow out are examined during the warm water season of that site. Abalone held in cooler waters may be chronically infected without showing any signs of disease. It is also recommended that PCR examination of faeces or bioassay of smaller abalone (e.g. 1–4 cm) commingled with broodstock for at least 6 weeks at >17°C is also used.

REFERENCES


* *

NB: There is an OIE Reference Laboratory for Infection with Xenohaliotis californiensis (see Table at the end of this Aquatic Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.2.9.

ABALONE VIRAL MORTALITY

CHAPTER IN PREPARATION

* *
* *
SECTION 2.3.

DISEASES OF CRUSTACEANS

CHAPTER 1.3.

GENERAL INFORMATION

1. DISEASES OF CRUSTACEANS LISTED BY THE OIE

Crustaceans are adversely affected by a number of diseases. This is especially evident in penaeid shrimp from aquaculture. All of the crustacean diseases that have significant social or economic impact are infectious diseases. The crustacean diseases and their aetiological agents that are included in the *Aquatic Animal Health Code* (the *Aquatic Code*) have a restricted geographical range, have no therapeutic remedies or treatments, are potentially excludable, and are of significant social and economic importance. For the current OIE list of diseases of crustaceans, please consult the current edition of the *Aquatic Code*.

Because of the size and importance of the penaeid shrimp aquaculture industry, the principles and methods discussed in this chapter will, of necessity, emphasise penaeid shrimp. The taxonomy of the penaeid shrimp was revised in 1997 (14). Penaeid subgenera were raised to being full genera. Because this change in penaeid taxonomy has not been universally accepted, for the purposes of this *Aquatic Manual*, the taxonomy of the penaeids as outlined by Holthuis (8) will be used in this *Aquatic Manual*.

2. DIAGNOSTIC METHODS

The methods available for diagnosis of the above-listed diseases include the traditional methods of morphological pathology (direct light microscopy, histopathology, and electron microscopy), bioassay methods with susceptible indicator hosts, and molecular methods (gene probes and polymerase chain reaction [PCR]). While tissue culture is considered to be a standard tool in medical, veterinary, and fish diagnostic laboratories, it has yet to be developed as a usable, routine diagnostic tool for crustacean pathogens. Clinical chemistry has not become a routinely used diagnostic tool by crustacean pathologists.

2.1. Diagnostic methods for diseases of crustaceans

At the time of writing this section of the *Aquatic Manual*, the available diagnostic methods that may be selected for diagnosis of the OIE listed crustacean diseases or detection of their aetiological agents are based on:

- Gross and clinical signs

- Direct bright-field, phase-contrast or dark-field microscopy with whole stained or unstained tissue wet-mounts, tissue squashes, and impression smears; and wet-mounts of faecal strands
Chapter I.3. - Diseases of crustaceans: General information

- Histology of fixed specimens
- Bioassays of suspect or subclinical carriers using a highly susceptible host (life stage or species) as the indicator for the presence of the pathogen
- Transmission or scanning electron microscopy
- Antibody-based tests for pathogen detection using immune sera polyclonal antibodies (PAbs) or monoclonal antibodies (MAbs)
- Molecular methods
  - DNA probes in dot-blot hybridisation assays directly with fresh tissue samples or with extracted DNA
  - DNA probes or RNA probes for in situ hybridisation assays with histological sections of fixed tissues
  - Standard and real-time PCR and reverse-transcription (RT)-PCR for direct assay with fresh tissue samples or with extracted DNA or RNA.

The detailed procedures for each of the available methods (screening, presumptive, and confirmatory) for diagnosis of each of the OIE listed crustacean diseases are outlined in the individual disease chapters of this Aquatic Manual.

There is a paucity of antibody-based diagnostic tests available for the pathogens that cause crustacean diseases. As crustaceans do not produce antibodies, antibody-based diagnostic tests are limited in their application to pathogen detection. While a number of antibody-based diagnostic methods have been developed and are described in the literature, these were developed with mouse or rabbit antibodies generated to viruses purified from infected hosts. Because crustacean viruses (and the necrotising hepatopancreatitis [NHP] bacterium) cannot be routinely cultured in vitro (i.e. produced in tissue culture), purified virus from infected hosts must be used to produce antibody. This has severely limited the development and availability of this diagnostic tool. The recent application of MAb technologies to this problem has begun to provide a few antibody-based tests. MAbs are available for the agents of several of the OIE listed crustacean diseases (White spot syndrome virus [WSSV], Taura syndrome virus [TSV], yellowhead virus [YHV], infectious hypodermal and haematopoietic necrosis virus [IHHNV], and the necrotising hepatopancreatitis bacterium [NHP-B]). Antibody-based diagnostic kits/reagents for TSV, WSSV, YHV, NHP-B infections are currently available from commercial sources.

Molecular methods have been developed and some methods are in widespread use for the detection of many of the viral, bacterial, and protozoan pathogens of the penaeid shrimp. Nucleic acid-based detection methods are readily available from the literature and some are available in kit form from commercial sources for the OIE listed pathogens TSV, WSSV, and yellowhead disease virus (YHV/GAV), IHHNV, *Panaeus monodon*-type baculovirus (MBV), *Baculovirus penaei* (BP), and infectious myonecrosis virus (IMNV). PCR or RT-PCR methods are available for all of these viruses and are in routine use by certain sectors of the crustacean aquaculture industry. For the agents of other OIE listed diseases, specific DNA probes tagged with nonradioactive labels are either reported in the literature or available commercially for application in dot-blot formats with haemolymph or tissue extracts, or for use with routine histological sections using in-situ hybridisation.

3. SAMPLING

There are at least three purposes for which crustacean stocks may be sampled with regard to the OIE listed crustacean diseases. These are: 1) surveillance; 2) stock or facility 'certification'; and 3) disease
diagnosis. The number and type of samples to be taken for analysis varies greatly according to which of these purposes applies.

A general approach to surveillance and sampling is given in chapter 1.1.4 of this Aquatic Manual. The sampling should be designed to enable detection, at a 95% confidence level, of infected animals. The following section gives information relevant to sampling crustaceans. For those diagnostic tests where the sensitivity and specificity have been established, sample size may be determined using methods such as FreeCalc (www.ausvet.com.au) or similar programs as outlined in Chapter 1.1.4 Requirements for surveillance for international recognition of freedom from infection. However, for those diagnostic tests where the values for sensitivity and specificity have not been established, the default sample size should be determined from Table 1 in the present chapter.

3.1. Diagnosis in disease situations

In clinical disease episodes, carefully selected quality specimens with representative lesions should be obtained from live or moribund crustaceans. Every effort should be made to sample those specimens for diagnosis that are representative of the disease(s) that is (are) affecting the crustacean stock of interest, and that are moribund or clinically diseased. Collection of dead specimens should be avoided. When cultured or wild crustacean stocks are presenting clinical signs of an active disease that are consistent with, or suggestive of, any one of the OIE listed crustacean diseases, care should be taken to ensure that the samples collected are preserved appropriately for the anticipated diagnostic tests (see sample preservation section for recommended methods).

The recommended minimum numbers of specimens to collect for diagnostic testing are 100 for the larval stages of most crustaceans; 50 for the postlarval stages; and 10 for juveniles and adults. Sample numbers may be greater if clinically diseased specimens are readily apparent and collected. Nonetheless, these recommended ‘minimum’ sample numbers are provided as guidelines, and it must be emphasised that carefully selected, quality specimens are far more valuable (and cost-effective) diagnostic specimens than dozens or hundreds of specimens taken at random to ‘fill out’ the sample.

3.2. Diagnosis in subclinical crustacean carriers

When samples are to be taken for surveillance, for testing of subclinical carriers of previous disease epizootics, for ‘certification’ of specific pathogen free (SPF) status, or for freedom of particular disease within a country, zone, or facility the sample size to be taken should be determined using a statistical table or a program such as FreeCalc. The minimum sample size for each lot tested should provide a 95% level of confidence that infected specimens, if present, will be in the sample, assuming a defined minimum prevalence of infection equal or greater than 2%, 5% or 10%. For surveillance and certification purposes for OIE listed diseases, the samples taken for diagnostic tests at any given aquaculture site or from wild stocks, should include the appropriate number of specimens from each lot to be tested according to Table 1 (or to the number calculated by FreeCalc, if applicable). For the OIE listed diseases it is highly recommended that the scheduling of sampling be planned (i.e. by farm schedule, season, etc.) so that the particular life-stage(s) are sampled at a time when the pathogen of concern is most likely to be detected. This is especially important when the available diagnostic methods are dependent on simple microscopy or histological methods and do not include molecular methods. For the baculoviruses BP and MBV larval and early postlarval are the most appropriate samples; for TSV, IHNV, WSSV, IMNV, NHP-B and YHV/GAV, juveniles and subadults provide the best samples; and for crayfish plague, juveniles and adults are suitable samples.

Samples taken for molecular or antibody-based tests for OIE-listed crustacean diseases may be combined as pooled samples of no more than five specimens per pooled sample.
3.3. Testing for verification or maintenance of freedom from specific diseases

Once a crustacean production facility has been recognised to be free of all or certain diseases listed in the Aquatic Code after 2 years of surveillance with laboratory tests and in the absence of any suspect clinical signs, twice-yearly inspections should continue. However, collection of specimens for testing may be reduced to 30 crustaceans (shrimp), including especially broodstock. Moribund shrimp observed during inspection visits must, however, be collected for further laboratory examination.

<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>
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<td>100,000 or more</td>
<td>150</td>
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</table>

Table 1. Random sample size based on assumed pathogen prevalence in lot and assuming 100% sensitivity and specificity of the technique


4. Sample Type and Preservation

4.1. Samples for direct microscopy

Samples for direct microscopic examination should be examined as soon as possible after collection. Use live specimens whenever possible, or use fresh, chilled, or 10% buffered formalin-fixed specimens when live specimens are not practical. If an adequate field laboratory is available, it should be used to process and examine samples near the site of collection.

4.2. Samples for histology

Collect shrimp by whatever means are available with a minimum of handling stress. Transport the shrimp to the laboratory via a well oxygenated water-filled utensil. Supply adequate aeration to the container if the shrimp are to be left for a short period of time before actual fixation. For the study of presumably diseased shrimp, select those shrimp that are moribund, discoloured, displaying abnormal behaviour, or otherwise abnormal, except in the case of intentional random sampling for estimation of disease prevalence.

i) Have ready an adequate supply of fixative. A general rule is that a minimum of ten volumes of fixative should be used for one volume of tissue sample (i.e. a 10 g sample of shrimp would require 100 ml of fixative).

ii) Davidson’s AFA (alcohol, formalin, acetic acid) fixative
Davidson’s AFA fixative is recommended for most histological applications. The fixative is rapid, reduces autolytic changes in tropical crustaceans (i.e. the penaeid shrimp), and its acidic content decalcifies the cuticle. The formulation for Davidson’s AFA is (for 1 litre):

330 ml 95% ethyl alcohol
220 ml 100% formalin* (a saturated 37–39% aqueous solution of formaldehyde gas)
115 ml glacial acetic acid**
335 ml tap water (for marine crustaceans, sea water may be substituted)

Store the fixative in glass or plastic bottles with secure caps at room temperature.

* Do not use previously made 10% formalin to prepare Davidson’s AFA because the formalin content of the Davidson’s AFA will be inadequate to provide satisfactory fixation.

** Do not substitute other acids, such as HCl, for acetic acid. Histological sections prepared from HCl–Davidson’s solution are not suitable for routine haematoxylin and eosin histological staining.

iii) Fixation procedures with Davidson’s AFA

- For larvae and postlarvae that are too small to be easily injected with fixative using a tuberculin syringe:
  Using a fine mesh screen or a Pasteur pipette, select and collect specimens. Immerse shrimp selected for sampling directly in the fixative. Fix for 12–24 hours in fixative, then transfer to 50–70% ethyl alcohol for storage.

- For larger postlarvae and very small juveniles that are too small to be injected:
  Select and collect specimens as described in Section 3. Use a needle or fine-pointed forceps to incise the cuticle. Immerse shrimp selected for sampling directly in the fixative. Fix for 12–24 hours in fixative, then transfer to 50–70% ethyl alcohol for storage.

- For larger postlarvae, juveniles, and adults:
  Inject fixative (use 5–10% volume: weight) via needle and syringe (needle gauge dependent on shrimp size, i.e. 27 gauge needle for postlarvae and small juveniles) into the living shrimp. The hepatopancreas (HP) should be injected first and at two or more sites, with a volume sufficient to change the HP to a white to orange colour; then inject fixative into adjacent regions of the cephalothorax, into the anterior abdominal region, and into the posterior abdominal region. The fixative should be divided between the different regions, with the cephalothoracic region, specifically the HP, receiving a larger share than the abdominal region. A good guide to insure adequate fixation is to inject an equivalent of 5–10% of the shrimp’s (or other crustacean’s) body weight; all signs of life should rapidly cease, and visible colour change should occur in the injected areas. Immediately following injection, slit the cuticle, with dissecting scissors, from the sixth abdominal segment to the base of the rostrum, being particularly careful not to cut deeply into the underlying tissue. The incision in the cephalothoracic region should be just lateral to the dorsal midline, while that in the abdominal region should be approximately mid-lateral.

- For shrimp (and most other crustaceans) larger than ~12 g:
  After injection of fixative, the body should then be transversely bisected, at least once, just posterior to the abdomen/cephalothorax junction, and (optional) again mid-abdominally.

- For very large crustaceans and crabs:
  The organs of interest may be excised after injection of fixative. Completion of fixation of these tissue samples is then handled as outlined previously. Following injection, incisions and bisection/trisection, or excision of key organs, immerse the specimen in the fixative (use 10:1 fixative:tissue ratio).
Allow fixation to proceed at room temperature for 24–72 hours depending on the size of shrimp (or crustacean) being preserved. Longer fixation times in Davidson’s AFA may be used to thoroughly decalcify the shell of crabs, lobsters, crayfish, etc.

Following fixation, the specimens should be transferred to 70% ethyl alcohol, where they can be stored for an indefinite period.

Record a complete history of the specimens at the time of collection: gross observations on the condition of the shrimp (or other crustacean), species, age, weight, source (wild, or if culture pond or tank number, stock number, etc.), and any other pertinent information that may be needed at a later time.

The label should stay with the specimens in the same container during fixation, storage and transport to the laboratory. Always use No. 2 soft-lead pencil on water-resistant paper (plastic paper is recommended; never use ink or marking pens as the ink is dissolved by alcohol).

iv) Transport and shipment of preserved samples

Because large volumes of alcohol should not be posted or shipped, the following methods are recommended: Remove the specimens from the 70% ethyl alcohol. For larvae, postlarvae, or small juveniles, use leak-proof, screw-cap plastic vials if available; if glass vials must be used, pack to prevent breakage. For larger specimens, wrap samples with white paper towels to completely cover (do not use raw cotton). Place towel-wrapped specimens in a sealable plastic bag and saturate with 70% ethyl alcohol. Insert the label and seal the bag. Place the bag within a second sealable bag. Multiple small sealable bags can again be placed within a sturdy, crush-proof appropriately labelled container for shipment (see Chapter 1.5.6 of the Aquatic Code for details).

4.3. Preservation of samples for antibody, DNA probe dot-blot tests, or polymerase chain reaction

For routine diagnostic testing by PCR, RT-PCR or for dot-blot tests with DNA probes, samples must be prepared to preserve the pathogen’s nucleic acid. Likewise, samples intended for testing with antibody-based methods must be preserved to retain reactive antigenic sites for the antibodies used.

• 4.3.1. Sample types

Samples selected for nucleic acid-based or antibody-based diagnostic tests should be handled and packaged (in new plastic sample bags or bottles) with great care to minimise the potential for cross contamination among the sample set taken from different (wild or farmed) stocks, from tanks, ponds, farms, etc. New plastic sample bags or bottles must be used. A water-resistant label, with the appropriate data filled out in No. 2 pencil, should be placed within each package or container for each sample set.

Some suitable methods for preservation and transport of samples taken for molecular or antibody-based tests are:

• Live specimens: These may be processed in the field or shipped to the diagnostic laboratory for testing.

• Haemolymph: This tissue is the preferred sample for certain molecular and antibody-based diagnostic tests. Samples may be collected by needle and syringe by cardiac puncture, from the haemocoel (i.e. the ventral sinus in penaeids), or from a severed appendage.

• Iced or chilled specimens: This is for specimens that can be transported to the laboratory for testing within 24 hours. Pack samples in sample bags surrounded by an adequate quantity
of wet ice around the bagged samples in a Styrofoam™-insulated box and ship to the laboratory.

- **Frozen whole specimens:** Select live specimens according to the criteria listed in Section 3, quick freeze in the field using crushed dry-ice, or freeze in the field laboratories using a mechanical freezer at $-20^\circ C$ or lower temperature. Prepare and insert the label into the container with the samples, pack samples with an adequate quantity of dry-ice in a Styrofoam™-insulated box, and ship to the laboratory.

- **Alcohol-preserved samples:** In regions where the storage and shipment of frozen samples is problematic, 90–95% ethanol may be used to preserve, store, and transport certain types of samples. Whole crustaceans (any life stage provided the specimen is no larger than 2–3 g), excised tissues (i.e. pleopods) from large crustaceans, or haemolymph may be preserved in 90–95% ethanol, and then packed for shipment according to the methods described in Section 4.2.v (see Chapter 1.5.6 of the Aquatic Code for details).

- **Preservation of RNA and DNA in tissues using RNAlater:** Tissue is cut to be less than 0.5 cm in one dimension and submerged in 5 volumes of RNAlater (e.g. a 0.5 g sample requires about 2.5 ml of RNAlater). Small organs such as kidney, liver and spleen can be stored whole in RNAlater. These samples can be stored at 4°C for one month, at 25°C for 1 week or at $-20^\circ C$ indefinitely. Archive RNAlater-treated tissues at $-20^\circ C$.

**KEY REFERENCES**


4. BROCK J.A. & MAIN K. (1994). A Guide to the Common Problems and Diseases of Cultured Penaeus vannamei. Published by the Oceanic Institute, Makapuu Point, P.O. Box 25280, Honolulu, Hawaii, USA.


* *
CHAPTER 2.3.1.

TAURA SYNDROME

1. CASE DEFINITION

For the purpose of this chapter, Taura syndrome (TS) is considered to be infection with Taura syndrome virus (TSV).

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

   a) Agent factors

   • Aetiological agent: TSV as described by Bonami et al. (3) and Mari et al. (40, 41).
   • TSV was listed as an unassigned species in the Family Dicistroviridae in the most recent report of the International Committee on Taxonomy of Viruses (the ICTV; 14).
   • TSV particles are 32 nm, nonenveloped icosahedrons with a buoyant density of 1.338 g/ml. The genome of TSV consists of a linear, positive-sense single-stranded RNA of 10,205 nucleotides, excluding the 3’ poly-A tail, and it contains two large open reading frames (ORFs). ORF 1 contains the sequence motifs for nonstructural proteins, such as helicase, protease and RNA-dependent RNA polymerase. ORF 2 contains the sequences for TSV structural proteins, including the three major capsid proteins VP1, VP2 and VP3 (55, 40, and 24 kDa, respectively). The virus replicates in the cytoplasm of host cells (3, 40, 41, 51).
   • Agent strains:
     • Genotypic variants of TSV: at least three genotypic groups of TSV have been identified based on the sequence of the VP1 (= CP2), the largest and presumably the dominant structural protein of the virus. Based on the sequence of VP1 (= CP2) these genotypic groups are: 1) the Americas group; 2) the South-East Asian group; and 3) the Belize group (8, 43, 54).
     • Antigenic variants of TSV: using the monoclonal antibody MAb 1A1 produced to a reference isolate from the Americas (TSV USA-HI94 – GenBank AF277675) (41, 48) at least two distinct variants have been demonstrated: Type A are those that react to MAb 1A1 (in enzyme-linked immunosorbent assay [ELISA], Western blots and in-situ hybridization [IHC] with infected tissues) and those that do not. The MAB 1A1 non-reactors were subdivided into Types B (TSV 98 Sinaloa, Mexico) and Type C (TSV 02 Belize) based on host species and virulence. All TSV isolates of the Americas and most, if not all, South-East Asian genotypes react with MAB 1A1. In marked contrast, none of the Belize genotype group reacts with MAb1A1 (12, 13), nor does a TSV isolate from the 2005 epizootic in Venezuelan shrimp farms.
     • Other reported causes of TS: Taura syndrome in Ecuador was initially linked to pesticide contamination of shrimp farms, a contention that was supported by litigation for ~8 years after the disease was scientifically shown to have a viral aetiology (3, 20, 29). Hence, several papers in the literature propose a toxic aetiology for TS (21–23).
b) Host factors

- Susceptible species: the principal host species for TSV are the Pacific White Shrimp *Litopenaeus vannamei* and the Pacific Blue Shrimp *L. stylirostris*. While the principal host species for TSV all belong to the penaeid genus *Litopenaeus*, other penaeid species can be infected with TSV by direct challenge, although disease signs do not develop. Documented natural and experimental hosts for TSV include: *L. setiferus*, *L. schmitti*, *Peneaus monodon*, *Metapenaeus ensis*, *Fenneropenaeus chinensis*, *Marsupenaeus japonicus*, *Farfantepenaeus aztecus* and *Fa. duorarum* (4, 5, 7, 8, 26, 27, 46, 53).

- In on-farm epizootics of TS involving unselected stocks of *L. vannamei*, the principal host species for TSV, typical cumulative mortalities range from 40 to >90% in cultured populations of postlarval (PL), juvenile, and subadult life stages. Survivors of TSV infections may carry the virus for life (7, 19, 26–28, 33, 34, 37).

- Selected lines of *L. stylirostris* were found to be resistant to TSV (genotype 1, MAb 1A1 type A) and these became the dominant stocks farmed in western Mexico after TSV reached Mexico in 1994. However, in 1998–1999, a new ‘strain’ of TSV (Type B; 13, 15, 28, 29, 62) emerged and caused massive epizootics in *L. stylirostris*. The emergence of this new ‘strain’ of TSV was soon followed in late 1999 by the introduction of White spot syndrome virus (WSSV) in shrimp farms in western Mexico, to which *L. stylirostris* had no resistance, effectively ending any interest in the culture of *L. stylirostris*.

- Susceptible life stages of the host species: TSV has been documented in all life stages (i.e. PL, juveniles and adults) of *L. vannamei* except in eggs, zygotes and larvae (26).

- Target organs: TSV infects and has been shown to replicate (using ISH with specific DNA probes) in principally the cuticular epithelium (or hypodermis) of the general exoskeleton, foregut, hindgut, gills and appendages, and often in the connective tissues, the haematopoietic tissues, the lymphoid organ, and antennal gland. The enteric organs (endoderm-derived hepatopancreas, midgut and midgut caeca mucosal epithelia) and smooth, cardiac, striated muscle, and the ventral nerve cord, its branches and its ganglia typically show no histological signs of infection by TSV and are usually negative for TSV by ISH (4, 17–19, 23, 26, 30–32, 53).

- Persistent infection and lifelong carriers: some members of populations of *L. vannamei* or *L. stylirostris* that survive TSV infections and/or epizootics may carry the virus for life and, although not documented, pass the virus on to their progeny by vertical transmission (18, 19).

- Vectors and sources of contamination:
  - Sea birds: TSV has been demonstrated to remain infectious for up to 48 hours (after ingestion of TSV infected shrimp carcasses) in the faeces passed by wild or captive sea gulls (*Larus atricilla*) and chickens (*Gallus domesticus*, used as a laboratory surrogate for all shrimp-eating birds). These findings implicate birds as being an important mechanical vector for the transmission of the virus within affected farms or farming regions (16, 57).
  - Aquatic insects: the water boatman (*Trichocorixa reticulata* [Corixidae], an aquatic insect that feeds on shrimp carcasses in shrimp farm ponds), has also been shown to serve as a mechanical vector of TSV (5, 25–27).
  - Frozen TSV-infected commodity products: TSV has been found in frozen commodity shrimp (*L. vannamei*) products in samples from markets in the USA that originated in Latin America and South-East Asia. Improper disposal of wastes (liquid and solid, i.e. peeled shells, heads, intestinal tracts, etc.) from value-added reprocessing of TSV-infected shrimp at coastal locations may provide a source of TSV that may contaminate wild or farmed stocks near the point of the waste stream discharge (27, 45).
  - Zoonotic potential: TSV was reported to infect human and monkey cell lines, suggesting a zoonotic potential for this virus and that penaeid shrimp could serve as reservoirs for TSV and other members of the ‘picornavirus superfamily’ that infect humans (2). Because of the experimental design and the improbable results reported by Audelo-del-Valle *et al.* (2), two
other laboratories repeated the study and both found that TSV does not infect or replicate in primate or human cell lines with known susceptibility to human picornaviruses (39, 47), effectively refuting the contention that TSV has zoonotic potential.

c) Disease pattern

- TS is best known as a disease of nursery- or grow-out-phase *L. vannamei* that occurs within ~14–40 days of stocking PLs into grow-out ponds or tanks, hence, shrimp with TS are typically small juveniles of from ~0.05 g to <5 g. Larger shrimp may also be affected, especially if they are not exposed to the virus until they are larger juveniles or adults (5, 6, 26, 27, 34).

- Transmission mechanisms: transmission of TSV can be by horizontal or vertical routes. Horizontal transmission by cannibalism or by contaminated water has been demonstrated (5, 20, 26, 27, 58). Vertical transmission from infected adult broodstock to their offspring is strongly suspected but has not been experimentally confirmed.

- Prevalence: in regions where the virus is enzootic in farmed stocks, the prevalence of TSV has been found in various surveys to range from 0 to 100% (5, 23, 24).

- Geographical distribution: TS is widely distributed in the shrimp-farming regions of the Americas and South-East Asia (4, 5, 8, 18, 26, 27, 35, 43, 54, 56, 61).

  - The Americas: following its recognition in 1992 as a distinct disease of cultured *L. vannamei* in Ecuador (6, 22), TS spread rapidly throughout many of the shrimp-farming regions of the Americas through shipments of infected PL and broodstock (5, 7, 18, 26, 27). Within the Western Hemisphere, TS and TSV have been reported from virtually every penaeid shrimp-growing region in the Americas and Hawaii (1, 5, 51). TSV is enzootic in cultured penaeid shrimp stocks on the Pacific coast of the Americas from Peru to Mexico, and it has been occasionally found in some wild stocks of *L. vannamei* from the same region (30, 32). TSV has also been reported in farmed penaeid stocks from the Atlantic, Caribbean, and Gulf of Mexico coasts of the Americas, but it has not been reported in wild stocks from these regions (18, 26, 27, 29).

  - Asia: TSV was introduced into Taipei China in 1999 with infected imported Pacific white shrimp, *L. vannamei*, from Central and South American sources (56, 61). Since that original introduction, the virus has spread with movements of broodstock and PL to China (People’s Rep. of), Thailand, Malaysia, and Indonesia where it has been the cause of major epizootics with high mortality rates (8, 43, 54).

d) Control and prevention

- Vaccination: no effective vaccines for TSV are available.

- Chemotherapy: no scientifically confirmed reports.

- Immunostimulation: no scientifically confirmed reports.

- Resistance breeding: TSV-resistant domesticated stocks of *L. vannamei* and *L. stylirostris* have been developed. Some domesticated lines of TSV-resistant *L. vannamei* (that are also TSV-free) are in widespread use by the shrimp-farming industries of the Americas and South-East Asia (9, 42, 58). After the appearance of TS in Central America, improved TS resistance was reported in wild caught *L. vannamei* PLs used to stock shrimp farms in the region (24).

- Restocking with resistant species: in regions where TS is enzootic, the use of TSV-resistant domesticated lines of *L. vannamei* has been highly successful in preventing losses due to TS (29, 42, 60). TSV-resistant domesticated lines of *L. stylirostris* were successfully used to stock Mexican farms that previously grew only TSV-susceptible *L. vannamei* following the introduction of TSV into Mexico in 1994 (9, 27, 62). However, a new ‘strain’ of TSV emerged in these *L. stylirostris* stocks in 1998–1999, and this ‘Type B TSV’ caused severe epizootics in the formerly TSV-resistant *L. stylirostris* stocks (13, 51).
• Blocking agents: resistance to TSV infection was reported by expression of the TSV coat protein antisense RNS in _L. vannamei_ zygotes. Transgenic juveniles reared from zygotes protected in this manner showed improved resistance to TSV challenge by _per os_ or intramuscular (IM) injection routes (38). Similar results have been produced by introducing random dsRNA sequences into juvenile _L. vannamei_ (50).

• General husbandry practices: some husbandry practices have been successfully applied to the prevention of TSV infections and TS disease. These include the application of polymerase chain reaction (PCR) prescreening of wild or pond-reared broodstock and/or their spawned eggs/nauplii and discarding those that test positive for the virus (15), fallowing and restocking of entire culture regions with TSV-free stocks (11), and the development of specific pathogen free (SPF) shrimp stocks of _L. vannamei_ and _L. stylirostris_ (27, 29, 36, 42, 49, 59, 60). The adoption of the latter technology (SPF stocks) has proven to be among the most successful husbandry practices for the prevention and control of TS. Unfortunately, there is a misconception in the industry that SPF is a genetic trait rather than a condition of health status. The development of SPF _L. vannamei_ that were free not only of TSV, but also of all the major known pathogens of penaeid shrimp, has resulted in the introduction of the species to Asia and to its challenging _P. monodon_ by 2004–2005 as the dominant farmed shrimp species in Asia, as well as the Americas where the SPF stocks were developed (29, 52).

3. DIAGNOSTIC METHODS

a) Field diagnostic methods

• Gross signs: TS disease has three distinct phases, acute, transition, and chronic, which are grossly distinguishable (18, 19, 26, 27, 32). Gross signs presented by shrimp in the acute and, especially, the transition phases of TS are unique and can provide a provisional diagnosis of the disease 5, 19, 26, 28).

• Acute phase: gross signs displayed by moribund _L. vannamei_ with acute-phase TS include expansion of the red chromatophores giving the affected shrimp a general, overall pale reddish coloration and making the tail fan and pleopods distinctly red; hence ‘red tail’ disease was one of the names given by farmers when the disease first appeared in Ecuador (32). In such shrimp, close inspection of the cuticular epithelium in thin appendages (such as the edges of the uropods or pleopods) with a ×10 hand lens reveals signs of focal epithelial necrosis. Shrimp showing these gross signs of acute TS typically have soft shells, an empty gut and are often in the late D stages of the molt cycle. Acutely affected shrimp usually die during ecdysis. If the affected shrimp are larger than ~1 g, moribund shrimp may be visible to sea birds at the pond edges and surface. Thus, during the peak of severe epizootics, hundreds of sea birds (gulls, terns, herons, cormorants, etc.) may be observed feeding on affected moribund shrimp that accumulate at the surface of the affected pond and edges (5–7, 16, 26, 27, 32, 57).

• Transition (recovery) phase: although only present for a few days during TS epizootics, the gross signs presented by shrimp in the transition phase can provide a tentative diagnosis of TSV infection. During the transition phase (which may be occurring while many shrimp in the affected populations are still in the acute phase and daily mortalities are high), fair to moderate numbers of shrimp in affected ponds show random, multifocal, irregularly shaped melanised cuticular lesions. These melanised spots are haemocyte accumulations indicating the sites resolving TS lesions in the cuticular epithelium. Such shrimp may or may not have soft cuticles and red-chromatophore expansion, and may be behaving and feeding normally (5, 19, 26).

• Chronic phase: after successfully molting, shrimp in the transition phase move into the chronic phase of TS in which persistently infected shrimp show no obvious signs of disease (5, 19, 26, 27, 32). However, _L. vannamei_ that are chronically infected with TSV may be less resistant to normal environmental stressors (i.e. sudden salinity reductions) than uninfected shrimp (36).
b) Clinical methods

- Gross pathology: see Section 3.a above.

- Direct microscopy: simple unstained wet-mounts of excised pieces of the gills, appendage tips, etc., examined by phase- or reduced-light microscopy may be used to demonstrate (and make a tentative diagnosis of acute phase TS) focal lesions of acute phase TS in cuticular epithelial cells. Preparations presenting TS acute phase lesions will contain numerous spherical structures (see Histopathological methods, below), which are pyknotic and karyorrhectic nuclei and cytoplasmic remnants of necrotic cells.

- Histopathology methods:
  - Acute phase of TS: diagnosis of TS in the acute phase of the disease is dependent on the histological demonstration (in haematoxylin and eosin [H&E] stained preparations), of multifocal areas of necrosis in the cuticular epithelium of the general body surface, appendages, gills, hindgut, and foregut (the esophagus, anterior and posterior chambers of the stomach). Cells of the subcuticular connective tissues and adjacent striated muscle fibres basal to affected cuticular epithelium are occasionally affected. In some severe cases of acute-phase TS, the antennal gland tubule epithelium is also destroyed. Prominent in the multifocal cuticular lesions are conspicuous foci of affected cells that display an increased eosinophilia of the cytoplasm and pyknotic or karyorrhectic nuclei. Cytoplasmic remnants of necrotic cells are often extremely abundant in these TS acute-phase lesions and these are generally presented as spherical bodies (1–20 µm in diameter) that range in staining from eosinophilic to pale basophilic. These structures, along with pyknotic and karyorrhectic nuclei, give acute-phase TS lesions a characteristic ‘peppered’ or ‘backshot-riddled’ appearance, which is considered to be pathognomonic for TS disease when there is no concurrent necrosis of the parenchymal cells of the lymphoid organ tubules. The absence of necrosis of the lymphoid organ in acute-phase TSV infections distinguishes TS disease from acute phase Yellow head disease in which similar patterns of necrosis to those induced by TSV may occur in the cuticular epithelium and gills (26).

In TSV-infected tissues, pyknotic or karyorrhectic nuclei give a positive (for DNA) Feulgen reaction, which distinguishes them from the less basophilic to eosinophilic cytoplasmic inclusions that do not contain DNA. The absence of haemocytic infiltration or other signs of a significant host-inflammatory response distinguishes the acute phase of TS from the transitional phase of the disease (4, 5–7, 12, 13, 18–20, 26, 32).

- Transition (recovery) phase of TS: in the transitional phase of TS, typical acute-phase cuticular lesions decline in abundance and severity and are replaced by conspicuous infiltration and accumulation of haemocytes at the sites of necrosis. The masses of haemocytes may become melanised giving rise to the irregular black spots that characterise the transition phase of the disease. In H&E sections, such lesions may show erosion of the cuticle, surface colonisation and invasion of the affected cuticle and exposed surface haemocytes by Vibrio spp. (19, 26). Sections of the lymphoid organ (LO) during the transition phase of TS may appear normal with H&E staining. However, when sections of the LO are assayed for TSV by ISH with a specific cDNA probe (or by IHC with MAb 1A1 for TSV type A, genotype 1), large quantities of TSV are shown accumulating in the more peripheral parenchymal cells of the LO tubules (19, 53).

- Chronic phase of TS: shrimp in the chronic phase of TS display no gross signs of infection, and histologically the only sign of infection is the presence of numerous prominent lymphoid organ spheroids (LOS), which may remain associated with the main body of the paired LO, or which may detach and become ectopic LOS bodies that lodge in constricted areas of the haemocoel (i.e. the heart, gills, in the subcuticular connectives tissues, etc.). Such LOS are spherical accumulations of LO cells and haemocytes and may be distinguished from normal LO tissues by their spherical nature and the lack of the central vessel that is typical for normal lymphoid organ tubules. When assayed by ISH with a cDNA probe for TSV (or with MAB 1A1 using IHC) some cells in the LOS give positive reactions for the virus, while no other target tissues react (19, 26, 27).
c) Agent detection and identification methods

i) Direct microscopic methods: none applicable.

ii) Agent isolation and identification

• Bioassay method: confirmation of TSV infection may be accomplished by bioassay of TSV-suspect animals with SPF juvenile *L. vannamei* serving as the indicator for the virus (7, 16, 19, 20, 26, 34, 46). Oral or injection protocols may be used. The oral method is relatively simple to perform and is accomplished by feeding chopped carcasses of suspect shrimp to SPF juvenile *L. vannamei* in small tanks (58). The use of a negative control tank of indicator shrimp, which receive only a normal feed, is required. When the carcass feeding (*per os*) protocol is used to bioassay for TSV, TS-positive indicator shrimp (by gross signs and histopathology) are typically apparent within 3–4 days of initial exposure, and significant mortalities occur by 3–8 days after initial exposure. The negative control shrimp must remain negative (for at least 10–15 days) for gross or histological signs of TS disease and unusual mortalities (19, 26, 58).

With the injection bioassay protocol, a variety of sample types may be tested for TSV. Whole shrimp are used if they were collected during a TSV epizootic. Heads only should be used if shrimp display gross transition-phase lesions (multifocal melanised spots on the cuticle) or no clinical signs of infection (chronic phase) as the virus, if present, will be concentrated in the lymphoid organ (19, 26). For nonlethal testing of broodstock, haemolymph samples may be taken and used to expose the indicator shrimp by IM injection (26).

To perform the IM (injection) bioassay for TSV:

i) Prepare a 1:2 or 1:3 ratio of TSV-suspect shrimp heads or whole shrimp with TN buffer (see Chapter 2.3.6 Infectious hypodermal and haematopoietic necrosis (IHNN) for the composition of this buffer) or sterile 2% saline prepared with distilled water.

ii) Homogenise the mixture using a tissue grinder or blender. Do not permit the mixture to heat up by excessive homogenisation or grinding. Tissues and resulting homogenate should be kept cool during the entire protocol by maintaining on ice.

iii) Clarify the homogenate by centrifugation at 3000 $g$ for 10 minutes. Decant and save the supernatant fluid. Discard the pellet.

iv) Centrifuge the supernatant fluid at 27,000 $g$ for 20–30 minutes at 4°C. Decant and save the supernatant fluid. Discard the pellet.

v) Dilute the supernatant fluid from step iv to 1/10 to 1/100 with sterile 2% saline. This solution may now be used as the inoculum to inject indicator shrimp (or filtered sterilised as described in step vi).

vi) Filter the diluted supernatant fluid from step v using a sterile syringe (size depends on the final volume of diluted supernatant) and a sterile 0.45 µm syringe filter. Multiple filters may have to be used as they clog easily. Filtrate should be collected in a sterile test tube or beaker. The solution can now be stored frozen (recommend −20°C for short-term [weeks] storage and −80°C for long-term [months to years] storage) or used immediately to inject indicator shrimp.

vii) Indicator shrimp should be from TSV-susceptible stocks of SPF *P. vannamei* (such as the ‘Kona stock’) (42), which are commercially available from a number of
sources in the Americas, and not from selected lines of known TSV-resistant stocks.

viii) Inject 0.01 ml per gram of body weight using a 1 ml tuberculin syringe. Indicator shrimp should be injected intramuscularly into the third tail segment. If the test shrimp begin to die within minutes post-injection, the inoculum contains excessive amounts of proteinaceous material and should be further diluted prior to injecting additional indicator shrimp. Sudden death occurring post-injection is referred to as ‘protein shock’, and is the result of systemic clotting of the shrimp’s haemolymph in response to the inoculum.

ix) Haemolymph samples may be diluted (1/10 or 1/20 in TN buffer), filter sterilised (if necessary), and injected into the indicator shrimp without further preparation.

x) If TSV was present in the inoculum, the indicator shrimp should begin to die within 24–48 hours post-injection. Lower doses of virus may take longer to establish a lethal infection and shrimp should be monitored for at least 10–15 days post-injection.

xi) The presence (or absence) of TSV in the indicator shrimp should be confirmed by histological analysis (and ISH by gene probe if available) of Davidson’s fixed moribund shrimp.

As a variation to the bioassay technique, a ‘sentinel shrimp’ system may be used. For example, TSV-sensitive stocks of small juvenile SPF L. vannamei may be held in net-pens in tanks, or in the same water system, with other shrimp of unknown TSV status to bioassay for the presence of infectious agents such as TSV.

- Antibody-based antigen detection methods: MAb for detection of TSV may be used to assay samples of haemolymph, tissue homogenates, or Davidson’s AFA fixed tissue sections from shrimp (12, 13, 48). TSV MAb 1A1 may be used to distinguish some variants or ‘strains’ of TSV from other strains (12, 13). (See Section 2.a of this chapter for more information on TSV types as determined by their reaction to MAb 1A1.)

Dot-blot immunoassay method:

i) For the dot-blot immunoassay method, 1 µl of test antigen (purified virus, infected shrimp haemolymph or SPF shrimp haemolymph) is dotted on to the surface of MA-HA-N45 assay plates (Millipore, South San Francisco, California [CA], USA).

ii) After air drying, the wells are blocked for 1 hour at room temperature with 200 µl of a buffer containing phosphate buffered saline and 0.05% Tween 20 (PBST) mixed with 10% normal goat serum (Life Technologies, Gibco BRL) and 2% Hammersten casein (Amersham Life Sciences, Arlington Heights, Illinois, USA).

iii) The wells are washed three times with PBST and then reacted with 100 µl primary antibody (MAb or mouse polyclonal antibodies) for 30 minutes at room temperature.

iv) Alkaline-phosphatase-labelled goat anti-mouse IgG, γ chain specific, secondary antibody (Zymed, South San Francisco, CA) diluted 1/1000 in PBST plus 10% normal goat serum is used for detection (30 minutes at room temperature).

v) After washing three times with PBST, once with PBS and once with distilled water, the reactions are visualised by development for 15 minutes at room temperature with nitroblue tetrazolium and bromo-chloro-indoyl phosphate (Roche Diagnostics, Corp.) in Tris-NaCl (100 mM each) buffer containing 50 mM MgCl2, pH 9.5.

vi) Reactions are stopped with distilled water.
vii) The reactions are graded using a scale from 0 to +4, with the highest intensity reaction being equivalent to the reaction generated using the MAb against the reference control consisting of semipurified TSV. A negative reaction is one in which no coloured spot is visible in the well.

Other antibody-based methods:

The TSV MAb 1A1 may be applicable to other antibody-based test formats (i.e. indirect fluorescent antibody [IFAT] or immunohistochemistry [IHC] tests with tissue smears, frozen sections, or deparaffinised fixed tissues). MAb 1A1 is applicable for use in an IHC format using Davidson’s AFA fixed tissue sections (12, 13).

It is recommended that unexpected results from MAb-based tests for TSV should be interpreted in the context of clinical signs, case history, and in conjunction with other test results (e.g. reverse-transcription [RT]-PCR test results, or findings from histology or ISH with a TSV-specific DNA probe – see appropriate sections in this chapter).

- Molecular methods: ISH and RT-PCR tests for TSV have been developed and varieties of both methods are commercially available. The dot-blot method for TSV detection is not available.

- DNA probes for ISH applications with nonradioactive cDNA probes. Nonradioactive, DIG-abellled cDNA probes for TSV may be produced in the laboratory or obtained from commercial sources. The ISH method provides greater diagnostic sensitivity than does more traditional methods for TSV detection and diagnosis that employ classic histological methods (18, 26, 28, 31, 40). The ISH assay of routine histological sections of acute- and transition-phase lesions in the cuticular epithelium, other tissues, and of LOS in transition and chronic phase with a specific DIG-labelled cDNA probe to TSV, provides a definitive diagnosis of TSV infection (18, 19, 26, 27). Pathognomonic TSV-positive lesions display prominent blue to blue-black areas in the cytoplasm of affected cells when reacted with the cDNA probes. Not reacting to the probe are the prominent karyorrhectic nuclear fragments and pyknotic nuclei that contribute to the pathognomonic ‘buckshot riddled’ appearance of TS lesions (26, 27, 40). (See Chapter 2.3.6 IHHN for details of the ISH method. See Chapter I.3 Section 4.2 for detailed information on the use of Davidson’s AFA fixative.)

False-negative ISH results may occur with Davidson’s fixed tissues if tissues are left in fixative for more than 24–48 hours. The low pH of Davidson’s fixative causes acid hydrolysis of the TSV single-stranded RNA genome, resulting in false-negative probe results. This artefact can be avoided through the use of neutral fixatives, including an ‘RNA-friendly’ fixative developed for shrimp, or by the proper use (avoiding fixation times over 24 hours) of Davidson’s fixative (17, 26, 31).

- RT-PCR method: tissue samples (haemolymph, pleopods, whole small shrimp, etc.) may be assayed for TSV using RT-PCR. Primers designated as 9195 and 9992, amplify a 231 base pair (bp) sequence of the TSV genome (44). The fragment amplified is from a conserved sequence located in the intergenic region and ORF 2 of TSV. Primer 9992F is located near the 3’ end of intergenic region and 9195R is located ORF 2 within VP2 (= CP1) (41, 44).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Product</th>
<th>Sequence</th>
<th>G-C ratio</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>9992F</td>
<td>231 bp</td>
<td>5’-AAG-TAG-ACA-GCC-GCG-CTT-3’</td>
<td>55°C</td>
<td>69°C</td>
</tr>
<tr>
<td>9195R</td>
<td>5’-TCA-ATG-AGA-GCT-TGG-TCC-3’</td>
<td>50</td>
<td>63°C</td>
<td></td>
</tr>
</tbody>
</table>
The RT-PCR method outlined below for TSV follows generally the method used in Nunan et al. (44).

i) Preparation of RNA template: RNA can be extracted from fresh, frozen and ethanol preserved tissues. Extraction of RNA should be performed using commercially available RNA tissue extraction kits, such as the High Pure RNA Tissue Kit (Roche, Penzberg, Germany) and following the manufacturer’s procedures for production of quality RNA templates.

ii) The RT-PCR assay is done in solution, using 10 µl of total RNA extracted from haemolymph, frozen shrimp tissues, ethanol fixed tissue as the template (concentration of RNA = 1–100 ng/ml).

iii) The following controls should be included in every RT-PCR assay for TSV: a) known TSV-negative tissue sample; b) a known TSV-positive sample (tissue or purified virus); and c) a ‘no-template’ control.

iv) The GeneAmp® EZ rTth RNA PCR kit (Applied Bioscience, Forster City, CA) is used for all amplification reactions described here.

v) The optimised RT-PCR conditions (final concentrations in 50 µl total volume) for detection of TSV in shrimp tissue samples are: primers (0.46 µM each), dNTPs (300 µM each), rTth DNA polymerase (2.5 U/50 µl), manganese acetate (2.5 mM), in 5 × EZ buffer (25 mM Bicine, 57.5 mM potassium acetate, 40% [w/v] glycerol, pH 8.2).

vi) If the thermal cycler does not have a heated lid, then light mineral oil (50 µl) is overlaid on the top of the 50 µl reaction mixtures to prevent condensation or evaporation during thermal cycling.

vii) The RNA template and all the reagents are combined and reverse transcription is allowed to proceed at 60°C for 30 minutes, followed by 94°C for 2 minutes.

Note: The reaction conditions described here were optimized using an automatic Thermal Cycler GeneAmp 980 (Applied Biosystems). The conditions should be optimized for each thermal cycler using known positive controls.

viii) At the completion of reverse transcription, the samples are amplified for 40 cycles under the following conditions: denaturation at 94°C for 45 seconds, and then annealing/extension at 60°C for 45 seconds. A final extension step for 7 minutes at 60°C follows the last cycle and the process is terminated in a 4°C soak file.

ix) Following the termination of RT-PCR, the amplified cDNA solutions are drawn off from beneath the mineral oil and placed into clean 0.5 ml microfuge tubes.

x) A 10 µl sample of the amplified product can then be added to the well of a 2.0% agarose gel, stained with ethidium bromide (0.5 g/ml), and electrophoresed in 0.5 × TBE (Tris, boric acid, ethylene diamine tetra-acetic acid [EDTA]).

xi) A 1 kb DNA ladder (Invitrogen, Carlsbad, CA) is used as a marker.

xiii) Details of the composition of the reagents and buffers used here may be found in Chapter 2.3.6 IHHN.

• **Real-time PCR method for TSV:** Real-time RT-PCR methods have been developed for the detection of TSV. These methods have the advantages of speed, specificity and sensitivity. The sensitivity of real-time RT-PCR is ~100 copies of the target sequence from the TSV genome (10, 55).

The real-time RT-PCR method using TaqMan chemistry described below for TSV follows generally the method used in Tang et al. (55).
The PCR primers and TaqMan probe were selected from the ORF1 region of the TSV genomic sequence (GenBank AFAF277675) that encodes for nonstructural proteins. The primers and TaqMan probe were designed by the Primer Express software (Applied Biosystems). The upstream (TSV1004F) and downstream (TSV1075R) primer sequences are: 5'-TTG-GGC-ACC-AAA-CGA-CAT-T-3' and 5'-GGG-AGC-TTA-AAC-TGG-ACA-CAC-TGT-3'), respectively. The TaqMan probe, TSV-P1 (5'-CAG-CAC-TGA-CGC-ACA-ATA-TTC-GAG-CAT-C-3'), which corresponds to the region from nucleotide 1024 to 1051, is synthesised and labelled with fluorescent dyes 5-carboxyfluoroscein (FAM) on the 5' end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end (Applied Biosystems, catalog no. 450025).

Preparation of RNA template: the extraction and purification of RNA template from haemolymph, or shrimp tissue, is the same as that described in the section for traditional RT-PCR.

The RT-PCR reaction mixture contains: TaqMan One-step RT-PCR Master Mix (Applied Biosystems, part no. 4309169), 0.3 µM of each primer, 0.1 µM of TaqMan probe, 5–50 ng of RNA, and water in a reaction volume of 25 µl. For optimal results, the reaction mixture should be vortexed and mixed well.

Amplification is performed with the GeneAmp 5700 Sequence Detection System (Applied Biosystems; ABI PRISM 7000, 7300, or 7500 can also be used). The cycling consists of reverse transcription at 48°C for 30 minutes and initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute.

At the end of reaction, real-time fluorescence measurements will be taken with a built in charge-coupled device (CCD) camera. A threshold will be set to be above the baseline that begins to detect the increase in signal associated with an exponential increase of PCR product. Samples will be defined as negative if the Ct (threshold cycle) value is 40 cycles. Samples with Ct value lower than 40 cycles are considered to be positive. To confirm the real-time RT-PCR results, an aliquot of RT-PCR product can be subjected to electrophoresis on a 4% ethidium bromide-agarose gel and photographed. A 72-bp DNA fragment can be visualised in the samples that are positive for TSV.

It is necessary to include a ‘no template control’ in each reaction run. This is to rule out the presence of fluorescence contaminants in the reaction mixture or in the heat block of thermal cycler. A positive control should also be included, and this can be an in vitro transcribed RNA containing the target sequence, purified virions, or RNA extracted from TSV-infected tissue.

4. RATING OF TESTS AGAINST PURPOSE OF USE

The methods currently available for surveillance, detection, and diagnosis of TSV are listed in Table 1. The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic sensitivity and specificity; C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and D = the method is presently not recommended and/or not available for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation (see Chapter 1.1.2), their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
Table 1. TSV surveillance, detection and diagnostic methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance</th>
<th></th>
<th>Presumptive</th>
<th>Confirmatory</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Larvae</td>
<td>PLs</td>
<td>Juveniles</td>
<td>Adults</td>
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<tr>
<td>Gross signs</td>
<td>D</td>
<td>D</td>
<td>C</td>
<td>C</td>
</tr>
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<td>D</td>
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<td>Direct LM</td>
<td>D</td>
<td>D</td>
<td>C</td>
<td>D</td>
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<td>Histopathology</td>
<td>D</td>
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<td>B</td>
<td>B</td>
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<td>TEM</td>
<td>D</td>
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<td>Antibody-based assays</td>
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<td>C</td>
<td>C</td>
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<tr>
<td>DNA probes <em>in situ</em></td>
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<td>B</td>
<td>B</td>
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<tr>
<td>RT-PCR</td>
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</tr>
<tr>
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</tbody>
</table>

PLs = postlarvae; LM = light microscopy; TEM = Transmission electron microscopy; RT-PCR = reverse-transcription polymerase chain reaction

5. CORROBORATIVE DIAGNOSTIC CRITERIA

a) Definition of suspect case

Sudden high mortalities in late PL, juvenile or subadult *L. vannamei* or *L. stylirostris* in regions where TSV is enzootic.

The sudden presence of numerous sea birds (gulls, cormorants, herons, terns, etc.) ‘fishing’ one or more shrimp culture ponds.

Samples of cultured *L. vannamei* or *L. stylirostris* from ponds with feeding sea birds that present gross signs indicative of acute or transition phase TS, such as a general reddish coloration, lethargy, soft shells, empty guts, and the presence of numerous irregular black spots on the cuticle.

Demonstration of foci of necrosis in the cuticular epithelium using low magnification (i.e. a ×10 hand lens or by direct microscopic examination of wet mounts) to examine the edges of appendages such as uropods or pleopods, or the gills.

b) Definition of confirmed case

Any combination of at least two of the following three methods (with positive results):

Histological demonstration of diagnostic acute phase TSV lesions in (especially) the cuticular epithelia of the foregut (oesophagus, anterior, or posterior chambers of the stomach) and/or in the gills, appendages, or general cuticle. Such TSV lesions are pathognomonic for TSV only when they occur without accompanying severe acute necrosis (with nuclear pyknosis and karyorrhexis) of the parenchymal cells of the lymphoid organ tubules.

ISH (with a TSV-specific cDNA probe) or IHC (with MAb 1A1) positive histological signal to TSV-type lesions in the acute, transition, or chronic phase of infection. However negative ISH results with MAb 1A1 should be interpreted cautiously as not all strains of TSV react with MAb 1A1.

RT-PCR positive results for TSV.
6. DIAGNOSTIC/DETECTION METHODS TO DECLARE FREEDOM

Two years of history of negative test results for TSV using the methods described in Section 5.b on samples of the appropriate type and size (see Chapter I.3, Section 3).

REFERENCES


*   *
*   *

**NB:** There is an OIE Reference Laboratory for Taura syndrome (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: [www.oie.int](http://www.oie.int)).
CHAPTER 2.3.2.

WHITE SPOT DISEASE

1. CASE DEFINITION

For the purpose of this chapter, White spot disease (WSD) is considered to be infection with white spot syndrome virus (WSSV).

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

   a) Agent factors

   • Aetiological agent, agent strains: the viral species *White spot syndrome virus 1*, as defined in Vlak *et al.* 2004 (66).
   • Stability of the agent: the agent is inactivated in <120 minutes at 50°C and <1 minute at 60°C (49); is viable for at least 30 days at 30°C in seawater under laboratory conditions (46); and is viable in ponds for at least 3–4 days (5, 29, 43, 49).
   • Replication cycle: *in-vitro* studies with primary cell culture and *in-vivo* studies with post-larvae show that the replication cycle is approximately 20 hours at 25°C.

   b) Host factors

   • Susceptible host species: all decapod (order Decapoda) crustaceans from marine and brackish or freshwater sources (3, 13, 17, 23, 26, 27, 35, 40–42, 57, 58, 69).
   • Susceptible stages of the host: all life stages potentially susceptible, from eggs to brood stock (37, 38, 63).
   • Species or sub-population predilection (probability of detection): higher probability of detection of the virus in crabs and shrimp. Best life stages for detection are late post-larval stages, juveniles and adults. Probability of detection can be increased by exposure to stressful conditions (e.g. eye stalk ablation, spawning, changes in salinity and temperature).
   • Target organs and infected tissue: ectodermal and mesodermal tissues, especially the cuticular epithelium and subcuticular connective tissues (38, 47, 68, 72).
   • Persistent infection with lifelong carriers: Persistent infection occurs commonly and lifelong infection has been shown (61). Viral loads during persistent infection can be extremely low and potentially undetectable by any available diagnostic test.
   • Vectors: rotifers (73), bivalves, polychaete worms (65) and non-decapodal crustacea including *Artemia salina* and the copepods; also, non-crustacean aquatic arthropods such as sea slaters (*Isopoda*) and *Euphydraeidae* insect larvae. All these species can accumulate high concentrations of viable WSSV, although there is no evidence of virus replication (7, 34, 40, 73).

   c) Disease pattern

   • Transmission mechanisms: vertical (trans-ovum), horizontal by consumption of infected tissue (e.g. cannibalism, predation, etc.), and by water-borne routes. Transmission of infection can occur from apparently healthy animals in the absence of disease. Dead and moribund animals can be a source of disease transmission (11, 37, 38).
• Prevalence: highly variable, from <1% in infected wild populations to up to 100% in captive populations (23, 40, 41, 54, 62).

• Geographical distribution: throughout East, South-East and South Asia, North, South and Central America. WSD-free zones and compartments are known within these regions (1–3, 8, 17–19, 37, 44, 50, 67, 75).

• Mortality and morbidity: all farmed penaeid shrimp species are highly susceptible to infection, often resulting in high mortality. Crabs, crayfish, freshwater prawns, spiny lobsters and clawed lobsters are susceptible to infection, but morbidity and mortality as a consequence of infection is highly variable (2, 9, 22, 24, 27, 47, 50, 56, 59, 74). High level infections are known in some decapods in the absence of clinical disease. Disease outbreaks may be induced by stressors, such as rapid changes in salinity. Water temperature has a profound effect on disease expression with average water temperatures of below ~30°C being conducive to WSD outbreaks (20, 21, 28, 64).

• Economic and/or production impact of the disease: value of lost production and trade approaching 10 billion USD has been reported since 1993.

d) Control and prevention

• Vaccination: no consistently effective vaccination methods have been developed.

• Chemotherapy: no scientifically confirmed reports.

• Immunostimulation: reports that beta-glucan, vitamin C, seaweed extracts (fucoidan) and other immunostimulants may improve resistance to WSD (4, 10, 15, 32, 51, 71).

• Resistance breeding: no significant improvements reported.

• Restocking with resistant species: not applicable for WSD.

• Blocking agents: not reported.

• General husbandry practices: a number of husbandry practices have been used successfully to manage WSD, e.g. avoiding stocking in the cold season, use of specific pathogen free (SPF) or polymerase chain reaction (PCR)-negative seed stocks, and use of biosecure water and culture systems (14, 25, 48, 54, 70).

3. DIAGNOSTIC METHODS

a) Field diagnostic methods

• Gross signs: presence of white spots under the cuticle and a high degree of colour variation with a predominance of reddish or pinkish discoloured shrimp, reduction in feed intake, increasing lethargy, movement of moribund shrimp to the water surface and pond/tank edges and consequent attraction of shrimp-eating birds.

b) Clinical methods

• Gross pathology: see Section 3.a above.

• Microscopic examination

  • Wet mounts: demonstration of hypertrophied nuclei in squash preparations of the gills and/or cuticular epithelium, which may be stained or unstained.

  • Demonstration of WSSV aggregates in unstained wet-mount preparations of haemolymph by dark field microscopy.

  Please Note: This is the simplest of the microscopic techniques and is recommended for people with limited expertise in WSSV. The aggregates appear as small reflective spots of 0.5 µm diameter (45).
• Fixed sections: histological demonstration of pathognomonic inclusion bodies in target tissues.

• *In situ* hybridisation: use of WSSV-specific DNA probes with histological sections to demonstrate the presence of WSSV nucleic acid in infected cells.

• Immunohistochemistry: use of WSSV-specific antibodies with histological sections or wet mounts to demonstrate the presence of WSSV antigen in infected cells.

• Electron microscopy: demonstration of the virus in tissue sections or in semi-purified virus preparations (e.g. from haemolymph).

c) Agent detection and identification methods

• Direct detection methods

  i) Microscopic methods

    • *Wet mounts*: see Section 3.b above.

    • *Fixed sections*: see Section 3.b above.

  ii) Agent isolation and identification

    • *Molecular techniques:*

      a) *Polymerase chain reaction* (PCR)

      The protocol described here is according to Lo *et al.* (38, 39), and is recommended for all situations where WSSV diagnosis is required. A positive result in the first step of this standard protocol implies a serious WSSV infection; when a positive result is obtained in the second amplification step only, a latent or carrier-state infection is indicated. Alternative assays have also been described (30, 31, 33, 36, 52, 55, 60), but are not recommended using unless they have first been compared with the protocol described here.

      **DNA extraction**

      i) Collect 100–200 mg shrimp tissue (pleopod of live juvenile to subadult shrimp, post-larvae 11 upwards [PL11 up] with removed heads, or whole PL10, or use 100 µl of haemolymph) in a 1.5 ml microfuge tube with 600 µl of lysis solution (100 mM NaCl, 10 mM Tris/HCl, pH 8, 25 mM EDTA [ethylene diamine tetra-acetic acid], 0.5% SLS [sodium N-laurylsarcosinate] or 2% SDS [sodium dodecyl sulfate], and 0.5 mg/ml proteinase K added just before use).

      ii) Using a disposable stick, homogenise the tissue in the tube thoroughly.

      iii) After homogenisation, incubate at 65°C for 1 hour.

      iv) Add 5 M NaCl to a final concentration of 0.7 M. Next slowly add 1/10 volume of N-cetyl N,N,N-trimethylammonium bromide (CTAB)/NaCl solution (10% CTAB in 0.7 M NaCl) and mix thoroughly.

      Please note: the CTAB extraction method described here is the only extraction method that has proved to be reliable. Commercial extraction kits are not recommended.

      v) Incubate at 65°C for 10 minutes, and then, at room temperature, add an equal volume of chloroform/isoamyl alcohol (24/1) and mix gently. Centrifuge at 13,000 *g* for 5 minutes and then transfer the aqueous solution (upper layer) to a fresh 1.5 ml tube and add an equal volume of phenol.

      vi) Mix gently and centrifuge at 13,000 *g* for 5 minutes. Collect the upper layer solution and repeat the phenol extraction process once or twice.
vii) Transfer the final upper layer to a new tube, mix gently with two volumes of chloroform/isoamyl alcohol (24/1) and centrifuge at 13,000 g for 5 minutes.

viii) Transfer the upper layer to a new tube and precipitate DNA by adding two volumes of 95% or absolute ethanol followed by standing at –20°C for 30 minutes or –80°C for 15 minutes.

ix) Centrifuge at 13,000 g for 30 minutes and discard the ethanol. Wash the DNA pellet with 70% ethanol, dry and resuspend in 100 µl sterilised double-distilled water at 65°C for 15 minutes.

x) Use 1 µl of this DNA solution for one PCR reaction.

Please note: The following nested PCR procedures are well established and provide reliable diagnostic results under the specified conditions. Care should be taken, however, to ensure that DNA samples are prepared from the recommended organs, and that the PCR temperature (particularly for annealing, the recommended temperature is 55°C) is accurately applied. To prevent the possibility of false positive results, it is important to adhere to the specified procedures, especially when they are used to test new candidate hosts such as Cherax quadricarinatus (12). For diagnosed incidences of WSSV in a new host or in a previously free zone, DNA sequencing should be used to confirm the positive results.

First-step PCR reaction

i) Add 1 µl DNA template solution (containing about 0.1–0.3 µg DNA) to a PCR tube containing 100 µl of reaction mixture (10 mM Tris/HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 200 µM of each dNTP, 100 pmol of each primer, 2 units of heat-stable DNA polymerase).

ii) The outer primer sequences are 146F1, 5’-ACT-ACT-AAC-TTC-AGC-CTA-TCTAG-3’ and 146R1, 5’-TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A-3’.

iii) The PCR profile is one cycle of 94°C for 4 minutes, 55°C for 1 minute, and 72°C for 2 minutes, followed by 39 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes and a final 5-minute extension at 72°C. The WSSV-specific amplicon from this reaction is 1447 bp. The sensitivity is approximately 20,000 copies of a plasmid template.

Second step of the (nested) PCR reaction: This second step is necessary for the detection of WSSV in shrimp at the carrier stage.

i) Add 10 µl of the first-step PCR reaction product to 90 µl of a PCR cocktail with the same composition as above except that it contains the second (inner) primer pair: 146F2 (5’-GTA-ACT-GCC-CCT-TCC-ATC-TCC-A-3’) and 146R2 (5’-TAC-GGC-AGC-TGC-TGC-ACC-TTG-T-3’).

ii) Use the same PCR amplification protocol as above. The WSSV-specific amplicon from this reaction is 941 bp. The overall sensitivity of both steps is approximately 20 copies of a WSSV plasmid template.

iii) To visualise, electrophorese 10 µl of PCR reaction products on 1% agarose gels containing ethidium bromide at a concentration of 0.5 µg/ml.

iv) Decapod-specific primers (143F 5’-TGC-CTT-ATC-AGCTNT-CGA-TTG-TAG-3’ and 145R 5’-TTC-AGN-TTT-GCA-ACC-ATA-CTT-CCC-3’) yielding an 848 bp amplicon; N represents G, A, T, or C) should be used in control reactions to verify the quality of the extracted DNA and the integrity of the PCR reaction. In the penaeid shrimp Penaeus aztecus, the PCR product generated by this decapod-specific primer pair corresponds to nucleotide sequence 352–1200 of the 18s rRNA (39). The decapod 18s RNA sequence is highly conserved and produces a similar sized PCR product in almost all...
decapods. A positive control (WSSV DNA template) and negative controls (no template and shrimp DNA template) should be included in every assay.

b) **DNA sequencing of PCR products**

For confirmation of suspected new hosts of WSSV, the DNA fragment amplified from the two-step nested diagnostic PCR should be sequenced. The cloning and sequencing protocols described here are according to Claydon et al. (12).

**Please note:** to save time and money, it is acceptable to sequence the PCR amplicon directly. If a positive result is obtained, then go to step iv below. In the event of a negative result, then the sample should be tested again using the cloning and sequencing procedures described below.

i) Excise the DNA fragments selected for further analysis from the agarose gels and purify them using Promega’s Wizard SV Gel and PCR clean-up system according to the manufacturer’s instructions.

ii) Ligate amplicons into pGEM®-T easy vector plasmid (Promega) and clone the construct according to the manufacturer’s instructions.

iii) DNA sequencing may be carried out using M13 universal primers (Promega) and a Beckman Coulter CEQ dye terminator cycle sequencing quick start kit.

iv) Compare the sequences so obtained to available databases using the Basic Local Alignment Search Tool (BLAST) to determine approximate phylogenetic affiliations.

c) **In situ hybridisation (ISH) method**

The protocol described here is based on that developed by Nunan & Lightner (52), although an alternative protocol has been described by Chang et al. (6)

i) Fix moribund shrimp with Davidson’s AFA fixative for 24–48 hours.

ii) Embed the tissues in paraffin and cut into 5 µm sections. Place sections on to Superfrost Plus positively charged microscope slides.

iii) Heat the slide on a hot plate at 65°C for 30 minutes.

iv) Deparaffinise, rehydrate and then treat for 2–30 minutes (depending on tissue type) with 100 µg/ml proteinase K in Tris/NaCl/EDTA (TNE) buffer at 37°C.

v) Post-fix the slides by chilling in pre-cooled 0.4% formaldehyde for 5 minutes at 4°C and wash the slides in 2 × standard saline citrate (SSC; 1 × SSC = 150 mM NaCl, 15 mM tri-sodium citrate, pH 7.0) at room temperature.

vi) Prehybridise the slides with prehybridisation solution (50% formamide, 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 5 × SSC, 1 mM EDTA, 50 mM Tris/HCl, pH 8) for 30 minutes at 42°C.

vii) Follow with hybridisation with the 1447 bp WSSV-specific PCR amplicon (see “First-step PCR reaction” above) that has been labelled with digoxigenin (DIG; Roche). It is recommended that the probe be labelled by incorporating DIG-dNTP in the PCR duplicating cycle. Optimum concentration should be determined by testing and adjusting until a high specific signal is obtained against a low background.

viii) For hybridisation, boil the probe for 10 minutes and immediately place on ice. Dilute the probe to 30–50 ng/ml in prehybridisation solution and apply 500 µl to each slide.

ix) Put the slide on a hotplate at 85–95°C for 6–10 minutes (make sure that it does not reach boiling point), quench slides on ice for 5 minutes and then transfer to a humid chamber for 16–20 hours at 42°C.
x) After hybridisation, wash the slides twice for 15 minutes each time with 2 × SSC at room temperature, twice for 5 minutes with 1 × SSC at 37°C, and twice for 5 minutes with 0.5 × SSC at 37°C.

xi) For hybridisation detection, wash slides with maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 5 minutes at room temperature.

xii) Block the slides with blocking solution (2% normal goat serum and 0.3% Triton X-100 in maleic acid buffer) for 30 minutes at 37°C.

xiii) Add 250 µl anti-DIG alkaline phosphatase (AP)-conjugated antibody solution (1 µl/ml anti-DIG/AP-Fab fragment in maleic acid buffer containing 1% normal goat serum and 0.3% Triton X-100) to each slide, and incubate at 37°C for 30 minutes.

xiv) Wash the slides twice with maleic acid buffer for 10 minutes each and once with detection buffer (100 mM Tris/HCl, 100 mM NaCl, pH 9.5) at room temperature.

xv) Add 500 µl development solution (prepare immediately before use by adding 45 µl NBT salt solution [75 mg/ml in 70% dimethylformamide], 35 µl 5-bromo-4-chloro-3-indoyl phosphate, toluidinium salt [X-phosphate] solution [50 mg/ml in dimethylformamide] and 1 ml 10% PVA to 9 ml of detection buffer) to each slide and incubate in the dark in a humid chamber for 1–3 hours.

xvi) Stop the reaction by washing the slides in TE buffer (10 mM Tri-HCl, 1 mM EDTA, pH 8.0) for 15 minutes at room temperature. Wash the slides in distilled water for ten dips, counterstain the slides in 0.5% aqueous Bismarck Brown Y for 30–90 seconds and then rinse with water. Wet mount using aqueous mounting media for observation immediately or dehydrate the slides and mount with Permount mounting media for long-term preservation.

xvii) Mount the slides with cover-slips and examine with a bright field microscope. Positive hybridisation appears as a dark blue to black precipitate against the yellow to brown counterstain.

d) Bioassay method:

If SPF shrimp are available, the following bioassay method is based on Nunan et al. (53) and Durand et al. (16), is suitable for WSSV diagnosis.

i) For bioassay, remove the pleopods from shrimp suspected of WSSV infection and homogenise in TN buffer (0.02 M Tris/HCl, 0.4 M NaCl, pH 7.4).

ii) Following centrifugation at 1000 g for 10 minutes, dilute the supernatant fluid 1/10 with 2% NaCl and filter (0.2 µm filter).

iii) Inject 0.2 ml of inoculum into the dorso-lateral aspect of the fourth abdominal segment of indicator shrimp (e.g. SPF Litopenaeus vannamei at the juvenile stage), injecting between the tergal plates into the muscle of the third abdominal segment.

iv) Examine moribund shrimp grossly or by using the methods described above. If at 3–5 days after inoculation there are still no moribund shrimp and all test results are negative, then it is safe to conclude that the bioassay results are negative.

4. RATING OF TESTS AGAINST PURPOSE OF USE

The methods currently available for surveillance, detection, and diagnosis of WSSV are listed in Table 1. The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard
method with good diagnostic sensitivity and specificity; C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and D = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation (see Chapter 1.1.2), their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 1. WSV surveillance, detection and diagnostic methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance</th>
<th>Presumptive</th>
<th>Confirmatory</th>
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<tbody>
<tr>
<td></td>
<td>Larvae</td>
<td>PLs</td>
<td>Juveniles</td>
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<tr>
<td>Gross signs</td>
<td>D</td>
<td>D</td>
<td>C</td>
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<tr>
<td>Bioassay</td>
<td>D</td>
<td>D</td>
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<tr>
<td>Direct LM</td>
<td>D</td>
<td>D</td>
<td>C</td>
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<tr>
<td>Histopathology</td>
<td>D</td>
<td>C</td>
<td>C</td>
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<tr>
<td>Transmission EM</td>
<td>D</td>
<td>D</td>
<td>D</td>
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<tr>
<td>Antibody-based assays</td>
<td>D</td>
<td>D</td>
<td>C</td>
</tr>
<tr>
<td>DNA Probes <em>in situ</em></td>
<td>D</td>
<td>D</td>
<td>C</td>
</tr>
<tr>
<td>PCR</td>
<td>D</td>
<td>B</td>
<td>A</td>
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</table>

PLs = postlarvae; LM = light microscopy; EM = electron microscopy; PCR = polymerase chain reaction.

5. CORROBORATIVE DIAGNOSTIC CRITERIA

a) Definition of suspect case

For juvenile and adult shrimp: gross signs of WSD (see Section 3.a above). For shrimp at any life stage (larva to adult): mortality. For shrimp and crab at ay life stage (larva to adult): hypertrophied nuclei in squash preparations of gill and/or cuticular epithelium; unusual aggregates in haemolymph by dark-field microscopy; inclusion bodies in histological sections in target tissues.

b) Definition of confirmed case

Suspect cases should first be checked by PCR. If in a previously WSSV-free country/zone/compartment, PCR results are positive, they should be confirmed by sequencing.

6. DIAGNOSTIC/Detection methods to declare freedom

Two-step PCR and sequencing are the prescribed methods for declaring freedom. Two-step PCR negative results are required. Where a two-step PCR positive result cannot be confirmed by sequencing, this also counts as a negative result.

REFERENCES


*   *
*   *

**NB:** There are OIE Reference Laboratories for White spot disease (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: [www.oie.int](http://www.oie.int).
1. **CASE DEFINITION**

For the purpose of this chapter, yellowhead disease (YHD) is considered to be infection with yellowhead virus (YHV).

2. **INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES**

   a) **Agent factors**

   - Aetiological agent, agent strains: yellowhead virus (genotype 1) is one of six known genotypes in the yellowhead complex of viruses and is the only known agent of yellowhead disease. Gill-associated virus (GAV) is designated as genotype 2. GAV and four other known genotypes in the complex (genotypes 3–6) occur commonly in healthy *Penaeus monodon* in East Africa, Asia and Australia and are rarely or never associated with disease (29, 32). YHV and other genotypes in the yellowhead complex are classified by the International Committee on Taxonomy of Viruses as a single species in the genus *Okavirus*, family *Roniviridae*, order *Nidovirales* (28). There is evidence of genetic recombination between genotypes (32).

   - Survival outside the host: YHV remains viable in aerated seawater for up to 72 hours (8).

   - Stability of the agent: YHV can be inactivated by heating at 60°C for 15 minutes (8). Little information is available on other inactivation methods but the virus appears to be susceptible to treatment with chlorine at 30 ppm (6).

   - Life cycle: high multiplicity YHV infections in cell culture have not been reported. Infection at a multiplicity of infection of 0.001 in primary lymphoid organ cell culture indicated maximum viral titre is obtained at 4 days post-infection. Clinical signs of YHD occur in *P. monodon* within 7–10 days of exposure.

   b) **Host factors**

   - Susceptible host species: natural and/or experimental infections have been reported to occur in the following species of penaeid and palemonid shrimp and prawns and krill: black tiger prawn (*Peneaus monodon*), brown tiger prawn (*Peneaus esculentus*), Kuruma prawn (*Marsupenaeus japonicus*), white banana prawn (*Fenneropenaeus merguiensis*), Pacific white prawn (*Litopenaeus vannamei*), Pacific blue prawn (*Litopenaeus stylirostris*), white prawn (*Litopenaeus setiferus*), brown prawn (*Farfantepenaeus aztecus*), pink prawn, hopper and brown-spotted prawn (*Farfantepenaeus duorarum*), red endeavour prawn (*Metapenaeus ensis*), greentail prawn (*Metapenaeus bennettae*), Sunda river prawn (*Macrobrachium sintangense*), mysid shrimp (*Palaemon styliferus*), barred estuarine shrimp (*Palaemon serrifer*), paste prawn (*Ascetes sp.*), Antarctic krill (*Euphausia superba*). There are variations in the susceptibility of different species to disease. Laboratory trials have shown that YHV can cause high mortality in *P. monodon*, *L. vannamei*, *L. stylirostrus*, *Fa. aztecus*, *Fa. duorarum*, *M. sintangense*, *P. styliferus* and *P. serrifer* (12, 15–17).

   - Susceptible stages of the host: *P. monodon* are susceptible to YHV infection beyond PL15 (10). Experimental infections with GAV indicate that larger (~20 g) *M. japonicus* are less susceptible to disease than smaller (~6–13 g) shrimp of the same species (23).
• Species or sub-population predilection (probability of detection): amongst susceptible species of shrimp, viruses in the yellowhead complex (genotypes 2–6) are only known to occur commonly (prevalence up to 100%) in healthy *P. monodon*, which appears to be the natural host (29, 32). However, YHV (genotype 1) infections and are usually detected only in the event of disease and do not occur commonly in healthy *P. monodon*. Natural YHV infections have been detected in *M. japonicus*, *Fe. merguiensis*, *L. setiferus*, *M. ensis*, *P. styliferus* and *E. superba* (7, 8).

• Target organs and infected tissue: YHV targets tissues of ectodermal and mesodermal origin including lymphoid organ, haemocytes, haematopoietic tissue, gill lamellae and spongy connective tissue of the subcutis, gut, anterior gland, gonads, nerve tracts and ganglia (3, 11).

• Persistent infection with lifelong carriers: GAV persists as a chronic infection in surviving *P. esculentus* for at least 50 days following experimental challenge (24). The high prevalence of infection of GAV and other yellowhead complex viruses (genotypes 2–6) in all life stages of healthy *P. monodon* suggests that lifelong chronic infections occur commonly (29, 32). There is also evidence of persistence of YHV (genotype 1) in survivors of experimental infection (T.W. Flegel, pers. comm.).

• Vectors: there are no known vectors of YHV.

c) Disease pattern

• Transmission mechanisms: YHV can be transmitted horizontally by injection, ingestion of infected tissue, immersion in membrane-filtered tissue extracts, or by co-habitation with infected shrimp (8, 11). Transmission has also been demonstrated by injection of extracts of paste prawns (*Acetes* sp.) collected from infected ponds (7). For GAV, vertical transmission has been shown to occur from both male and female parents, probably by surface contamination or infection of tissue surrounding the fertilised egg (5).

• Prevalence: the overall prevalence of yellowhead complex viruses in healthy *P. monodon* (as detected by nested polymerase chain reaction [PCR]) is very high (50–100%) in most sampled farmed and wild populations in Australia, Asia, and East Africa. The prevalence of individual genotypes varies according to the geographic origin of the shrimp. The prevalence of YHV (genotype 1) may be low (>1%) in healthy wild or farmed *P. monodon*, but would be very high (approaching 100%) in disease outbreak ponds. By other less sensitive detection methods (e.g. histology, immunoblot, dot-blot, in-situ hybridisation), the prevalence of infection in healthy shrimp would be lower.

• Geographical distribution: YHD has been reported in China (People's Rep. of), India, Indonesia, Malaysia, the Philippines, Sri Lanka Taipei China, Thailand, and Vietnam (1, 11, 18, 29, 30, 31). GAV and other genotypes in the yellowhead complex have been detected in healthy *P. monodon* from Australia, India, Indonesia, Malaysia, Mozambique, the Philippines, Taipei China, Thailand, and Vietnam (29, 32).

• Mortality and morbidity: YHD can cause up to 100% mortality in infected *P. monodon* ponds within 3 days of the first appearance of clinical signs. GAV has been associated with mortalities up to 80% in *P. monodon* ponds in Australia.

• Economic and/or production impact of the disease: The economic impact of YHV in Thailand in 1996 has been estimated to be USD 30 million in 1992 and USD 40 million in 1993. Although this represents only 3–4% of the gross value of production, total crop loss during grow-out has a very significant impact on individual farmers.

d) Control and prevention

• Vaccination: no consistently effective vaccination methods have been developed.

• Chemotherapy: no scientifically confirmed reports.

• Immunostimulation: no scientifically confirmed reports.
Chapter 2.3.3. - Yellowhead disease

- Resistance breeding: not reported.
- Restocking with resistant species: not reported.
- Blocking agents: not reported.
- General husbandry practices: specific pathogen free (SPF) or PCR-negative seed stocks and biosecure water and culture systems may be used to reduce the risk of disease.

3. DIAGNOSTIC METHODS

a) Field diagnostic methods

- Clinical signs: YHV can infect cultured shrimp from late postlarval stages onwards, but mass mortality usually occurs in early to late juvenile stages. Exceptionally high feeding activity followed by an abrupt cessation of feeding may occur within 2–4 days of the appearance of gross clinical signs of disease and mortality (3, 13). Moribund shrimp may congregate at pond edges near the surface. Moribund shrimp may exhibit a bleached overall appearance and a yellowish discoloration of the cephalothorax caused by the underlying yellow hepatopancreas, which may be exceptionally soft when compared with the brown hepatopancreas of normal shrimp. In many cases, total crop loss occurs within a few days of the first appearance of shrimp showing gross signs of YHD. While cessation of feeding, congregate at pond edges and a generally bleached appearance are always seen in YHD outbreaks, these disease features are not particularly distinctive for YHD. The other more pathognomonic gross signs are not always seen and thus they are not reliable, even for preliminary diagnosis of YHD.

Gross signs of GAV disease include swimming near the surface and at the pond edges, cessation of feeding, a reddening of body and appendages, and pink to yellow coloration of the gills (22). However, these signs occur commonly in diseased shrimp are not considered pathognomonic for GAV disease.

b) Clinical methods

- Gross pathology: see Section 3.a above.
- Clinical chemistry: none described.
- Microscopic examination

Moribund shrimp collected from pond edges during a disease outbreak are the preferred source of material for examination. Apparently normal shrimp should also be collected from the same ponds.

- Wet mounts: fix whole shrimp or gill filaments in Davidson’s fixative (11) overnight. After fixation, wash some gill filaments thoroughly with tap water to remove the fixative, and stain with Meyer’s haematoxylin and eosin (H&E) (11). After staining and dehydration, when the tissue is in xylene, place a gill filament on a microscope slide in a drop of xylene and, using a fine pair of needles (a stereo microscope is helpful), break off several secondary filaments. Replace the main filament in xylene where it can be stored indefinitely as a permanent reference in a sealed vial. Being careful not to let the xylene dry, tease apart the secondary filaments on a microscope slide and remove any large fragments or particles that would thicken the mount unnecessarily. Finally, add a drop of mounting fluid and a cover-slip. Use light pressure to flatten the mount as much as possible. This procedure may also be used with thin layers of subcuticular tissue. Examine under a light microscope using a ×40 objective lens. For YHD outbreak samples, moderate to large numbers of deeply basophilic, evenly stained, spherical, cytoplasmic inclusions (approximately 2 µm in diameter or smaller) will be observed (6). This evidence should be used together with the results from haemolymph smears (see below) in making a presumptive diagnosis of a YHD outbreak. As for the fixed tissues and the filaments in xylene, these whole-mount slides can be kept as a permanent record.
If rapid results are required, the fixation step can be shortened to only 2 hours by changing the acetic acid portion of the Davidson’s fixative formula to 50% concentrated HCl. For best results, this fixative should not be stored for more than a few days before use. After fixation, wash thoroughly to remove the fixative and check that the pH has returned to near neutral before staining. Do not fix for longer periods or above 25°C as this may result in excessive tissue damage that will make interpretation difficult or impossible.

- Smears: for moribund shrimp from YHD outbreaks, haemolymph smears are not useful because haemocytes are usually depleted in advanced stages of the disease. Haemolymph samples should be collected from grossly normal shrimp from a suspect pond where moribund shrimp have also been collected. Draw the haemolymph into a syringe containing two volumes of either 25% formalin or Davidson’s fixative in which the acetic acid of the formula has been replaced by either water or formalin. Mix thoroughly, ignore clots in the syringe, place a drop on a microscope slide, smear and then air-dry before staining with H&E or other standard blood smear stains. Dehydrate, add mounting fluid and a cover-slip. Examine under a light microscope using a ×40 objective lens. For YHD outbreak samples, some of the smears will show moderate to large numbers of haemocytes with karyorhectic or pycnotic nuclei (19). It is important that the slides with these nuclei show no evidence of concomitant bacterial infection, as bacterial infections may cause similar changes in haemocytes. When making a presumptive diagnosis of a YHD outbreak, the results from haemolymph smears should be used together with the results from rapidly stained whole mounts (see above) or stained tissue sections.

- Fixed sections: fix moribund shrimp from a suspected YHV outbreak in Davidson’s fixative and process for preparation of standard H&E-stained tissue sections (2, 11). Examine the sections by light microscopy for the presence of moderate to large numbers of deeply basophilic, evenly stained, spherical, cytoplasmic inclusions approximately 2 µm in diameter or smaller in tissues of ectodermal and mesodermal origin (3). Tissues of the lymphoid organ, stomach subcuticulum and gills are particularly useful.

Lymphoid organ spheroids are commonly observed in healthy P. monodon chronically infected with GAV and lymphoid organ necrosis often accompanies GAV disease (22, 25). However, spheroid formation and degeneration of lymphoid organ tissue also occur during infection with other shrimp viruses (11, 21).

- Electron microscopy: not applicable

**c) Agent detection and identification methods**

- **Direct detection methods**

  - **Microscopic methods**

    - Transmission electron microscopy (TEM): for TEM, the most suitable tissues of moribund shrimp suspected of YHV infection are those of the lymphoid organ and gills. For screening or surveillance of grossly normal shrimp, the most suitable tissue is from the lymphoid organ.

    Stun live shrimp by immersion in iced water until just immobilised or kill by injection of fixative. Quickly dissect and remove small portions of target tissue (no larger than a few mm in diameter) and fix in at least 10 volumes of 6% glutaraldehyde held at 4°C and buffered with sodium cacodylate (Na[CH3]2AsO2·3H2O) solution (8.6 g Na cacodylate, 10 g NaCl, distilled water to make 100 ml, adjusted to pH 7 with 0.2 N HCl) or phosphate solution (0.6 g NaH2PO4·H2O, 1.5 g Na2HPO4, 1 g NaCl, 0.5 g sucrose, distilled water to make 100 ml, adjusted to pH 7 with 0.2 N HCl). Fix for at least 24 hours prior to processing. For long-term storage in fixative at 4°C, reduce glutaraldehyde to 0.5–1.0%. Processing involves post-fixation with 1% osmium tetroxide, dehydration, embedding, sectioning and staining with uranyl acetate and lead
citrate according to standard TEM methods. The reagents used for this procedure have been described elsewhere (11).

In YHV infected cells, both nucleocapsid precursors and complete enveloped virions are observed. Nucleocapsid precursors are long filaments approximately 15 nm in diameter and of variable length (80–450 nm) that occur in the cytoplasm, sometimes densely packed in paracrystalline arrays. Virions are rod-shaped, enveloped particles (40–60 nm × 150–200 nm) with rounded ends and prominent projections (8–11 nm) extending from the surface. Virions are commonly seen in the cytoplasm of infected cells and in association with intracellular vesicles. Virions may also be seen budding at the cytoplasmic membrane and in interstitial spaces. GAV virions and nucleocapsids are indistinguishable from YHV by TEM.

ii) Agent isolation and identification

- **Cell culture/artificial media:** although primary shrimp cell culture methods are available, they are not recommended for YHV isolation/identification because of the high risk of contamination with adventitious agents.

- **Bioassay:** the bioassay procedure is based on that described by Spann et al. (22) but similar procedures have been described by several other authors (16, 20, 30). Bioassay should be conducted in susceptible shrimp (see Section 2.b above) that have been certified as specific pathogen free and have been obtained from a biosecure breeding facility. Alternatively, susceptible wild or farmed shrimp to be used for bioassay should be screened by nested reverse-transcription (RT) PCR on haemolymph samples to confirm the absence of pre-existing chronic infections with YHV, GAV or related viruses. Shrimp should be maintained throughout the procedure under optimal conditions for survival of the species in laboratory culture.

Collect moribund shrimp from a disease outbreak or shrimp suspected of being carriers of infection and maintain at 4°C or on ice. Remove and discard the tail and appendages. If necessary, the whole shrimp or the retained cephalothorax may be snap-frozen and stored at −80°C or in liquid nitrogen until required. Thaw stored samples rapidly in a 37°C water bath within two snap-seal plastic bags and then maintain at 4°C or on ice during all procedures. Remove the carapace and calciferous mouth-parts. Suspend the remaining tissues in six volumes of TN buffer (0.02 M Tris/HCl, pH 7.4, 0.4 M NaCl) and homogenise in a tissue grinder to form a smooth suspension. Clarify the homogenate at 1300 g for 20 minutes at 4°C. Remove the supernatant fluid below the lipid layer and pass through a 0.45 µm filter. Maintain the filtrate at 4°C for immediate use or snap-freeze and store in aliquots at −80°C or in liquid nitrogen. Thaw the filtrate rapidly at 37°C and maintain on ice prior to use.

Inject at least twelve juvenile (1–5 g) shrimp of a known susceptible species (*P. monodon*, *P. esculentus*, *M. japonicus*, *F. merguiensis*, *L. vannamei*, *L. stylirostris*), with 5 µl of filtrate per gram body weight into the second abdominal segment using a 26-gauge needle. Inject two equivalent groups of at least 12 shrimp with TN buffer and a filtered tissue extract prepared from uninfected shrimp. One additional group of at least 12 shrimp should be injected last with a known and calibrated positive control inoculum from shrimp infected with YHV or GAV (as required). Maintain each group of shrimp in a separate covered tank with a separate water supply for the duration of the bioassay. Ensure no inadvertent transfer of water between tanks by good laboratory practice. Observe the shrimp and record mortalities for at least 21 days or until the test and positive control groups reach 100% mortality. Collect at least one moribund shrimp from each of the four groups for examination by histology, TEM, in-situ nucleic acid hybridisation, and PCR or Western-blot analysis to confirm the presence of YHV or GAV (as required) in the sample (refer to the Sections below for test procedures).
Note that shrimp to be tested that are suspected of being carriers of low level chronic infections may produce an inoculum containing a very low dose of virus. In bioassay, such an inoculum may not necessarily cause mortalities, gross signs of disease or histology characteristic of a lethal infection. In this event, molecular tests or TEM must be applied to the bioassay shrimp.

- **Antibody-based antigen detection methods:**

  **Immunoblot detection:**

  Prepare reagents and carry out assays according to the protocols of Lu et al. (16) and Loh et al. (14). This includes purification of YHV virions from laboratory-infected shrimp, generation of immunoglobulins (Igs) in New Zealand white rabbits, purification of the IgG using recombinant bacterial protein-G columns and removal of cross-reacting normal shrimp antigens by adsorption on to acetone-dried, ground shrimp muscle tissue and haemolymph. For assay, remove 0.1 ml of haemolymph from live shrimp specimens and dilute in an equal volume of citrate buffer for immediate use or storage at –80°C until used. For Western blotting, use 200 µl sample, clarify at 8000 g for 5 minutes and then pellet the supernatant at 140,000 g for 5 minutes. Resuspend pellets in 100 µl 2 × loading buffer (2.5 ml 0.5 mM Tris/HCl, pH 6.8, 4 ml 10% sodium dodecyl sulfate [SDS], 2 ml glycerol, 1 µl beta-mercaptoethanol, and 0.5 ml deionised distilled water) and heat at 95°C for 5 minutes. Load a 10 µl subsample on to 5% SDS/polyacrylamide gel, and conduct electrophoresis at 200 V. Blot the gel on to a nitrocellulose membrane (pore size, 0.1 mm) in blotting buffer (3.03 g Tris base, 14.4 g glycine, and 200 ml mehanol per litre) at 100 V for 1 hour. Rinse the membrane with phosphate buffered saline (PBS), pH 7.4, soak in 5% skim milk (in PBS) for 1 hour, and rinse with PBS for 5 minutes. Next, treat the membrane with a 1/1000 dilution of the primary antibody (IgG) for 1 hour, rinse three times with PBS for 5 minutes, and then treat with a 1/2500 dilution of goat anti-rabbit IgG-horseradish-peroxidase conjugate for 1 hour. Rinse again three times with PBS for 5 minutes and then treat with the substrate, 3,3',5,5'-tetramethylbenzidine, until a bluish/purple colour develops. Stop the reaction by soaking the membrane in distilled water. All incubations should be carried out at 25°C ± 2°C. Use a purified viral preparation as a positive control and identify 2–4 major protein bands characteristic of YHV at 116, 64 and 20 kDa. The sensitivity is 0.4 ng of YHV protein (≈ 10⁶ YHV virions).

- **Molecular techniques:**

  **Reverse-transcription polymerase chain reaction (RT-PCR):**

  Three RT-PCR methods are described. The first protocol is a 1-step RT-PCR adapted from Wongteerasupaya et al. (33) that can be used for confirmation of YHV in shrimp collected from suspected YHD outbreaks. This protocol will detect only YHV and not GAV or other genotypes. The second protocol is a more sensitive multiplex RT-nested PCR procedure adapted from Cowley et al. (4). It can be used for differential detection of YHV and GAV in disease outbreak shrimp or for screening of healthy carriers. This test will not detect all known genotypes in the yellowhead complex, and genotype 3 may react as GAV. The test is available in a suitably modified form from a commercial source (Farming Intelligen Technology Corporation, Taipei China). OIE requires a formal process of validation and certification commercial tests. A list of certified test kits and manufacturers is available at the OIE website. The third protocol is a sensitive multiplex RT-nested PCR procedure provided by Wijegoonawardane, Cowley & Walker (unpublished). This test can be used for screening healthy shrimp for viruses in the yellowhead complex. It will detect all six currently known genotypes (including YHV and GAV) but will not discriminate between genotypes. Assignment of genotype can be achieved by nucleotide sequence analysis of the RT-PCR product.
Sample preparation:

For juvenile or adult shrimp, lymphoid organ, gill tissue or haemolymph may be used to prepare total RNA. Fresh tissue is preferred. Lymphoid organ and gill tissue preserved in 95% analytical-grade ethanol or RNA later (various manufacturers), or stored frozen at −70°C are also suitable for total RNA preparation. Disrupt 10–20 mg lymphoid organ or gill tissue or 50 µl haemolymph in 500 µl Trizol™ reagent (Invitrogen, Carlsbad, California, USA) and extract total RNA according to the product manual. Resuspend RNA in 25 µl DEPC (diethylpyrocarbonate)-treated water, heat at 55°C for 10 minutes, cool on ice and use immediately or store at −70°C until required. Ideally, a 1/200 dilution (i.e. 2.5 µl RNA in 500 µl DEPC-treated water) should be prepared, and absorbances A_{260} nm and A_{280} nm (UV spectrophotometer required) should be determined to quantify and check the quality of the RNA. RNA yield varies with the type and freshness of tissues as well as the quality of the preservative used and the length of preservation. However, approximate RNA yield from fresh tissues varies from 0.2 to 2.0 µg/µl and alcohol-preserved tissues yield approximately 0.1–1 µg/µl.

From a nursery or hatchery tank containing 100,000 postlarvae (PL) or more, sample approximately 1000 PL from each of five different points. Pool the samples in a basin, gently swirl the water in the basin and then select an assay sample from live PL that collect at the centre of the basin. Choose the sample number according to the assumed or target prevalence. Homogenise the sample in an appropriate volume of Trizol™ reagent and extract RNA according to the product manual.

For each set of RNA samples to be tested, DEPC-treated water and extracts known to contain YHV RNA and/or GAV RNA (as appropriate to the test) should be included as negative and positive controls, respectively.

Protocol 1: RT-PCR for specific detection of YHV in diseased shrimp

Mix 2 µl RNA in 20 µl of PCR buffer (10 mM Tris/HCl, pH 8.3, 50 mM KCl) containing 2.5 U of M-MLV (Moloney murine leukaemia virus) reverse transcriptase, 1.0 U of ribonuclease inhibitor, 0.75 µM of antisense primer (144R, below), 1 mM each of dATP, dTTP, dCTP, and dGTP, and 5 mM of MgCl₂, and incubate at 42°C for 15 minutes to synthesise cDNA. Next, incubate the mixture at 100°C for 5 minutes to inactivate the reverse transcriptase and allow the mixture to cool to 5°C. Add the PCR mixture (10 mM Tris/HCl, pH 8.3, 50 mM KCl) containing 2.5 U of Taq DNA polymerase (Perkin Elmer Cetus), 2 mM MgCl₂ and 0.75 µM of sense primer (10F, below) to give a final volume of 100 µl. Overlay the tubes with 100 µl of mineral oil and conduct PCR amplification for 40 cycles at 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, and finishing at 72°C for 10 minutes. Apply 20µl of the amplified PCR product to a 2% agarose/TAE (Tris-acetate-EDTA [ethylene diamine tetra-acetic acid]) gels containing 0.5 µg/ml ethidium bromide alongside a suitable DNA ladder and detect using a UV transilluminator.

A positive reaction will be indicated by a 135 bp product. The sensitivity of the assay is approximately 0.01 pg of purified YHV RNA (≈ 10³ genomes).

PCR primers sequences:

10F: 5’-CCG-CTA-ATT-TCA-AAA-ACT-ACG-3’
144R: 5’-AAG-GTG-TTA-TGT-CGA-GGA-AGT-3’

Protocol 2: RT-nested PCR for differential detection of YHV and GAV in healthy or diseased shrimp

For cDNA synthesis, 2 µl of RNA (ideally 1.0 µg total RNA, if quantified) and 0.7 µl of 50 pmol/µl primer GY5 to a total to 6 µl in DEPC-treated water, incubate at 70°C for 10 minutes and chill on ice. Add 2 µl Superscript II buffer × 5 (250 mM Tris/HCl,
pH 8.3, 375 mM KCl, 15 mM MgCl₂, 1 µl 100 mM DTT and 0.5 µl 10 mM dNTP stock mixture (i.e. 10 mM dATP, 10 mM dTTP, 10 mM dCTP, 10 mM dGTP) and mix gently. Preheat to 42°C for 2 minutes, add 0.5 µl of 200 U/µl Superscript II reverse transcriptase (Gibco-BRL) and incubate at 42°C for 1 hour. Then heat the reaction at 70°C for 10 minutes, chill on ice and spin briefly in a microcentrifuge to collect the contents of the tube. For the first PCR step, prepare a 50 µl reaction mixture containing 1 × Taq buffer (10 mM Tris/HCl, pH 8.3, 50 mM KCl, 0.1% Triton X-100), 1.5 mM MgCl₂, 35 pmol each primer GY1 and GY4, 200 µM each of dATP, dTTP, dCTP and dGTP and 2.5 U Taq polymerase (Promega) in a 0.5 ml thin-walled tube. Overlay the reaction mixture with 50 µl liquid paraffin, heat at 85°C for 2–3 minutes and then add 1 µl cDNA. Conduct PCR amplification using 35 cycles at 95°C for 30 seconds, 66°C for 30 seconds, and 72°C for 45 seconds, followed by final extension at 72°C for 7 minutes. For the second PCR step, prepare a 50 µl reaction mixture containing 2 µl of the first step PCR product, 1 × Taq buffer (above), 1.5 mM MgCl₂, 35 pmol each primer GY2, Y3 and G6, 200 µM each of dATP, dTTP, dCTP and dGTP and 2.5 U Taq polymerase (Promega) in a 0.5 ml thin-walled tube overlaid with liquid paraffin. Conduct PCR using amplification conditions as described above. Apply 10 µl of the amplified PCR product to 2% agarose/TAE gels containing 0.5 µg/ml ethidium bromide alongside a suitable DNA ladder and detect using a UV transilluminator.

If the viral load is sufficiently high, a 794 bp DNA will be amplified from either GAV or YHV in the first PCR step. In the second PCR step, a 277 bp product indicates detection of YHV and a 406 bp product indicates detection of GAV. The presence of both 406 bp and 277 bp products indicates a dual infection with GAV and YHV. The detection sensitivity of the second-step PCR is ~1000-fold greater than the first-step PCR and GAV or YHV RNA can be detected to a limit of 10 fg lymphoid organ total RNA.

The sequences of RT-PCR primers generic for GAV and YHV (GY) or specific for GAV (G) or YHV (Y) are as follows:

GY1: 5’-GAC-ATC-ACT-CCA-GAC-AAC-ATC-TG-3’
GY2: 5’-CAT-CTG-TCC-AGA-AGG-CGT-CTA-TGA-3’
GY4: 5’-GTG-AAG-TCC-ATG-TGT-GTG-AGA-CG-3’
GY5: 5’-GAG-CTG-GAA-TTC-AGT-GAG-AGA-ACA-3’
Y3: 5’-ACG-CTC-TGT-GAC-AAG-CAT-GAA-GTT-3’
G6: 5’-GTA-GTA-GAG-ACG-AGT-GAC-ACC-TAT-3’

Protocol 3: RT-nested PCR for detection of all currently known genotypes in the yellowhead complex (including YHV and GAV)

For cDNA synthesis, mix 2 µl of RNA (ideally 1.0 µg total RNA, if quantified), 50 ng random hexamer primers and 1.0 µl 10 mM dNTP and make up to a total volume of 14 µl in sterile DEPC-treated water, incubate at 65°C for 5 minutes and chill on ice. Add 4.0 µl Superscript III buffer × 5 (Invitrogen), 1.0 µl 100 mM DTT, 1.0 µl 40 U/µl RNaseOUT™ (Invitrogen) and 1.0 µl 200 U/µl Superscript III reverse transcriptase (Invitrogen) and mix gently. Incubate at 25°C for 5 minutes and then at 42°C for 55 minutes, stop the reaction by heating at 70°C for 15 minutes, chill on ice and spin briefly in a microcentrifuge to collect the contents of the tube. For the first PCR step, add 1 µl cDNA to a total 25 µl reaction mixture containing 1 × Taq buffer (10 mM Tris/HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 1.5 µl 25 mM MgCl₂, 0.35 µl primer mix containing 25 pmol/µl of each primer pool (see below) YC-F1ab and YC-R1ab, 0.5 µl 10 mM dNTP mix and 0.25 µl 5 U/µl Taq DNA polymerase (Fisher Biotech). Conduct PCR amplification using denaturation at 95°C for 1 minute followed by 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 40 seconds, followed by a final extension at 72°C for 7 minute. For the second PCR step, use 1 µl of the first PCR product in the reaction mixture as prepared above but substituting primer
pools YC-F2ab and YC-R2ab. Conduct PCR amplification using denaturation at 95°C for 1 minute followed by 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, followed by a final extension at 72°C for 7 minute. Apply 8 µl of the amplified PCR product to 2% agarose/TAE gels containing 0.5 µg/ml ethidium bromide alongside a suitable DNA ladder and detect using a UV transilluminator.

If the viral load is sufficiently high, a 358 bp DNA is amplified in the first PCR step. The second (nested) PCR step amplifies a 146 bp product. The detection of these products indicates detection of one of the six genotypes in the yellowhead complex. Further assignment of genotype (if required) is possible by nucleotide sequence analysis of either PCR product followed by comparison with sequences of the known genotypes by multiple sequence alignment and phylogenetic analysis. The detection sensitivity limits of the first step PCR and nested PCR are 2,500 and 2.5 RNA templates, respectively.

PCR primer sequences (each primer comprises a pool of equal quantities of two related oligonucleotide sequences):

YC-F1ab pool:  5'-ATC-GTC-GTC-AGC-TAC-CGC-AAT-ACT-GC-3'
5'-ATC-GTC-GTC-AY-TAY-CGT-AAC-ACC-GC-3'

YC-R1ab pool: 5'-TCT-TCR-CGT-GTG-AAC-ACY-TTC-TTR-GC-3'
5'-TCT-GCG-TGG-GTG-AAC-ACC-TTC-TTG-GC-3'

YC-F2ab pool:  5'-CGC-TTC-CAA-TGT-ATC-TGY-ATG-CAC-CA-3'
5'-CGC-TTY-CAR-TGT-ATC-TGC-ATG-CAC-CA-3'

YC-R2ab pool: 5'-RTC-DGT-GTA-CAT-GTT-TGA-GAG-TTT-GTT-3'
5'-GTC-AGT-GTA-CAT-ATT-GGA-GAG-TTT-RTT-3'

Mixed base codes:  R(AG), Y(CT), M(AC), K(GT), S(GC), W(AT), H(ACT), B(GCT), V(AGC), D(AGT), N(AGCT).

In situ hybridisation

The protocol of Tang & Lightner (26) is described here. The method is suitable for detection of YHV or GAV (27). To preserve the viral RNA, fix live shrimp with neutral-buffered, modified Davidson’s fixative without acetic acid (RF-fixative) (9). Process the fixed shrimp using standard histological methods and prepare 4 µm thick sections on Superfrost Plus slides (Fisher Scientific, Pennsylvania, USA). Prior to hybridisation, incubate sections at 65°C for 45 minutes, remove paraffin with Hemo de (Fisher Scientific, Pennsylvania, USA), and rehydrate through an ethanol series to water. Digest sections with proteinase K (100 µg/ml, in 50 mM Tris/HCl, pH 7.4, 10 mM NaCl, 1 mM EDTA) for 15 minutes at 37°C, followed by post-fixation in formaldehyde (0.4%) for 5 minutes. Rinse in 2 × SSC (standard saline citrate), then prehybridise with 500 µl prehybridisation solution (4 × SSC, 50% formamide, 1 × Denhardt’s, 0.25 mg/ml yeast RNA, 0.5 mg/ml sheared salmon sperm DNA, 5% dextran sulfate) at 42°C for 30 minutes. For hybridisation, overlay the sections with 250 µl hybridisation solution containing a digoxigenin-labelled probe (20–40 ng/ml) at 42°C overnight. The next day, wash the sections as follows: 2 × SSC once for 30 minutes at room temperature; 1 × SSC twice for 5 minutes at 37°C; 0.5 × SSC twice for 5 minutes at 37°C. Incubate the sections with sheep anti-digoxigenin-alkaline phosphatase conjugate (Roche) at 37°C for 30 minutes. Wash with 0.1 M Tris/HCl, pH 7.5, 0.15 M NaCl twice for 10 minutes at room temperature and rinse with 0.1 M Tris/HCl, pH 9.5, 0.1 M NaCl. Incubate with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate in the dark for 1–2 hours for colour development. Counterstain with Bismarck Brown Y (0.5%), dehydrate through a series of ethanol and Hemo de, add Permount (Fisher Scientific, Pennsylvania, USA) and cover with a cover-slip. YHV-infected cells give a blue to purple-black colour against the brown counter-stain. Include positive controls of
YHV-infected tissue and negative controls of uninfected shrimp tissue. The diagnostic probe can be prepared by PCR labelling using the following primers:

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

4. RATING OF TESTS AGAINST PURPOSE OF USE

The methods currently available for surveillance, detection, and diagnosis of YHV are listed in Table 1. The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic sensitivity and specificity; C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and D = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation (see Chapter 1.1.2), their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 1. YHV surveillance, detection and diagnostic methods

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<th>Method</th>
<th>Surveillance</th>
<th>Presumptive</th>
<th>Confirmatory</th>
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<tr>
<td></td>
<td>Larvae</td>
<td>PLs</td>
<td>Juveniles</td>
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<td>Gross signs</td>
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<td>D</td>
<td>C</td>
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<tr>
<td>Histopathology</td>
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<td>D</td>
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<td>Transmission EM</td>
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<tr>
<td>Bioassay</td>
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<tr>
<td>Sequence</td>
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</table>

PLs = postlarvae; LM = light microscopy; EM = electron microscopy; PCR = polymerase chain reaction.

5. CORROBORATIVE DIAGNOSTIC CRITERIA

a) Definition of suspect case

Yellowhead disease is primarily a disease of farmed Penaeus monodon caused by yellowhead virus. Yellowhead disease usually affects early to late juvenile stages and may appear as a cessation of feeding and congregation of shrimp at pond edges. Moribund shrimp may exhibit a bleached overall appearance and a yellowish discoloration of the cephalothorax caused by the underlying yellow hepatopancreas. Mortality can reach up to 100%, but subclinical infections also occur. Corroborative diagnostic criteria are summarised in Section 3 of this chapter.

b) Definition of confirmed case

Yellowhead disease may be confirmed by the detection of high levels of disseminated infection in tissues of ectodermal and mesodermal origin by in situ hybridisation in conjunction with the detection of amplified products of the prescribed size using discriminatory RT-PCR assays as
described in Section 3 of this chapter. As low-level chronic infections with yellowhead complex viruses are common in some regions, detection of the presence of virus is not, in itself, evidence of aetiology.

6. DIAGNOSTIC/DETECTION METHODS TO DECLARE FREEDOM

Two-step PCR and sequencing are the prescribed methods for declaring freedom. Two-step PCR negative results are required. Where a two-step PCR positive result cannot be confirmed by sequencing, this also counts as a negative result.

REFERENCES


* * *

**NB:** There is an OIE Reference Laboratory for Yellowhead disease (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: [www.oie.int](http://www.oie.int)).
CHAPTER 2.3.4.

TETRAHEDRAL BACULOVIRILOSIS
(Baculovirus penaei)

1. CASE DEFINITION

For the purpose of this chapter, tetrahedral baculovirosis is considered to be infection with Baculovirus penaei. Synonyms: PvSNPV (singly enveloped nucleopolyhedrovirus from Litopenaeus vannamei).

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

a) Agent factors

• Aetiological agent: Baculovirus penaei (BP) as described by Couch (14–17), Summers (40), Overstreet (34) and Bonami et al. (3).
• The International Committee on Virus Taxonomy lists the related virus MBV (spherical baculovirosis) as a tentative species in the genus Nucleopolyherdovirus (19). Therefore, BP should also be considered as a tentative species in this genus.
• Agent strains: at least three geographical strains have been demonstrated. These are: 1) southeast Atlantic and Gulf of Mexico coasts of the USA and the Caribbean; 2) Pacific Coast of South, Central and North America; and 3) Hawaii (7, 10, 18, 29).

b) Host factors

• Susceptible species: BP infections have been reported in one or more species of the following penaeid genera: Litopenaeus, Farfantepenaeus, Fenneropenaeus, Melicertus, Penaenaeus, Trachypenaeus and Protrachypene (11, 18, 24, 27, 31–34). All penaeid species may be potential hosts (27, 28, 34).
• Susceptible stages of the host: all life stages, except eggs and nauplii, are susceptible to infection by BP.
• Target organs: BP is strictly enteric infecting mucosal epithelial cells of the hepatopancreas tubules and the anterior midgut (5, 14, 16, 17, 21, 27).
• Persistent infection and lifelong carriers: persistent infection occurs commonly in penaeid hosts of BP. Wild adult L. vannamei females that are heavily infected with BP have been shown to excrete BP-contaminated faeces when spawning, thereby contaminating the eggs and passing the virus to the next generation (21, 27).
• Vectors: none is known in natural infections, but the rotifer Brachionus plicatilis and Artemia salina nauplii were used as passive carriers of BP to deliver the virus to larval stages of L. vannamei in experimental infections (35, 39).

c) Disease pattern

• Transmission mechanisms: transmission of BP is horizontal by ingestion of infected tissue (cannibalism), faeces, occlusion bodies, or contaminated detritus or water (21, 27, 35).
• Prevalence: highly variable, from <1% in wild and cultured populations up to 100% in cultured populations in larval rearing tanks and nursery ponds (5, 27).
**Geographical distribution:** BP is enzootic in wild penaeids in the Americas and Hawaii. It has not been reported in wild or cultured penaeid shrimp in the eastern hemisphere despite numerous introductions of American penaeids to Asia and the Indo-Pacific (4, 7, 27).

**Mortality and morbidity:** the larval stages (specifically protozoa and mysis) and early postlarval (PL) stages are the most easily infected in laboratory challenge studies (8, 20, 22, 35, 37–39) and are the stages where the highest mortalities are likely to occur in penaeid shrimp hatcheries. High mortality rates are unusual as a consequence of BP infection in the juvenile or adult stages, but infection may cause poor growth and reduced survival in nursery or grow-out ponds at shrimp farms (6, 27, 34).

**Economic and/or production impact of the disease:** BP has caused serious disease with high mortality rates in hatcheries and significant production losses in farms in the Americas. While not usually causing high mortality rates in infected juvenile or adult stages, BP has been documented to cause reduced growth and lower harvest production in populations that are persistently infected with the virus (6, 27, 34).

d) **Control and prevention**

- Vaccination: no effective vaccination methods for BP have been developed.
- Chemotherapy: no scientifically confirmed reports.
- Immunostimulation: no scientifically confirmed reports.
- Resistance breeding: the potential for selective breeding for BP resistance has been demonstrated (1).
- Restocking with resistant species: not applicable to BP.
- Blocking agents: not reported.
- Disinfectants: Inactivation of BP by disinfectants, low pH, heat and UV irradiation has been reported (23).
- General husbandry practices:
  - **Hatchery:** a number of husbandry practices have been applied for the prevention of BP infections and disease. Prescreening of broodstock for BP has been somewhat effective in detecting heavily infected carriers of the virus and thereby reducing the transmission of the disease from parent to offspring. With nonlethal testing methods, this is accomplished by simple light microscopic examination of faecal strands (or by polymerase chain reaction [PCR] testing of faecal strands if PCR testing facilities are readily available). Alternatively, spent broodstock may be killed after spawning and simple light microscopic examination of an hepatopancreas squash can be run (or the excised hepatopancreas may be tested by PCR) to determine the spawner’s BP infection status. Because BP is transmitted from adults to their offspring by faecal contamination of the spawned eggs, prevention of infection in hatcheries may be achieved by taking additional steps to eliminate faecal contamination of spawned eggs and larvae by thoroughly washing nauplii or eggs with formalin, iodophores, and clean sea water (12). The routine disinfection of spawned eggs from infected or potentially infected broodstock has reduced the incidence of BP epizootics in hatcheries (30).
  - **Nursery and grow-out ponds:** BP infections remain common in earthen-bottom ponds in regions of the Americas where the virus is enzootic (4, 13, 25), but incidence and prevalence of BP infections may be reduced in lined nursery and grow-out ponds.

3. **Diagnostic methods**

a) **Field diagnostic methods**

- Gross signs: Protozoa, mysis and early PL stages with severe BP infections may present a whitish midgut (due to the presence of occlusion bodies and cell debris in the faecal material).
Juveniles and adults present no gross signs of diagnostic value, nor do larvae with less severe infections.

b) Clinical methods

- Gross pathology: see Section 3.a above.

- Direct microscopic examination
  
  - *Wet mounts of fresh tissue*: diagnosis of BP infections is made by the demonstration of single or multiple tetrahedral occlusion bodies in epithelial cell nuclei in squash preparations of hepatopancreas or midgut examined by phase-contrast or bright-field microscopy. Occlusion bodies are tetrahedral or pyramidal in three-dimensional form, and range in size from less than 0.1 µm to nearly 20 µm from pyramidal base to peak, with a modal, vertical length of 8 µm. In some publications, the occlusion bodies of BP are referred to as PIBs (polyhedral inclusion bodies) (4, 6, 27).

  - *Wet mounts of faecal strands*: this nonlethal method may be used to screen for carriers of BP. The method can be applied to juvenile or older shrimp, and it is perhaps most useful as a nonlethal method for screening valuable broodstock. Faecal samples from shrimp to be tested may be obtained by placing the shrimp in an aquarium, spawning tank, or other suitable tanks for a few hours until faecal strands are present on the tank bottom. The faecal strands are best collected using a clear plastic siphon hose (an air line fitted with a section of plastic pipette as a tip is ideal) and placed in a beaker, cup, or suitable container. The faecal strands may be made into wet mounts and examined directly for occlusion bodies. BP occlusion bodies are prominent, refractive tetrahedrons that range from just resolvable to nearly 20 µm in height (4, 6, 27).

  - Collected faeces may also be used as the sample for nonlethal testing for BP by PCR. PCR will provide greater diagnostic sensitivity for low-grade infections than will direct microscopic examination (4, 30).

  - Histopathology: histology may be used to provide a definitive diagnosis of BP infection. Because 10% buffered formalin and other fixatives provide, at best, only fair fixation of the shrimp hepatopancreas (the principal target organ for BP and other baculovirus infections of penaeid shrimp), the use of 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) is highly recommended for all routine histological studies of shrimp (2, 4, 30). Routine histological stains such as Mayer-Bennett’s or Harris’ haematoxylin and eosin (H&E) may be used for the demonstration of pathognomonic (for BP) tetrahedral occlusion bodies in hepatopancreatoocytes, gut epithelial cells, or gut lumen (3, 4, 27). Typically, BP-infected hepatopancreatic (or occasionally midgut) cells will present markedly hypertrophied nuclei with single or, more often, multiple eosinophilic occlusion bodies, chromatin diminution and margination (4, 27). Occlusion bodies may be stained bright red with H&E stains, and intensely, but variably, with Gram’s tissue stains. For example, Brown and Brenn’s 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 decolourising, etc.) than the surrounding tissue, which may aid in demonstrating their presence in low-grade infections (4, 5, 26, 27).

  - Autofluorescence method with phloxine stain: another method for detecting BP occlusion bodies is based on the fluorescence of phloxine-stained occlusion bodies. Aqueous 0.001% phloxine may be added to tissue squash preparations to make wet-mounds of hepatopancreas or faeces for direct examination. Histological sections stained with routine H&E containing 0.005% phloxine, are also suitable for this procedure. BP occlusions in wet mounts of tissue squashes, in faeces, or in histological sections fluoresce bright yellow-green against a pale green background under epi-fluorescence (barrier filter of 0–515 nm and a 490 nm exciter filter). Other objects in the tissues and insect baculovirus occlusion bodies do not fluoresce with this method. Hence, the method can provide a rapid and specific diagnosis (4, 27, 41).

  - *In situ* hybridisation (see Section 3.c below).
• Antibody-based methods: polyclonal antibodies for detection of BP have been developed and reported (25, 27), but none is available for routine diagnosis of BP infections.

• Electron microscopy: BP infection can be confirmed by demonstration of the virus (or pathognomonic occlusion bodies) in sections, or demonstration of the virus in semi-purified virus preparation prepared from the hepatopancreas (14, 15, 17, 21).

c) Agent detection and identification methods

• Direct detection methods

  i) Microscopic methods

    • Wet mounts: see Section 3.b above.

    • Histological sections: see Section 3.b above.

  ii) Agent isolation and identification

    • Antibody-based antigen detection methods: see Section 3.b above.

    • In situ hybridisation: the in situ hybridisation method using a DIG-labelled DNA probe for BP follows generally the methods outlined in Poulos et al. (36) and Lightner (27) and given below.

      i) Embed Davidson's fixed tissue in paraffin and cut sections at 4 µm or less thickness. Place sections on to positively charged microscope slides. Do not use gelatin in water to float sections; use only distilled or deionised water.

      ii) Heat the slides for 30–45 minutes at 60°C. Rehydrate the tissue as follows:

        | Step                        | Time/Volume |
        |-----------------------------|-------------|
        | Xylene (or suitable substitute) | 3 × 5 minutes each |
        | Absolute alcohol            | 2 × 1 minute each |
        | 95% alcohol                 | 2 × 10 dips each |
        | 80% alcohol                 | 2 × 10 dips each |
        | 50% alcohol                 | 1 × 10 dips each |
        | Distilled water             | 6 × 10 dips each | (do not let slides dry out)

      iii) Pipette 500 µl of 100 g/ml proteinase K prepared fresh in TNE buffer. Incubate for 15 minutes at 37°C.

      iv) Wash slides in cold 0.4% formaldehyde for 5 minutes.

      v) Wash slides in 2 × SSC for 5 minutes at room temperature.

      vi) Prehybridise slides using 500 µl of hybridisation buffer and incubate for 30 minutes at 42°C in hybridisation chamber.

      vii) Dilute DIG-labelled specific probe in hybridisation buffer to appropriate concentration and boil for 10 minutes. Quench on ice for 5 minutes.

      viii) Pipette 500 µl of probe on to slide. Place on 85°C heat block for 6–7 minutes. Quench slides on ice for 5 minutes. Incubate overnight at 42°C in hybridisation chamber.

      ix) Wash slides as follows:

        | Step          | Time/Volume          |
        |---------------|----------------------|
        | 2 × SSC       | 2 × 15 minutes at room temperature |
        | 1 × SSC       | 2 × 5 minutes at 42°C |
        | 0.5 × SSC     | 2 × 15 minutes at 42°C |
        | Buffer I      | 1 × 5 minutes at room temperature |
x) Pipette 500 µl Buffer II (Blocking Buffer). Incubate at 37°C for 30 minutes.

xi) Pipette 250 µl of anti-DIG-AP antibody (dilute 1 µl in 1 ml Buffer II). Incubate at 37°C for 30 minutes.

xii) Wash slides as follows:

Buffer I 2 × 10 minutes at room temperature
Buffer III 1 × 5 minutes at room temperature

xiii) Mix 4.5 µl NBT (nitroblue tetrazolium) and 3.5 µl X-phosphate (bromochloro-indoxyl phosphate) for each 1 ml of Buffer III containing 1% polyvinyl alcohol. Pipette 500 µl onto each slide and incubate for 1–3 hours in the dark at room temperature in a humid chamber.

xiv) Stop the colour reaction in Buffer IV for 5 minutes at room temperature.

xv) Counter-stain and dehydrate the slides as follows:

Distilled water 1 × 10 dips
0.5% Bismarck brown 1 × 2–5 minutes
95% alcohol 3 × 10 dips each
Absolute alcohol 3 × 10 dips each
Xylene (or suitable substitute) 4 × 10 dips each


Notes:

• This protocol can be performed in an incubator designed for in-situ hybridisation or a food dehydrator can be used as the heat source for the in-situ incubation chamber. Slides can be placed into pipette boxes, with water in the bottom, closed and placed in the dehydrator. It is important to have a temperature control and a humid atmosphere.

• The hybridisation step (viii) can be performed using a cover-slip to reduce evaporation. If a cover-slip is used, the volume of probe can be reduced to 250 µl.

• The heating step at 85°C is necessary to denature the double-stranded DNA genome. If reactions do not develop, inadequate heating to denature the genome is the most likely reason.

• Proteinase K is needed to eliminate protein bound to nucleic acid and to increase probe binding to nucleic acid.

• See the Reagents Section (below) for formulas of buffers used in this procedure.

• Reagent and buffers for in-situ hybridisation method:

  i) 10 × Tris/NaCl/EDTA (ethylene diamine tetra-acetic acid) (TNE) buffer

  0.5 Tris base 60.57 g
  100 mM NaCl 5.84 g
  1 mM EDTA-2 H₂O 3.72 g
  DD H₂O 900 ml

  pH to 7.4 with concentrated or 5 M HCl. QS to 1 litre. Autoclave. Store at 4°C. To make 1 × TNE, dilute 100 ml 10 × stock in 900 ml DD H₂O; Filter 1 × solution through 0.45 µm filter.
ii) Lysozyme, 100 µg/ml (prepare just prior to use)

1 × TNE 10 ml
Lysozyme 1 mg

iii) Proteinase K, 100 µg/ml (prepare just prior to use)

1 × TNE 10 ml
Proteinase K 100 µl stock proteinase K (10 mg/ml)

Stock PK: add 100 mg proteinase K to 10 ml DDH₂O. Dispense 100 µl into tubes and store at –20°C. Prepare working concentration (100 µg/ml) just prior to use.

iv) 0.4% formaldehyde

37% formaldehyde 5.4 ml
DD H₂O 500 ml

Store at 4°C; can be reused five times or stored for 3 months before discarding.

v) Hybridisation buffer (50 ml final volume)

4 × SSC 10 ml 20 × SSC
50% formamide 25 ml 100% formamide
1 × Denhardt’s 2.5 ml 20 × Denhardt’s
0.5 mg/ml salmon sperm DNA 2.5 ml of 10 mg/ml solution
5% dextran sulfate 10 ml 25% dextran sulfate

Store at 4°C

vi) 20 × SSC buffer

3 M NaCl 175.32 g
0.3 M Na citrate-2H₂O 88.23 g
DD H₂O QS to 1000 ml
pH to 7.0; autoclave; store at 4°C.

To make 2 × SSC, dilute 100 ml 20 × SSC in 900 ml DD H₂O; To make 1 × SSC, dilute 50 ml 20 × SSC in 950 ml DD H₂O; To make 0.5 × SSC, dilute 50 ml 20 × SSC in 1950 ml DD H₂O. Filter solutions through 0.45 µm filter; store at 4°C.

vii) 20 × Denhardt’s solution

BSA (Fraction V) 0.4 g bovine serum albumin
Ficoll 400 0.4 g Ficoll
PVP 360 0.4 g polyvinylpyrrolidone
DD H₂O 100 ml

Filter through 0.45 µm filter; store at 4°C.

viii) 25% dextran sulfate

Dextran sulfate 25 g
DD H₂O QS to 100 ml

Warm on low heat with stirring until dissolved; store frozen.

ix) Salmon sperm DNA (10 mg/ml)

Salmon sperm DNA 0.25 g
DD H₂O 25 ml

Slowly add DNA to water in a beaker with a stir bar. Heat and stir to dissolve DNA, adding more until all the DNA is dissolved. Autoclave. Dispense into sterile tubes. Store at –20°C.
x) 10 × Buffer I

1 M Tris base 121.1 g
1.5 M NaCl 87.7 g
DD H2O QS to 1000 ml
pH to 7.5 with HCl. Autoclave; store at 4°C.

To make 1 × Buffer I, dilute 100 ml of 10 × stock in 900 ml DD H2O. Filter through 0.45 µm filter; store at 4°C.

xi) Buffer II (blocking buffer and Ab Dilution Buffer)

Genius reagent 11 0.5 g
Buffer I 100 ml
Warm on low heat with stirring 30 minutes to dissolve. Solution will be cloudy, but with no particulates. Store at 4°C for up to 2 weeks.

xii) Buffer III

100 mM Tris base 1.21 g
100 mM NaCl 0.58 g
DD H2O QS to 1000 ml
pH to 9.5 with HCl
Then add:
50 mM MgCl2.6H2O 16.10 g
Filter through 0.45 µm filter; store at 4°C.

xiii) 10% polyvinyl alcohol (PVA)

Polyvinyl alcohol 10 g
(30,000–70,000 MW) DD H2O 100 ml
Stir PVA and warm, if necessary, to get into solution. Dispense 10 ml/tube. Store at –20°C.

xiv) Development solution

Mix 90 ml Buffer III with 10 ml of 10% PVA and store at 4°C. Just prior to use, for each 1 ml of Buffer III with PVA add:
Nitroblue tetrazolium salt 4.5 µl NBT (75 mg/ml in 70% dimethylformamide)
5-bromo-4-chloro-3-indoyl phosphate, toluidinum salt 3.5 µl X-phosphate (50 mg/ml in dimethylformamide)

xv) 10 × Buffer IV

10 mM Tris base 1.21 g
1 mM EDTA.2H2O 3.7 g
DD H2O 1000 ml
pH to 8.0 with 5 N HCl. Autoclave; store at 4°C.

To make 1 × Buffer IV, dilute 100 ml of 10 × stock in 900 ml DD H2O. Filter through 0.45 µm filter; store at 4°C.

xvi) 0.5% Bismarck Brown Y

Bismarck Brown Y 2.5 g
DD H2O 500 ml

Dissolve the stain in water. Filter through a Whatman No. 1 filter; store at room temperature.
**Polymerase chain reaction methods:**

The protocol described here is modified from Wang *et al.* (42).

Appropriate samples: excised hepatopancreata, whole larvae or PLs (pooled), or faeces. Samples may be fresh, frozen, preserved in 90% ethanol, or other media designed for preservation of samples for DNA amplification.

Substances in the hepatopancreas and faeces of shrimp have been found to inhibit the DNA polymerase used in the PCR assay. Therefore, DNA extraction is required before PCR can be successfully used to detect BP.

**DNA extraction**

DNA extraction kits are convenient and commercially available. Otherwise, a suitable DNA extraction procedure is as follows:

i) A sample of faeces or hepatopancreas is added to digestion buffer (~1:10 ratio of sample:buffer in up to 400 µl of buffer) containing 50 mM KCl, 10 mM Tris/HCl, pH 8.3, 0.1 mg/ml gelatin, 0.45% Nonidet P-40, 0.45% Tween 20, and 80 µg/ml proteinase K, crushed, and dispersed with a wooden toothpick or a pipette tip.

ii) After dispersing the sample in the digestion buffer, heat to 60°C for 1 hour and then to 95°C for 10 minutes.

iii) Centrifuge at 12,000 g for 2 minutes, transfer the supernatant fluid to a new tube, and store on ice.

iv) Remove 50 µl of the digested sample and dilute with 150 µl of dilution buffer (10 mM Tris/HCl, pH 8.0, 0.1 mM EDTA) and extract with 200 µl of phenol/isoamyl alcohol/chloroform (PIC) (25/1/24).

v) After vortexing the sample for 5 seconds, leave the tube for 5 minutes and then centrifuge at 12,000 g for 2 minutes.

vi) Remove 160 µl of the aqueous (upper) phase and transfer to a new microcentrifuge tube.

vii) The extraction step may be repeated if necessary.

viii) Precipitate the DNA by adding 20 µg of glycogen (1 µl of a 20 mg/ml stock), 65 µl of a 7.5 M ammonium acetate and 390 µl of ethanol, store at –20°C for >1 hour, and then pellet the DNA by centrifugation at 12,000 g for 5 minutes.

ix) Rinse the DNA pellet with 200 µl of 70% ethanol to remove residual ammonium acetate, dry, and then dissolve the DNA pellet in 30 µl of dilution buffer or distilled water prior to adding the sample (template) to the PCR reaction mixture and beginning the PCR.

**PCR method (42)**

Primers: three forward and three reverse primers selected from a ~1430 base pair (bp) segment of the BP polyhedrin gene have been reported by Wang *et al.* (42). The sequences for these primers are:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPA</td>
<td>5’-GAT-CTG-CAA-GAG-GAC-AAA-CC-3’</td>
<td>Temperature 61°C</td>
</tr>
<tr>
<td>BPB</td>
<td>5’-ATC-GCT-AAG-CTC-TGG-CAT-CC-3’</td>
<td>Temperature 64°C</td>
</tr>
<tr>
<td>BPD</td>
<td>5’-TGT-TCT-CAG-CCA-ATA-CAT-CG-3’</td>
<td>Temperature 62°C</td>
</tr>
<tr>
<td>BPE</td>
<td>5’-TAC-ATC-TTG-GAT-GCC-TCT-GC-3’</td>
<td>Temperature 63°C</td>
</tr>
</tbody>
</table>
BPF 5'-TAC-CCT-GCA-TTC-CTT-GTC-GC-3'  Temperature 68°C
BPG 5'-ATC-CTG-TTT-CCA-AGC-TCT-GC-3'  Temperature 64°C

The combinations of these primers amplify segments from BP template DNA of:
BPA/BPF – 196 bp; BPA/BPB – 560 bp; BPA/BPG – 933 bp; BPD/BPB – 207 bp;
BPD/BPG – 580 bp; and BPE/BPG – 221 bp.

The following PCR procedure was adapted from Wang et al. (42):

i) For BP PCR, the DNA in each extracted sample is denatured by heating in a boiling water bath for 3 minutes followed by quick chilling in ice-water.

ii) 25 µl reaction mixture containing 5 mM of each primer, 1.5 mM MgCl₂, and 0.5–1 unit of DNA polymerase is added.

iii) After heating the reaction mixture for 3 minutes at 95°C, 30 PCR cycles (a DNA melting step at 94°C, a primer annealing step at 60°C, and an elongation step at 72°C) are performed followed by an elongation step of 5 minutes at 72°C.

iv) The resultant PCR products may be compared with molecular standards by 2% agarose gel electrophoresis or assayed for with a specific DNA probe for the fragment following a Southern transfer.

v) The following controls should be included in every PCR assay for BP: a known negative tissue or faecal sample; a known positive tissue or faecal sample (this can be the DNA clone from which a specific set of primers was designed); and a ‘no-template’ control.

Alternative method used by the OIE Reference Laboratory at the University of Arizona (unpublished). Use sample type and extraction methods as described above.

Primers: one forward and reverse primer pair (6581F/6582R) selected from clone IR36 (referred to as B1.23 in Bonami et al. (3) and deposited in GenBank with accession number DQ496179) produces a 644 bp amplicon. The sequences for these primers are:

6581 5'-TGT-AGC-AGC-AGA-GAA-GAG-3'
6582 5'-CAC-TAA-GCC-TAT-CTC-CAG-3'

Method:

PCR reaction mixture:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>25 µl reaction mix</th>
<th>25 µl PCR beads*</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>16.5 µl</td>
<td>23.0 µl</td>
<td></td>
</tr>
<tr>
<td>10 × buffer</td>
<td>2.5 µl</td>
<td></td>
<td>1 ×</td>
</tr>
<tr>
<td>dNTP’s</td>
<td>0.5 µl each</td>
<td></td>
<td>200 µM each</td>
</tr>
<tr>
<td>Primer A</td>
<td>0.5 µl</td>
<td>0.5 µl</td>
<td>0.31 µM</td>
</tr>
<tr>
<td>Primer B</td>
<td>0.5 µl</td>
<td>0.5 µl</td>
<td>0.31 µM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.5 µl</td>
<td></td>
<td>1.5 µM</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0.5 µl</td>
<td></td>
<td>2.5 U</td>
</tr>
<tr>
<td>Template</td>
<td>1.0 µl</td>
<td>1.0 µl</td>
<td></td>
</tr>
</tbody>
</table>

*PuReTaq™ Ready-To-Go PCR beads™, Amersham Biosciences, Buckinghamshire, UK
PCR cycling parameters:

<table>
<thead>
<tr>
<th>Primers</th>
<th>Mix/Beads</th>
<th>Time</th>
<th>Temp. °C</th>
<th>No. cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>6581/6582</td>
<td>Mix/Beads*</td>
<td>5 minutes</td>
<td>95</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 seconds, 30 seconds, 1 minute</td>
<td>95, 60, 72</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 minutes</td>
<td>72</td>
<td>1</td>
</tr>
</tbody>
</table>

4. RATING OF TESTS AGAINST PURPOSE OF USE

The methods currently available for surveillance, detection, and diagnosis of BP are listed in Table 1. The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic sensitivity and specificity; C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and D = the method is presently not recommended and/or not available for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation (see Chapter 1.1.2), their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

**Table 1. Baculovirus penaei surveillance, detection and diagnostic methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance</th>
<th>Presumptive</th>
<th>Confirmatory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Larvae</td>
<td>PLs</td>
<td>Juveniles</td>
</tr>
<tr>
<td>Gross signs</td>
<td>C</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Bioassay</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Direct LM</td>
<td>B</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Histopathology</td>
<td>B</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>TEM</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Antibody-based assays</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>DNA Probes <em>in situ</em></td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>PCR</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Sequence</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
</tbody>
</table>

PLs = postlarvae; LM = light microscopy; TEM = Transmission electron microscopy; PCR = polymerase chain reaction

5. CORROBORATIVE DIAGNOSTIC CRITERIA

   a) **Definition of suspect case**

   For larvae (especially protozoa, mysis and early PL stages): mortality with larvae presenting white midguts. For juveniles: poor growth in populations with a prior history of BP infection.

   b) **Definition of confirmed case**

   Any combination of at least two of the following three methods (with positive results):

   Microscopic demonstration of tetrahedral occlusion bodies in wet mounts of whole larvae or excised hepatopancreata. For older PLs, juveniles and adults: tetrahedral occlusion bodies evident in wet mount squashes and/or in histological sections of the hepatopancreas or faeces.
**6. Diagnostic/detection methods to declare freedom**

Two years of history of negative test results for BP using:

- PCR performed on samples of the appropriate type and sample size; and/or
- Wet mount and/or histological results in which no tetrahedral occlusion bodies are observed in samples of the appropriate type and sample size.

**REFERENCES**


* * *

NB: There is an OIE Reference Laboratory for tetrahedral baculovirosis (Baculovirus penaei) (see Table at the end of this Aquatic Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.3.5.

SPHERICAL BACULOVIROSIS
(Penaeus monodon-type baculovirus)

1. CASE DEFINITION

For the purpose of this chapter, spherical baculovirosis is considered to be infection with Penaeus monodon-type baculovirus. Synonyms: MBV from P. monodon was designated PmSNPV (for singly enveloped nuclear polyhedrosis virus from P. monodon) in accordance with the guidelines for virus nomenclature published by the International Committee on Taxonomy of Viruses (ICTV) (35), and it appears as the tentative species P. monodon NPV, or PemoNPV, in the 7th and 8th Reports of the ICTV (15, 46). Although PemoNPV may be the most correct name for the virus, the term P. monodon baculovirus (MBV) will be used in most instances to designate this virus in this Aquatic Manual.

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

a) Agent factors

• Aetiological agent: P. monodon baculovirus (MBV) as described by Lightner & Redman (28), Lightner et al. (30), Mari et al. (34).

• The International Committee on Virus Taxonomy lists MBV (Spherical Baculovirosis) as a tentative species named PemoNPV in the genus Nucleopolyhedrovirus (15).

• Agent strains: based on the wide geographical and host species range of MBV, the existence of different strains of MBV is likely. Polymerase chain reaction (PCR) tests designed for East and South-East Asian isolates of MBV have recently been shown to give false negative test results for MBV infected P. monodon from Africa (Lightner, unpublished data) further suggesting that MBV (or the species PemoNPV) is made up of more than one strain.

b) Host factors

• Susceptible species: MBV infections have been reported in one or more species of the following penaeid genera: Penaeus, Metapenaeus, Fenneropenaeus and Melicertus (14, 18, 21, 24, 28, 41). Experimental water-borne and per os challenge of the Japanese tiger shrimp, Marsupenaeus japonicus, 1-day-old postlarvae (PL) with MBV failed to produce detectable infections (17). Likewise, despite the simultaneous culture of MBV-infected P. monodon in a number of Western Hemisphere farms, and the consequent direct exposure of certain Western Hemisphere penaeids (i.e. specifically L. vannamei, L. stylirostris and Farfantepenaeus californiensis) to MBV, the virus did not produce infections in these species, nor has it become established in the shrimp farms or in wild stocks of exposed regions (24, 30).

• Susceptible stages of the host: all life stages, except eggs and nauplii, are susceptible to infection by MBV.

• Target organs: MBV is strictly enteric infecting mucosal epithelial cells of the hepatopancreas tubules and the anterior midgut (1, 6, 13, 20, 24, 30).

• Persistent infection and lifelong carriers: persistent infection occurs commonly in penaeid hosts of MBV. Wild adult P. monodon females that are heavily infected with MBV have been shown to excrete MBV-contaminated faeces when spawning, thereby contaminating the eggs and passing the virus to the next generation (20, 24).

• Vectors: none is known in natural infections.
c) Disease pattern

- Transmission mechanisms: transmission of MBV is horizontal by ingestion of infected tissue (cannibalism), faeces, occlusion bodies, or virus-contaminated detritus or water (20, 24). MBV has been experimentally transmitted in the laboratory by exposure of larval or early PL *P. monodon* by water-borne or *per os* challenge. MBV occlusion bodies were apparent by 2 days post-challenge in hepatopancreatic cells when PL-1 were challenged at 28°C in 33 ppt sea water (37–39).

- Prevalence: highly variable, from <1% in wild and cultured populations up to 100% in cultured populations in larval-rearing tanks and nursery ponds (1, 7, 9, 11, 12, 24, 37, 49).

- Geographical distribution: MBV is enzootic in wild penaeids in the following regions bordering on the Indo-Pacific: East and South-East Asia, Indian subcontinent, Middle East, Australia, Indonesia, New Caledonia, East Africa, and Madagascar. Outside the normal geographical range of *P. monodon*, MBV has not been reported in wild penaeid shrimp. However, MBV has been reported from sites where introduced *P. monodon* has been cultured in the Mediterranean, West Africa, Tahiti and Hawaii as well as several sites in North and South America and the Caribbean (5, 24).

- Mortality and morbidity: the larval stages (specifically protozoea and mysis) and early PL stages are the life stages where significant mortalities may occur and they are the most easily infected in laboratory challenge studies (11, 38, 39). In enzootic regions culturing *P. monodon*, MBV prevalence and infection severity may be high (from 50% to nearly 100%) in juveniles and adults, but without associated mortality or morbidity. MBV infections are apparently well tolerated by *P. monodon* unless they are severely stressed (7, 9, 11, 12, 24, 25, 37). Nonetheless, heavy MBV infections in farmed *P. monodon* may suppress growth rate, result in reduced survival and reduce overall culture performance (1, 2, 7, 16, 24, 36, 38).

- Economic and/or production impact of the disease: MBV has caused serious disease, sometimes with high mortality rates in hatcheries and significant production losses in farms in the Indo-Pacific. While not usually causing high mortality rates in infected juvenile or adult stages, MBV has been documented to cause reduced growth and lower harvest production in populations that are persistently infected with the virus (2, 24).

d) Control and prevention

- Vaccination: no effective vaccination methods for MBV have been developed.

- Chemotherapy: no scientifically confirmed reports.

- Immunostimulation: no scientifically confirmed reports.

- Resistance breeding: no MBV-resistant stocks of the susceptible species have been demonstrated.

- Restocking with resistant species: not applicable to MBV.

- Blocking agents: not reported.

- General husbandry practices:
  - Hatchery: a number of husbandry practices have been applied to the prevention of MBV infections and disease. Prescreening of broodstock for MBV has been somewhat effective in detecting heavily infected carriers of the virus and thereby reducing the transmission of the disease from parent to offspring. With nonlethal testing methods, this is accomplished by simple light microscopic examination of faecal strands (or by PCR testing of faecal strands if PCR testing facilities are readily available). Alternatively, spent broodstock may be killed after spawning and simple light microscopic examination of a hepatopancreas squash can be run (or the excised hepatopancreas may be tested by PCR) to determine the spawner's MBV infection status. Because MBV is transmitted from adults to their offspring by faecal contamination of the spawned eggs, prevention of infection in hatcheries may be achieved by taking additional steps to eliminate faecal contamination of spawned eggs and...
larvae by thoroughly washing nauplii or eggs with formalin, iodophores, and clean sea water (10).

- **Nursery and grow-out ponds:** MBV infections remain common in earthen-bottom ponds in regions of the Indo-Pacific where the virus is enzootic (5, 7, 24), but incidence and prevalence of MBV infections may be reduced in lined nursery and grow-out pond.

3. **DIAGNOSTIC METHODS**

a) **Field diagnostic methods**

- **Gross signs:** Protozoea, mysis and early PL stages with severe MBV infections may present a whitish midgut (due to the presence of occlusion bodies and cell debris in the faecal material) (24). Juveniles and adults present no gross signs of diagnostic value, nor do larvae or PLs with less severe infections.

b) **Clinical methods**

Infection of the hepatopancreas by *Penaeus monodon*-type baculovirus (MBV) is one of the most easy to diagnose diseases of the penaeid shrimps and prawns. The occlusion bodies formed by the virus are very conspicuous and easily demonstrated by direct light microscopy with fresh specimens or by routine histological methods with fixed specimens. Direct microscopic methods are most suitable for the PL stages, which are commonly moved in regional and international trade. Highly sensitive molecular methods for MBV are also available and provide the most sensitive methods for surveillance applications, especially for nonlethal testing of broodstock.

- **Gross pathology:** see Section 3.a above.

- **Direct microscopic pathology**

  - **Wet mounts of fresh tissue:** diagnosis of MBV infections is made by the demonstration of single or multiple generally spherical occlusion bodies in wet mounts of squash preparations of hepatopancreas or midgut examined by phase-contrast or bright-field microscopy. In carefully prepared unstained preparations, MBV occlusion bodies are visible as single or multiple, slightly refractive, greenish intranuclear inclusions that range in diameter from less than 0.1 µm to nearly 20 µm. Staining the tissue squash with 0.05% aqueous malachite green aids in demonstration of the occlusion bodies by staining them more intensely than other similar sized spherical objects, such as normal host cell nuclei, nucleoli, secretory granules, phagolysosomes, and lipid droplets (5, 23, 24, 30).

  - **Wet mounts of faecal strands:** this method may be used as a nonlethal method to screen for carriers of MBV. The method can be applied to juvenile or older shrimp, and it is perhaps most useful as a nonlethal method for screening valuable broodstock. Faecal samples from shrimp to be tested may be obtained by placing the shrimp in an aquarium, spawning tank, or other suitable tanks for a few hours until faecal strands are present on the tank bottom. The faecal strands are best collected using a clear plastic siphon hose (an air line fitted with a section of plastic pipette as a tip is ideal) and placed in a beaker, cup, or other suitable container. The faecal strands may be made into wet mounts and examined directly for occlusion bodies. MBV occlusion bodies are roughly spherical, refractive bodies, that may occur singly or in clusters. In very fresh faecal strands, they may occur in clusters held together by the nuclear membrane. The addition of a drop of 0.05% aqueous malachite green to the wet-mount preparation aids in demonstrating the MBV occlusion bodies by staining them more intensely green than other round objects in faeces (5, 24).

- **Collected faeces may also be used as the sample for nonlethal testing for MBV by PCR. PCR will provide greater diagnostic sensitivity for low-grade infections than will direct microscopic examination (5, 29).**

- **Histopathology:** histology may be used to provide a definitive diagnosis of MBV infection. Because 10% buffered formalin and other fixatives provide, at best, only fair fixation of the shrimp hepatopancreas (the principal target organ for MBV), the use of 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) is highly recommended for all routine histological studies of shrimp (4, 24). To obtain the best results, dead shrimp should not be used. Only live, moribund, or compromised shrimp should be selected for fixation and histological examination. Selected shrimp are killed by injection of fixative directly into the hepatopancreas; the cuticle over the cephalothorax and abdomen just lateral to the dorsal midline is opened with fine-pointed surgical scissors to enhance fixative penetration (the abdomen may be removed and discarded), the whole shrimp (or cephalothorax less the abdomen) is immersed in fixative for from 24 to no more than 48 hours, and then transferred to 70% ethyl alcohol for storage. After transfer to 70% ethyl alcohol, fixed specimens may be transported (via post or courier to the diagnostic laboratory) by wrapping in cloth or a paper towel saturated with 70% ethyl alcohol and packed in leak-proof plastic bags.

To begin histological processing, fixed shrimp are ‘cut-in’ (see Bell & Lightner [4] for a photographic guide to this procedure) to facilitate eventual sectioning of the hepatopancreas and midgut. After dehydration, the specimens are embedded in paraffin and sections of 4–6 µm thickness are cut. Routine histological stains such as Mayer Bennett’s or Harris’ haematoxylin and eosin (H&E) may be used for the demonstration of MBV diagnostic spherical occlusion bodies in hepatopancreatoocytes, gut epithelial cells, or gut lumen. Typically, MBV-infected hepatopancreatic (or occasionally midgut) cells will present markedly hypertrophied nuclei with single or, more often, multiple eosinophilic occlusion bodies along with chromatin diminution and margination. Occlusion bodies may be stained bright red with H&E stains, and intensely, but variably, with Gram’s tissue stains. For example, Brown and Brenn’s 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 decolourising, etc.) than the surrounding tissue, which may aide in demonstrating their presence in low-grade infections (5, 6, 21, 23, 24, 50).

- **Autofluorescence method with phloxine stain**: another method for detecting MBV occlusion bodies is based on the fluorescence of phloxine-stained occlusion bodies. Aqueous 0.001% phloxine may be added to tissue squash preparations to make wet mounts of hepatopancreas or faeces for direct examination. Histological sections stained with routine H&E containing 0.005% phloxine, are also suitable for this procedure. MBV occlusions in wet mounts of tissue squashes, in faeces, or in histological sections fluoresce bright yellow-green against a pale green background under epi-fluorescence (barrier filter of 0–515 nm and a 490 nm exciter filter). Other objects in the tissues and insect baculovirus occlusion bodies do not fluoresce with this method. Hence, the method can provide a rapid and specific diagnosis (5, 24, 44).

- **In-situ hybridisation** (see Section 3.c below).

- **Antibody-based methods**: polyclonal antibodies produced in rabbits for detection of Tetrahedral Baculovirosis (BP) polyhedrin (22) cross react with MBV using indirect fluorescent antibody test (IFAT) methods (24), but none is available for routine diagnosis of MBV infections.

- **Electron microscopy**: MBV infection can be confirmed by demonstration of the virus (or pathognomonic occlusion bodies with occluded virions) in sections, or demonstration of the virus in semi-purified virus preparation prepared from the hepatopancreas (13, 16, 20, 30, 31, 34).

### c) Agent detection and identification methods

- **Direct detection methods**
  
  - **Microscopic methods**
    
    - **Wet mounts**: see Section 3.b above.
    
    - **Histological sections**: see Section 3.b above.
ii) Agent isolation and identification

- Antibody-based antigen detection methods: see Section 3.b above.

- Molecular methods using DNA probes to MBV: non-radioactive DIG-labelled gene probes to MBV have been developed (27, 33, 34, 40). DIG-labelled DNA probes for MBV are commercially available as ShrimProbeTM kits from DiagXotics (Lawrenceville, New Jersey, USA). The probes are labelled with a non-radioactive label, digoxigenin-11-dUTP (DIG). These probes only work well with the in-situ hybridisation method with histological sections because there are substances present in the hepatopancreas and faeces of shrimp that provide both false-positive and false-negative results with samples that are blotted directly and not extracted prior to probing.

- Dot-blot hybridisation procedure for MBV: while specific DNA probes for MBV are available, their application to dot-blot hybridisation procedures is not recommended for most routine diagnostic applications. Pigments present in the hepatopancreas leave a coloured spot on the hybridisation membrane that can result in the masking of a positive test or in the false interpretation of a negative test. Likewise, bits of chitin (which nonspecifically bind DNA probes), pigments, and other materials present in the faecal sample may also result in false-positive or false-negative dot-blot hybridisation tests. Extraction of DNA from the hepatopancreas or faeces prior to blotting or the use of chemiluminescent or radioactively labelled probes may circumvent these problems and is recommended. Nonetheless, the adequacy of other test methods (i.e. direct wet mounts, histology, or PCR) has not indicated a need for the further refinement and application of the dot-blot method (24, 30).

- In-situ hybridisation procedure: the in-situ hybridisation protocol given in detail for Tetrahedral Baculovirosis (BP) in Section 3.c.ii of Chapter 2.3.4 uses the same method except that a DIG-labelled probe for MBV is used.

- Polymerase chain reaction for MBV

Several PCR methods have been developed for MBV and may be suitable for certain applications (8, 32, 45, 47, 48). However, more sensitive methods have been recently developed and demonstrated to detect MBV from several geographical regions (3, 43).

Substances in the hepatopancreas and faeces of shrimp have been found to inhibit the DNA polymerase used in the PCR assay. Therefore, DNA extraction is required before PCR can be successfully applied to the detection of this virus (3, 8, 19). DNA extraction kits are convenient and commercially available. Otherwise, refer to Section 3.c.ii in Chapter 2.3.4 Tetrahedral baculovirosis (BP) for a suitable DNA extraction procedure.

The following controls should be included in every PCR assay for MBV: a known negative tissue or negative faecal sample; a known positive tissue or faecal sample (this can be the DNA clone from which a specific set of primers was designed); and a ‘no-template’ control.

Nested PCR method for MBV (3): this nested PCR method is capable of detecting low concentrations of MBV (down to eight viral genome equivalents). Two external and two internal primers were designed using a DNA sequence derived from the plasmid p4Ec196, which was constructed from a 7.4 kb EcoRI fragment of an Australian isolate of MBV. The primer sequences are:
Chapter 2.3.5. - Spherical baculovirosis (Penaeus monodon-type baculovirus)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBV1.4F</td>
<td>5’-CGA- TTC-CAT-ATC-GGC-GCA-ATA-3’</td>
<td>62°C (68.9°C)</td>
</tr>
<tr>
<td>MBV1.4r</td>
<td>5’-TTG-GCA-TGC-ACT-CCC-TGA-GAT-3’</td>
<td>64°C (70.8°C)</td>
</tr>
<tr>
<td>MBV1.4NF</td>
<td>5’TCC-AAT-CGC-GTC-TGC-GAT-ACT-3’</td>
<td>64°C (70.8°C)</td>
</tr>
<tr>
<td>MBV1.4NR</td>
<td>5’-CGC-TAA-TGG-GGC-ACA-AGT-CTC-3’</td>
<td>66°C (72.8°C)</td>
</tr>
</tbody>
</table>

The melting temperatures of the primers are according to the formula $2(A+T) + 4(G+C)$, or according to the per cent GC method (values in parentheses).

**DNA extraction**

i) PCR inhibitors were noted by Belcher & Young (3) to be present in DNA samples prepared from whole MBV-infected PL *Penaeus monodon* when using the extraction method recommended by Wang *et al.* (51) for BP, which incorporates proteinase K. However, when hot phenol was used to extract the DNA, this inhibitory effect was removed.

ii) With the hot phenol method, the sample to be tested (PLs, shrimp hepatopancreas, faeces) is freeze-dried and ground to a powder in liquid nitrogen with a motor and pestle.

iii) Approximately 300 mg of the resulting material is added immediately to 400 µl of preheated (65°C) lysis buffer (100 mM Tris/HCl, 100 mM ethylene diamine tetraacetic acid [EDTA], 1% sodium dodecyl sulfate, pH 8.0) and incubated at 65°C for 5–10 minutes.

iv) The resulting suspension is coarsely homogenised by spot centrifugation and homogenisation with a microfuge tube pestle. Tris/HCl-buffered phenol, pH 8.0 (600 µl) is added and the mixture is incubated for 2 hours at 65°C with occasional inversion.

v) Following centrifugation at 12,000 $g$ for 10 minutes at room temperature, the aqueous layer is transferred to a fresh microfuge tube and extracted twice with an equal volume of phenol/chloroform (1/1). Then, a total of 50 µl of the aqueous layer is transferred to a fresh microfuge tube containing 150 µl dilution buffer and extracted once more with an equal volume of phenol/chloroform (1/1) followed by a straight chloroform extraction.

vi) Ammonium acetate is added to the aqueous layer to a final concentration of 2.5 M, mixed briefly, and two volumes of −20°C ethanol are added with 1 µl of 20 mg/litre glycogen to precipitate the DNA.

vii) DNA is precipitated by incubation at −20°C overnight or by incubation at −70°C for 1 hour.

viii) DNA is pelleted at 12,000 $g$ for 15 minutes at 4°C. The resulting DNA pellet is rinsed twice, first with 500 µl 80% cold ethanol and centrifuged at 12000 $g$ for 10 minutes at 4°C, followed by an identical rinse and centrifugation at room temperature.

ix) The final DNA pellet is dried *in vacuo*, resuspended in 100 µl dilution buffer (10 mM Tris/HCl, pH 8.0, 0.1 mM EDTA, pH 8.0) at room temperature overnight or at 37°C for 2 hours. Following spectrophotometric analysis, and prior to PCR, the DNA is diluted to 50 ng/µl in dilution buffer.
Nested PCR steps of Belcher & Young (3):

i) Prior to PCR, the extracted total DNA is denatured in boiling water for 3 minutes followed by quick chilling in ice-water.

ii) A total of 100 ng of extracted DNA is used as template.

iii) Each reaction tube contains 50 mM KCl, 10 mM Tris/HCl, pH 9, 0.1% Triton X-100, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 0.25 µM of each MBV1.4F and MBV1.4R, 2.5 U of Taq, and made up to a final volume of 50 µl.

iv) The reaction mixes are overlaid with mineral oil (as necessary).

v) The conditions for the first round of amplification are: one cycle of 96°C for 5 minutes; 40 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 60 seconds; and one cycle of 72°C for 7 minutes.

vi) The second step of the nested PCR is accomplished with 0.5 µl of the primary PCR reaction used as template with the internal primers.

vii) The second round of amplification reaction contains 50 mM KCl, 10 mM Tris/HCl, pH 9.0, 0.1% Triton X-100, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.25 µM of each of the primers MBV1.4NF and MBV1.4NR, and 2.5 U of Taq, and made up to a final volume of 50 µl.

viii) The reaction mixes are overlaid with mineral oil (as necessary).

ix) The conditions for the second round of amplification are: one cycle of 96°C for 5 minutes; 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 60 seconds; and one cycle of 72°C for 7 minutes.

x) Demonstration of the PCR products (533 bp first step and 361 bp second step) is accomplished by adding 1 µl of gel-loading buffer (0.25% [w/v] bromophenol blue, 15% [w/v] Ficoll-type 400, 100 mM EDTA, pH 8.0) to 10 µl of each reaction mixture and electrophoresis through a 0.8% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) containing 0.5 g/litre ethidium bromide.

An alternative single-step PCR method is used by the OIE Reference Laboratory at the University of Arizona because it is less prone to contamination (43). This method uses the sample type and extraction methods as described above in Section 3.c.ii above.

Primers: one forward and reverse primer pair (261F/261R) selected from clone GC7 and deposited in GenBank with accession number AY819785) produces a 261 bp amplicon (43).

The sequences for these primers are:

261F  5’-AAT-CCT-AGG-CGA-TCT-TAC-CA-3’
261R  5’-CGT-TCG-TTG-ATG-AAC-ATC-TC-3’

DNA templates:

i) Extracted from hepatopancreas (frozen or ethanol fixed);

ii) Extracted from whole PLs (frozen or ethanol fixed);

iii) Extracted from faeces (frozen or ethanol fixed).
Chapter 2.3.5. - Spherical baculovirosis (Penaeus monodon-type baculovirus)

**PCR reaction mixture:**

<table>
<thead>
<tr>
<th>Reagent (concentration)</th>
<th>25 µl PCR beads*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled H₂O</td>
<td>23.5 µl</td>
</tr>
<tr>
<td>Primer 261F (0.3 µM)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Primer 261R (0.3 µM)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>DNA template (50–450 ng of DNA)</td>
<td>0.5 µl</td>
</tr>
</tbody>
</table>

*PuReTaq™ Ready-To-Go PCR beads™, Amersham Biosciences, Buckinghamshire, UK

**PCR cycling parameters:**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Mix/Beads</th>
<th>Time</th>
<th>Temp. °C</th>
<th>No. cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>261F/261R</td>
<td>Beads*</td>
<td>5 minutes</td>
<td>95</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 seconds,</td>
<td>94, 60, 72</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 minutes</td>
<td>72</td>
<td>1</td>
</tr>
</tbody>
</table>

4. **RATING OF TESTS AGAINST PURPOSE OF USE**

The methods currently available for surveillance, detection, and diagnosis of MBV are listed in Table 1. The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic sensitivity and specificity; B = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and D = the method is presently not recommended and/or not available for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation (see Chapter 1.1.2), their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

**Table 1. MBV surveillance, detection and diagnostic methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance</th>
<th>Presumptive</th>
<th>Confirmatory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Larvae</td>
<td>PLs</td>
<td>Juveniles</td>
</tr>
<tr>
<td>Gross signs</td>
<td>C</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Bioassay</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Direct LM</td>
<td>B</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Histopathology</td>
<td>B</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>TEM</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Antibody-based assays</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>DNA probes <em>in situ</em></td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>PCR</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Sequence</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
</tbody>
</table>

PLs = postlarvae; LM = light microscopy; TEM = Transmission electron microscopy; PCR = polymerase chain reaction
5. CORROBORATIVE DIAGNOSTIC CRITERIA

a) Definition of suspect case
For larvae (especially protozoea, mysis and early PL stages) of the susceptible species: mortality with larvae presenting white midguts. For juveniles: poor growth or poor culture performance in populations with a prior history of MBV infection or in regions where MBV is prevalent.

b) Definition of confirmed case
Any combination of at least two of the following three methods (with positive results):

- Microscopical demonstration of spherical occlusion bodies in wet mounts of whole larvae or excised hepatopancreata. For older PLs, juveniles and adults: spherical occlusion bodies evident in wet-mount squashes and/or in histological sections of the hepatopancreas or faeces.

- In-situ hybridisation positive histological signal to MBV-type lesions (i.e. hypertrophied nuclei with or without pathognomonic spherical occlusion bodies.

- PCR positive results for MBV.

6. DIAGNOSTIC/DETECTION METHODS TO DECLARE FREEDOM

Two years of history of negative test results for MBV using:

- PCR performed on samples of the appropriate type and sample size.

- Wet mount and/or histological results in which no spherical occlusion bodies are observed in samples of the appropriate type and sample size.

REFERENCES


* * *

NB: There is an OIE Reference Laboratory for Spherical baculovirosis (Penaeus monodon-type baculovirus) (see Table at the end of this Aquatic Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
CHAPTER 2.3.6.

INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS

1. CASE DEFINITION

For the purpose of this chapter, infectious hypodermal and haematopoietic necrosis is considered to be infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV) (6, 7, 35, 36, 39, 56).

Synonyms: the International Committee on the Taxonomy has assigned IHHNV (a parvovirus) as a tentative species in the genus Brevidensovirus, family Parvoviridae with the species name of PstDNV (for Penaeus stylirostris densovirus) (19). For the purpose of this Aquatic Manual, most references to the viral agent of IHHN will be as IHHNV.

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

a) Agent factors

• Aetiological agent: IHHNV is the smallest of the known penaeid shrimp viruses. The IHHN virion is a 20–22 nm, nonenveloped icosahedron, with a density of 1.40 g/ml in CsCl, contains linear single-stranded DNA with an estimated size of 4.1 kb, and has a capsid with four polypeptides of molecular weight 74, 47, 39, and 37.5 kD (7, 45).

• Agent strains: at least four distinct genotypes of IHHNV have been identified (56, 57), but only two of the four genotypes have been demonstrated to be infectious to either Litopenaeus vannamei and/or Penaeus monodon. Although a portion of the IHHNV genome has been found in penaeid shrimp from East Africa and the western Indo-Pacific region, that putative agent may not be infectious to the representative host species L. vannamei and P. monodon.

b) Host factors

• Susceptible host species: most penaeid species can be infected with IHHNV, including the principal cultured species, P. monodon, L. vannamei, and L. stylirostris.

• IHHNV infections are most severe in the Pacific Blue Shrimp, L. stylirostris, where the virus can cause acute epizootics and mass mortality (> 90%). In L. stylirostris the juvenile and subadult life stages are the most severely affected (2, 3, 10, 11, 27, 34, 35).

• IHHNV causes the chronic disease ‘runt-deformity syndrome’ (RDS) in L. vannamei in which reduced, irregular growth and cuticular deformities, rather than mortalities, are the principal effects (9, 13, 15, 22, 27, 43). IHHNV infection in P. monodon is usually subclinical, but RDS, reduced growth rates and reduced culture performance has been reported in IHHNV infected stocks (16, 48).

• Susceptible life stages of the host species: IHHNV has been demonstrated in all life stages (i.e. eggs, larvae, postlarvae [PL], juveniles and adults) of L. vannamei. Eggs produced by IHHNV-infected females with high virus loads were found to generally fail to develop and hatch. Those nauplii produced from infected broodstock that do hatch had a high prevalence of IHHNV infection (42).
• Target organs: IHHNV infects and has been shown to replicate (using in-situ hybridisation [ISH] with specific DNA probes) in tissues of ectodermal and mesodermal origin from the embryo. Thus, the principal target organs include: the gills, cuticular epithelium (or hypodermis), all connective tissues, the hematopoietic tissues, the lymphoid organ, antennal gland, and the ventral nerve cord, its branches and its ganglia. The enteric organs (endoderm-derived hepatopancreas, midgut and midgut caeca mucosal epithelia) and smooth, cardiac, and striated muscle show no histological signs of infection by IHHNV and are usually negative for IHHNV by ISH (27, 32).

• Persistent infection and lifelong carriers: some members of populations of *L. stylirostris* and *L. vannamei* that survive IHHNV infections and/or epizootics, may carry the virus for life and pass the virus on to their progeny and other populations by vertical and horizontal transmission (2, 27, 28, 41, 43).

• Selected stocks of *L. stylirostris* that are resistant to IHHN disease have been developed (17, 58), with some stocks also refractory to infection (54). A genetic basis for IHHN susceptibility in *L. vannamei* has been reported (1).

• Vectors: none is known in natural infections.

c) Disease pattern

• Transmission mechanisms: transmission of IHHNV can be by horizontal or vertical routes. Horizontal transmission by cannibalism or by contaminated water has been demonstrated (27, 35–37, 54), as has vertical transmission via infected eggs (43).

• Prevalence: in regions where the virus is enzootic in wild stocks, the prevalence of IHHNV has been found in various surveys to range from 0 to 100%. Some reported mean values for IHHNV prevalence in wild stocks are: 26% and 46% in *L. stylirostris* in the lower and upper Gulf of California, respectively (47); 100% and 57%, respectively, in adult female and adult male *L. stylirostris* from the mid region of the Gulf of California (42); 28% in wild *L. vannamei* collected from the Pacific coast of Panama (44); and from 51 to 63% in *L. vannamei* collected from the Pacific coasts of Ecuador, Colombia and Panama (43). Other penaeids collected during some of these surveys and found to be IHHNV positive included the brown shrimp, *Farfantepenaeus californiensis* and the Western white shrimp *L. occidentalis*. In farms where IHHNV is present, its prevalence can range from very low to 100%, but high prevalence is typical (16, 25, 27, 28, 30, 35, 40).

• Geographical distribution: IHHNV appears to have a world-wide distribution in either wild or cultured penaeid shrimp (10, 27, 46). In the Western Hemisphere, IHHNV is commonly found in wild penaeid shrimp in the eastern Pacific from Peru to Mexico. Although IHHNV has been reported from cultured *L. vannamei* and *L. stylirostris* in most of the shrimp-culturing regions of the Western Hemisphere and in wild penaeids throughout their range along the Pacific coast of the Americas (Peru to northern Mexico), the virus has not been reported in wild penaeid shrimp on the Atlantic coast of the Americas (8, 12, 27, 28, 30, 34). IHHNV has been also reported in cultured penaeid shrimp from Pacific islands including the Hawaiian Islands, French Polynesia, Guam, and New Caledonia. In the Indo-Pacific region, the virus has been reported from cultured and wild penaeid shrimp in East Asia, South-East Asia, and the Middle East (8, 27). An IHHN-like virus has been reported from Australia (23 46).

• Four IHHNV genotypes have been documented: 1) from the Americas and East Asia (principally the Philippines); 2) from South-East Asia; 3) East Africa; and 4) the western Indo-Pacific region including Madagascar and Mauritius. The first two genotypes are infectious to the representative penaeids, *L. vannamei* and *P. monodon*, while the latter two genetic variants may not be infectious to these species (56, 57).

d) Control and prevention

• Vaccination: no effective vaccination methods for IHHNV have been developed.

• Chemotherapy: no scientifically confirmed reports.
Chapter 2.3.6. - Infectious hypodermal and haematopoietic necrosis

• Immunostimulation: no scientifically confirmed reports.
• Resistance breeding: some IHHNV resistant stocks of L. stylirostris have been developed and these have had some successful application in shrimp farms (17, 28, 58, 61). However, such stock have no increased resistance to diseases such as white spot syndrome virus (WSSV), and hence, their use has been limited.
• Restocking with resistant species: there has been some limited application and success with IHHNV resistant L. stylirostris (17, 28, 58, 61).
• Blocking agents: not reported.
• General husbandry practices: some husbandry practices have been successfully applied for the prevention of IHHNV infections and disease. Among these has been the application of PCR prescreening of wild or pond-reared broodstock and/or their spawned eggs/nauplii and discarding those that test positive for the virus (20, 43) and the development of specific pathogen free (SPF) shrimp stocks L. vannamei and L. stylirostris (28, 29, 38, 49, 60). The latter has proven to be the most successful husbandry practice for the prevention and control of IHHN (21, 29, 49). Unfortunately, there is a misconception in the industry that SPF is a genetic trait rather than a condition of health status. The development of SPF L. vannamei that were free not only of IHHNV, but also of all the major known pathogens of penaeid shrimp, has resulted in the introduction of the species to Asia and to its challenging P. monodon by 2004–2005 as the dominant farmed shrimp species in Asia, as well as the Americas where the SPF stocks were developed (29, 52).

3. DIAGNOSTIC METHODS

a) Field diagnostic methods
• Gross signs:
  • IHHN disease in L. stylirostris: IHHNV often causes an acute disease with very high mortalities in juveniles of the species. Vertically infected larvae and early PL do not become diseased, but in approximately 35-day-old or older juveniles, gross signs of the disease may be observed, followed by mass mortalities. In horizontally infected juveniles, the incubation period and severity of the disease is somewhat size and/or age dependent, with young juveniles always being the most severely affected. Infected adults seldom show signs of the disease or mortalities (2, 3, 8, 11, 12, 24–28, 35, 36). Gross signs are not IHHN specific, but juvenile L. stylirostris with acute IHHN show a marked reduction in food consumption, followed by changes in behaviour and appearance. Shrimp of this species with acute IHHN have been observed to rise slowly in culture tanks to the water surface, there to become motionless and then to roll-over and slowly sink (ventral side up) to the tank bottom. Shrimp exhibiting this behaviour may repeat the process for several hours until they become too weak to continue, or until they are attacked and cannibalised by their healthier siblings. Litopenaeus stylirostris at this stage of infection often have white or buff-coloured spots (which differ in appearance and location from the white spots that sometimes occur in shrimp with WSSV infections) in the cuticular epidermis, especially at the junction of the tergal plates of the abdomen, giving such shrimp a mottled appearance. This mottling later fades in moribund L. stylirostris as such individuals become more bluish. In L. stylirostris and in P. monodon with terminal phase IHHNV infections, moribund shrimp are often distinctly bluish in colour, with opaque abdominal musculature (8, 24–28, 35, 36).

  • IHHN disease in L. vannamei: the chronic disease, RDS, occurs in this species as a result of IHHNV infection. The severity and prevalence of RDS in infected populations of juvenile or older L. vannamei may be related to infection during the larval or early PL stages. RDS has also been reported in cultured stocks of L. stylirostris and P. monodon. Juvenile shrimp with RDS may display a bent (45° to 90° bend to left or right) or otherwise deformed rostrum, a deformed 6th abdominal segment, wrinkled antennal flagella, cuticular roughness, 'bubble-heads', and other cuticular deformities. Populations of juvenile shrimp
with RDS display disparate growth with a wide distribution of sizes and many smaller than expected (‘runted’) shrimp. The coefficient of variation (CV = the standard deviation divided by the mean of different size groups within a population) for populations with RDS is typically greater than 30% and may approach 90%, while IHHNV-free (and thus RDS-free) populations of juvenile *L. vannamei* and *L. stylirostris* usually show CVs of 10–30% (9–14, 27, 48, 49).

**b) Clinical methods**

- **Gross pathology:** see Section 3.a above.
- **Direct microscopic pathology:** no reliable methods have been developed.
- **Histopathology:** histological demonstration of prominent intranuclear, Cowdry type A inclusion bodies provides a provisional diagnosis of IHHNV infection. These characteristic IHHN inclusion bodies are eosinophilic and often halowed (with haematoxylin and eosin stains of tissues preserved with fixatives that contain acetic acid, such as Davidson’s AFA and Bouin’s solution) (4, 27), intranuclear inclusion bodies within chromatin-marginated, hypertrophied nuclei of cells in tissues of ectodermal (epidermis, hypodermal epithelium of fore- and hindgut, nerve cord and nerve ganglia) and mesodermal origin (haematopoietic organs, antennal gland, gonads, lymphoid organ, and connective tissue). Intranuclear inclusion bodies due to IHHNV may be easily confused with developing intranuclear inclusion bodies due to WSSV infection. *In-situ* hybridisation assay (see Section 3.c.ii of this chapter) of such sections with a specific DNA probe to IHHNV provides a definitive diagnosis of IHHNV infection (4, 10, 25, 27, 33).
- **Enhancement of infection:** the prevalence and severity of IHHNV infections may be ‘enhanced’ in a contained population by rearing shrimps in relatively crowded or stressful conditions. The ‘crowding stress’ factors may include high stocking densities and marginal water quality (i.e. low dissolved oxygen, elevated water temperature, or elevated ammonia or nitrite) in the holding tank water. These conditions may encourage expression of low-grade IHHNV infections and the transmission of the agent from carriers to previously uninfected hosts in the population resulting in increased prevalence and severity of infections that can be more easily detected using the available diagnostic and detection methods for IHHNV (27).

**c) Agent detection and identification methods**

- **Direct detection methods**
  
  i) **Direct microscopic methods:** no reliable methods have been developed.
  
  ii) **Agent isolation and identification**

  - *Antibody-based antigen detection methods:* none has been successfully developed.
  
  - *Molecular techniques:* dot-blot, ISH, and PCR tests for IHHNV have been developed and a number of methods and commercial products using these methods are readily available.

  - DNA probes for dot-blot and ISH applications: gene probe and PCR methods provide greater diagnostic sensitivity than do more traditional techniques for IHHN diagnosis that employ classic histological approaches. Furthermore, these methods have the added advantage of being applicable to nonlethal testing of valuable broodstock shrimp. A haemolymph sample may be taken with a tuberculin syringe, or an appendage (a pleopod for example) may be biopsied (5), and used as the sample for a direct dot-blot test.

  - Detection of IHHNV with nonradioactive DIG-labelled gene probes: the probe is labelled with a non-radioactive label, digoxigenin-11-dUTP (DIG-11-dUTP). The
system using DIG to label nucleic acid probes was developed by Boehringer Mannheim Biochemicals (this company now owned by Roche Diagnostic Corporation), which is described in the Roche DIG Nonradioactive Labeling and Detection Product Selection Guide and DIG Application Manual for Filter Hybridization System User’s Guide for Membrane Hybridization and from Boehringer Mannheim’s Nonradioactive In Situ Hybridization Application Manual (50, 51). The protocols given below use DIG-labelled probe to IHHNV produced by one of several methods. Probes may be produced using a fragment of cloned IHHNV DNA as the template by the random primed labelling method (27, 39). An alternative method for producing DIG-labelled probes using specific primers from the cloned IHHNV DNA and the Roche PCR DIG Probe Synthesis Kit™.

• Dot-blot hybridisation procedure: the dot-blot hybridisation method given below uses a DIG-labelled DNA probe for IHHNV and follows generally the methods outlined in Mari et al. (39) and Lightner (27). Formulas for the required reagents are given after the protocols.

i) Prepare a positively charged nylon membrane (Roche Diagnostics Cat. No. 1-209-299 or equivalent): cut pieces to fit samples and controls and mark with soft-lead pencil making 1 cm squares for each sample. Include a positive and a negative control on each filter. Lay out on to a piece of filter paper (Whatman 3MM).

ii) If necessary, dilute samples to be assayed in TE plus 50 µg/ml salmon sperm DNA, using 1 µl sample in 9 µl buffer in 1.5 ml microcentrifuge tubes. Samples for dot-blots can be haemolymph, tissues homogenised in TN buffer, or extracted DNA in 10 mM Tris/HCl.

iii) Boil samples for 10 minutes and quench on ice for 5 minutes. Briefly microfuge samples in the cold to bring down all liquid and to pellet any coagulated protein. Keep on ice until samples are dotted on to the membrane.

iv) Dot 1–3 µl of each sample on to an appropriate place on the filters. Allow to air-dry and then fix samples on to the membrane by baking at 80°C for 30 minutes or by UV cross-linking using a DNA transilluminator for 3 minutes.

v) Adjust a water bath to 68°C and prepare prehybridisation solution. For a 10 × 15 cm membrane, prepare 8 ml per membrane. Set a stirring hot plate set to ‘low’ and stir while warming the solution for 30 minutes until the blocking agent has dissolved and the solution is cloudy. Also prepare some heat-seal bags that are slightly larger in size than the membrane: five to six bags will be needed per membrane.

vi) Remove membranes from the oven or transilluminator and put into a heat-seal bag with 4 ml per membrane of prehybridisation solution. Seal the bags and put into a 68°C water bath for 0.5–1 hour.

vii) Boil the DIG-labelled probe for 10 minutes, quench on ice and then microfuge in the cold to bring all the liquid down in the microcentrifuge tube. Keep on ice. Remove the prehybridisation solution from the bags. Add 2 ml of fresh prehybridisation solution to each bag and then add the correct, predetermined amount of DIG-labelled probe to each, mixing well as it is being added. Seal the bags, place back in the 68°C water bath and incubate for 8–12 hours.

viii) Wash membranes well with:

\[
2 \times \text{standard saline citrate (SSC)/0.1\% sodium dodecyl sulfate (SDS)} \\
0.1 \times \text{SSC/0.1\% SDS} \\
\text{(use 4 ml/filter and seal in bags)}
\]

5 minutes at room temperature

\[
3 \times 15 \text{ minutes at } 68^\circ \text{C}
\]
Chapter 2.3.6. - Infectious hypodermal and haematopoietic necrosis

Buffer I  1 ×  5 minutes at room temperature
Buffer II  1 ×  30 minutes at room temperature
Buffer I  1 ×  5 minutes at room temperature
(Buffers are prepared ahead of time).

ix) React the membrane in bags with anti-DIG AP conjugate (Roche Diagnostics 1-093-274) diluted 1/5000 in Buffer I. Use 3 ml per membrane, incubate for 30–45 minutes at room temperature on a shaker platform.

x) Wash membrane well with:
Buffer I  2 ×  15 minutes at room temperature
Buffer III  1 ×  5 minutes at room temperature

xi) Develop the membranes in bags using 3 ml per membrane of development solution (nitroblue tetrazolium salt [NBT]/X-phosphate in Buffer III) made up just prior to use. React in the dark at room temperature for 1–2 hours. Stop the reactions in Buffer IV and dry the membranes on 3MM filter paper.

xii) Photograph the results. (Colour fades over time.)

xiii) Store dry membranes in heat-seal bags.

• The in-situ hybridisation method given below uses a DIG-labelled DNA probe for IHHNV and follows generally the methods outlined in Mari et al. (39) and Lightner (27). Formulas for the required reagents are given after the protocols.

i) Embed tissue in paraffin and cut sections at 4–6 µm thickness. Place sections on to positively charged microscope slides (do not put gelatin in water to float sections; use just water).

ii) Put slides in a slide rack, such as a Tissue-Tek rack. Heat the slides in an oven for 45 minutes at 60°C. In the staining centre, rehydrate the tissue as follows:
Xylene (or suitable substitute)  3 ×  5 minutes each
Absolute alcohol  2 ×  1 minute each
95% alcohol  2 ×  10 dips each
80% alcohol  2 ×  10 dips each
50% alcohol  1 ×  10 dips
Distilled water  six rinses (do not let slides dry out)

iii) Wash the slides for 5 minutes in phosphate buffered saline (PBS or TNE). Prepare fresh proteinase K at 100 µg/ml in PBS (or TNE). Place slides flat in a humid chamber, pipette on 500 µl of the proteinase K solution and incubate for 10–15 minutes at 37°C. Drain fluid on to blotting paper.

iv) Return slides to slide rack. Fix sections in 0.4% cold formaldehyde for 5 minutes at room temperature.

v) Incubate slides in 2 × SSC for 5 minutes at room temperature.

vi) With slides flat, add 0.5–1 ml prehybridisation buffer and incubate in a humid chamber for 15–30 minutes at 37°C.

vii) Boil the DIG-labelled probe for 10 minutes and quench on ice; spin briefly in the cold and keep on ice. Dilute the probe to 25 ng/ml in prehybridisation solution and cover the tissue with 250 µl of the solution. Incubate the slides for 2–4 hours at 42°C or overnight at 37°C in a humid chamber. Drain fluid on to blotting paper. During this incubation, prewarm the wash buffers at 37°C.
viii) Place slides in slide rack. Wash the slides as follows:

- $2 \times$ SSC: $2 \times 5$–30 minutes at $37^\circ$C
- $1 \times$ SSC: $2 \times 5$ minutes at $37^\circ$C
- $0.5 \times$ SSC: $2 \times 5$ minutes at $37^\circ$C

ix) Wash the slides for 5 minutes in Buffer I at room temperature. Put the slides flat in a humid chamber and block with 0.5 ml per slide of Buffer II. Incubate for 15 minutes at $37^\circ$C. Drain the fluid on to blotting paper.

x) Dilute the anti-DIG AP conjugate (Roche Applied Science cat. 10686322) 1/1000 in Buffer II (1 µl anti-DIG AP per 1 ml buffer). Cover tissue with 500 µl of diluted conjugate and incubate in a humid chamber for 30 minutes at $37^\circ$C.

xi) Place the slides in a slide rack. Wash in Buffer I twice for 5–10 minutes each time at room temperature. Wash one time with Buffer III for 5–10 minutes.

xii) Prepare the development solution by first adding 4.5 µl NBT per 1 ml buffer III. Mix well. Then add 3.5 µl X-phosphate per ml of solution and mix well. Pipette on 500 µl per slide and incubate in a humid chamber in the dark for 2–3 hours at room temperature.

xiii) Stop the reaction by returning the slides to a slide rack and washing in Buffer IV for 15 minutes at room temperature.

xiv) Counterstain the slides by dipping for 5 minutes in 0.5% aqueous Bismarck brown Y.

xv) Dehydrate the slides in the staining centre as follows:

- 95% alcohol: $3 \times$ 10 dips each
- Absolute alcohol: $3 \times$ 10 dips each
- Xylene (or suitable substitute): $4 \times$ 10 dips each

Do not allow the slides to dry out – leave them in the last xylene (or xylene substitute) container until ready for cover-slips.

xvi) Mount with cover-slips and mounting medium (Permount).

xvii) Examine the slides under bright-field for dark-blue or black precipitate that marks sites where IHHNV DNA is present. Pathodiagnostic intranuclear Cowdry type A inclusions are well marked with probe. Also often marked are host cell nuclei without obvious inclusions, cytoplasmic inclusions, and accumulation of free virus in the tissue spaces and haemolymph.

**NOTE:** Always run a known positive and negative control.

- Reagent formulas for *in-situ* hybridisation method

  i) $10 \times$ phosphate buffered saline

  \[
  \begin{align*}
  \text{NaCl} & \quad 160 \text{ g} \\
  \text{KH}_2\text{PO}_4 & \quad 4 \text{ g} \\
  \text{Na}_2\text{HPO}_4 & \quad 23 \text{ g} \\
  \text{KCl} & \quad 4 \text{ g} \\
  \text{DD H}_2\text{O} & \quad 1950 \text{ ml (qs to 2 litres)}
  \end{align*}
  \]

  pH to 8.2 with NaOH; autoclave to sterilise; store at room temperature. To make 1 × PBS, dilute 100 ml 10 × PBS in 900 ml DD H$_2$O; Filter 1 × solution through 0.45 µm filter; store at 4°C.
ii) 10 × Tris/NaCl/EDTA (TNE) buffer

Tris base  60.57 g
NaCl  5.84 g
EDTA  3.72 g
DD H₂O  900 ml (qs to 1 litre)

pH to 7.4 with concentrated or 5 M HCl. To make 1 × TNE, dilute 100 ml 10 ×
TNE in 900 ml DD H₂O; Filter 1 × solution through 0.45 µm filter; store at 4°C.

iii) Proteinase K, 100 µg/ml (prepare just prior to use)

PBS  10 ml 1 × PBS
Proteinase K  1 mg

iv) 0.4% formaldehyde

37% formaldehyde  5.4 ml
DD H₂O  500 ml

Store at 4°C; can be reused up to four times before discarding.

v) Prehybridisation buffer (50 ml final volume)

4 × SSC  10 ml 20 × SSC
50% formamide  25 ml 100% formamide
1 × Denhardt’s  2.5 ml 20 × Denhardt’s
5% dextran sulfate  10 ml 25% dextran sulfate
Warm to 60°C

Boil 2.5 ml of 10 mg/ml salmon sperm DNA and add to buffer for final
concentration of 0.5 mg/ml salmon sperm DNA; store at 4°C.

vi) 20 × SSC buffer

3M NaCl  175.32 g NaCl
0.3 M Na₃C₆H₅O₇·2H₂O  88.23 g Na citrate.2H₂O
DD H₂O  1000 ml (qs)
pH to 7.0; autoclave; store at 4°C.

To make 2 × SSC, dilute 100 ml 20 × SSC in 900 ml DD H₂O; To make 1 × SSC,
dilute 50 ml 20 × SSC in 950 ml DD H₂O; To make 0.5 × SSC, dilute 50 ml 20 ×
SSC in 1950 ml DD H₂O. Filter solutions through 0.45 µm filter; store at 4°C.

vii) 20 × Denhardt’s solution

BSA (Fraction V)  0.4 g bovine serum albumin
Ficoll 400  0.4 g Ficoll
PVP 360  0.4 g polyvinylpyrollidone
DD H₂O  100 ml

Filter solutions through 0.45 µm filter; store at 4°C. Aliquot 2.5 ml into small
tubes and store frozen.

viii) 25% dextran sulfate

Dextran sulfate  25 g
DD H₂O  100 ml

Mix to dissolve; store frozen in 10 ml aliquots.

ix) Salmon sperm DNA (10 mg/ml)

Salmon sperm DNA  0.25 g
DD H₂O  25 ml
To prepare, warm the water and slowly add the DNA with stirring until completely dissolved; boil for 10 minutes; shear the DNA by pushing through an 18-gauge needle several times; aliquot 2.5 ml into small tubes and store frozen; boil for 10 minutes just before using to facilitate mixing in the buffer.

x) 10 × Buffer I

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (g)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris/HCl</td>
<td>121.1</td>
<td>Tris base</td>
</tr>
<tr>
<td>1.5 M NaCl</td>
<td>87.7</td>
<td>NaCl</td>
</tr>
<tr>
<td>DD H₂O</td>
<td>1000 ml (qs)</td>
<td></td>
</tr>
</tbody>
</table>

pH to 7.5 with HCl. Autoclave; store at 4°C.

To make 1 × Buffer I, dilute 100 ml of 10 × stock in 900 ml DD H₂O. Filter through 0.45 µm filter; store at 4°C.

xi) Buffer II (blocking buffer)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking reagent</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Buffer I</td>
<td>50 ml 1 × Buffer I</td>
</tr>
</tbody>
</table>

Store at 4°C for up to 2 weeks.

xii) Buffer III

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (g)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM Tris/HCl</td>
<td>1.21 g</td>
<td>Tris base</td>
</tr>
<tr>
<td>100 mM NaCl</td>
<td>0.58 g</td>
<td>NaCl</td>
</tr>
<tr>
<td>DD H₂O</td>
<td>100 ml (qs)</td>
<td></td>
</tr>
</tbody>
</table>

pH to 9.5 with HCl

Then add:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM MgCl₂</td>
<td>1.02 g MgCl₂·6H₂O</td>
</tr>
</tbody>
</table>

Filter through 0.45 µm filter; store at 4°C.

xiii) 10% polyvinyl alcohol (PVA)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyvinyl alcohol</td>
<td>10 g</td>
</tr>
<tr>
<td>DD H₂O</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

To prepare, slowly add PVA to water while stirring on low heat. (It takes 2–3 hours for PVA to go into solution.) Dispense 10 ml per tube and store frozen at –20°C.

xiv) Development solution

Mix 90 ml Buffer III with 10 ml of 10% PVA. Store at 4°C. Just prior to use, for each 1 ml of Buffer III with PVA add:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5 µl NBT</td>
<td>75 mg NBT/ml in 70% dimethylformamide</td>
</tr>
<tr>
<td>(Roche Diagnostics 1-383-213)</td>
<td></td>
</tr>
<tr>
<td>3.5 µl X-phosphate</td>
<td>5-bromo-4-chloro-3-indoyl phosphate, toluidine salt</td>
</tr>
<tr>
<td>(50 mg/ml in dimethylformamide)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Roche Diagnostics 1-383-221)</td>
</tr>
</tbody>
</table>

xv) Buffer IV

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Tris/HCl</td>
<td>1.21 g Tris base</td>
</tr>
<tr>
<td>1 mM EDTA</td>
<td>0.37 g EDTA.2H₂O (disodium salt)</td>
</tr>
<tr>
<td>DD H₂O</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

pH to 8.0 with HCl. Filter through 0.45 µm filter; store at 4°C.
xvi) 0.5% Bismarck Brown Y

\[
\begin{align*}
\text{Bismarck Brown Y} & \quad 2.5 \text{ g} \\
\text{DD H}_2\text{O} & \quad 500 \text{ ml}
\end{align*}
\]

Dissolve the stain in water. Filter through a Whatman No. 1 filter; store at room temperature.

- Polymerase chain reaction

Several single step PCR methods are available for IHHNV detection (23, 45, 53–55, 57). A number of commercial PCR kits are available for IHHNV detection. A nested method is also available, but only as a kit from a commercial source (43).

There are multiple geographical variants of IHHNV, some of which are not detected by all of the available methods for IHHNV. Two primers sets 392F/R and 389F/R appear to be the most suitable for detecting all the known genetic variants of IHHNV (23, 56, 57, 59). Hence, confirmation of unexpected positive and/or negative PCR results for IHHNV with a second primer set, or use of another diagnostic method (i.e. real time PCR, bioassay, ISH) is advisable.

Table 1. Recommended primer sets for 1-step PCR detection of IHHNV

<table>
<thead>
<tr>
<th>Primer</th>
<th>Product</th>
<th>Sequence</th>
<th>G:C/Temp.</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>389F</td>
<td>389 bp</td>
<td>5'-CGG-AAC-ACA-ACC-CGA-CTT-TA-3'</td>
<td>50/72°C</td>
<td>GenBank</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AF218266</td>
</tr>
<tr>
<td>389R</td>
<td></td>
<td>5'-GGC-CAA-GAC-CAA-AAT-ACG-AA-3'</td>
<td>45/71°C</td>
<td></td>
</tr>
<tr>
<td>77012F</td>
<td>356 bp</td>
<td>5'-ATC-GGT-GCA-CTA-CTC-GGA-3'</td>
<td>50/68°C</td>
<td>GenBank</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AF218266</td>
</tr>
<tr>
<td>77353R</td>
<td></td>
<td>5'-TCG-TAC-TGG-CTG-TTC-ATC-3'</td>
<td>55/63°C</td>
<td></td>
</tr>
<tr>
<td>392F</td>
<td>392 bp</td>
<td>5'-GGG-CGA-ACC-AGA-ATC-ACT-3'</td>
<td></td>
<td>Tang et al.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(54)</td>
</tr>
<tr>
<td>392R</td>
<td></td>
<td>5'-ATC-CGG-AGG-AAT-CTG-ATG-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Primers 389F/R and 392F/R described above are from the nonstructural protein-coding region (ORF 1) of the IHHNV genome. Primers 77353/77012 are from a region in between the nonstructural and the structural (coat protein) protein coding regions of the genome. In the event that results are ambiguous using the 389F/R ‘universal’ primer set, it is recommended to use primers from a different region of the genome for confirmatory testing. In this case, that would mean primers 77012/77353 or the 392F/R primer sets.

- The PCR method described below for IHHNV follows generally the methods outlined in Nunan et al. (45). Cumulative experience with the technique has led to modifications with respect to template (DNA extraction of clinical specimens), choice of primers (Table 1), and volume of reaction

  i) Use as template, the DNA extracted from ground tissue homogenate (TN buffer, 0.4 M NaCl, 20 mM Tris, pH 7.4) or haemolymph (collected with a small amount of 10% sodium citrate) or from tissue or haemolymph that was fixed in 95%
ethanol and then dried. A control consisting of tissue or haemolymph from known negative animals should be included during the DNA extraction step. The DNA can be extracted by a variety of methods but excellent results have been obtained using kits from Roche Diagnostics (Cat. No. 1-796-828) or Qiagen (Cat. No. 51304), or reagents from Gibco Life Sciences (DNazol Cat. No. 10503-027). Spectrophotometric readings of the final DNA will indicate the purity of the DNA and the amount of total DNA extracted from the sample. Use 1–5 µl of extracted DNA per 50 µl reaction volume.

**Note:** Homogenised tissue material or haemolymph that has not had the DNA extracted may also be used directly as template, but the concentration of virus will be lower and there may be substances present that will inhibit the PCR.

ii) The following controls should be included in every PCR assay for IHHNV: a) DNA from a known negative tissue sample; b) DNA from a known positive sample (either from tissue or haemolymph or from a plasmid clone that contains the fragment that the specific set of primers amplifies; and c) a ‘no template’ control.

iii) Use as primers, primers 389F and 389R, which elicit a band 389 bp in size from IHHNV-infected material, or primers 77012F and 77353R, which elicit a band 356 bp in size from IHHNV-infected material. Prepare primers at 100 ng/µl in distilled water. Keep frozen at –70°C.

iv) Use a ‘hot start’ method for the polymerase: if you use Applied Biosystem’s AmpliTaq Gold, this involves a 5-minute step at 95°C to denature DNA prior to the primers binding and activation of the enzyme. This programme is then linked to the cycling programme (35 cycles) and an extension programme. The programme is set as follows:

<table>
<thead>
<tr>
<th>Hot start Programme 1</th>
<th>Linked to Programme 2</th>
<th>Linked to Programme 3</th>
<th>Linked to Programme 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 minutes 95°C</td>
<td>30 seconds 95°C</td>
<td>7 minutes 72°C</td>
<td>4°C until off</td>
</tr>
<tr>
<td></td>
<td>30 seconds 55°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 minute 72°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>35 cycles</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

v) Prepare a ‘master mix’ consisting of water, 10 × PCR buffer, the four dNTPs, the two primers, MgCl₂, AmpliTaq Gold and water (assume use of 1 µl of template; if using more, adjust water accordingly). Add mix to each tube. Use thin-walled tubes designed for PCR. Always run a positive and a negative control.

‘Master Mix’:

<table>
<thead>
<tr>
<th>DD H₂O</th>
<th>32.5 µl × number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 × PCR buffer</td>
<td>5 µl × number of samples</td>
</tr>
<tr>
<td>10 mM dTTP</td>
<td>1 µl × number of samples</td>
</tr>
<tr>
<td>10 mM dATP</td>
<td>1 µl × number of samples</td>
</tr>
<tr>
<td>10 mM dCTP</td>
<td>1 µl × number of samples</td>
</tr>
<tr>
<td>10 mM dGTP</td>
<td>1 µl × number of samples</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>4 µl × number of samples</td>
</tr>
<tr>
<td>Forward primer (100 ng/µl)</td>
<td>1.5 µl × number of samples</td>
</tr>
<tr>
<td>Reverse primer (100 ng/µl)</td>
<td>1.5 µl × number of samples</td>
</tr>
<tr>
<td>AmpliTaq Gold</td>
<td>0.5 µl × number of samples</td>
</tr>
</tbody>
</table>

Vortex this solution to mix all reagents well; keep on ice.

**Note:** The volume of the PCR reaction may be modified. Previously, the PCR reactions for IHHNV were run in 100 µl volumes, but it is not necessary to use that amount of reagents, therefore 50 µl volumes are described in this procedure.
Likewise, the PCR reactions can also be run in volumes as small as 25 µl. To do this, increase or decrease the volume of the reagents accordingly.

vi) For 50 µl reaction mix, add 49 µl Master Mix to each tube and then add 1 µl of the sample to be tested.

vii) Vortex each tube, spin quickly to bring down all liquid. If your thermal cycler does not have a heated lid to prevent condensation, then carefully overlay the top of each sample with 25–50 µl mineral oil and re-cap the tubes. Insert tubes into thermal cycler and start programme 1 (‘hot start’), which is linked to cycling, extension and soak cycles.

viii) If mineral oil was used, recover samples from under the mineral oil using a pipette set at 50 µl and transfer to a fresh tube. Using the long-tipped pipette tips (designed for loading gels) results in less oil being carried over with the sample.

ix) Run 10 µl of the sample in a 1.5% agarose gel (containing 0.5 µg/ml ethidium bromide to stain the DNA). Look for the 389 bp band (if using primers 389F and 389R) or for the 356 bp band (if using primers 77012F and 77353R). Bands are not always seen, as it is necessary to have at least 10 ng DNA/µl to see DNA in a gel. A Southern transfer of the gel or a dot-blot can be run for more sensitive detection. The DNA can also be precipitated (0.3 M sodium acetate and 2.5 volumes 100% ethanol, –70°C, for 1–3 hours, centrifuge for 20 minutes) and resuspended in 1/10th volume (i.e. 4 µl) TE (10 mM Tris, 1 mM EDTA, pH 7.5) or water and either re-run in the gel or tested in a dot-blot.

• Real-time PCR method for IHHNV

Real-time PCR methods have been developed for the detection of IHHNV. These methods offer extraordinary sensitivity that can detect a single copy of the target sequence from the IHHNV genome (18, 55).

The real-time PCR method using TaqMan chemistry described below for IHHNV follows generally the method used in Tang & Lightner (55).

i) The PCR primers and TaqMan probe are selected from a region of the IHHNV genomic sequence (GenBank AF218266) that encodes for nonstructural protein. The primers and TaqMan probe are designed by the Primer Express software (Applied Biosystems). The upstream (IHHNV1608F) and downstream (IHHNV1688R) primer sequences are: 5'-TAC-TCC-GGA-CAC-CCA-ACC-A-3' and 5'-GGC-TCT-GGC-AGC-AAA-GGT-AA-3', respectively. The TaqMan probe (5'-ACC-AGA-CAT-AGA-GCT-ACA-ATC-CTC-GCC-TAT-TTG-3'), which corresponds to the region from nucleotide 1632 to 1644, is synthesised and labelled with fluorescent dyes 5-carboxyfluoroscein (FAM) on the 5' end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end (Applied Biosystems, part no. 450025).

ii) Preparation of DNA template: the extraction of purification of DNA template is the same as that described in the section of traditional PCR.

iii) The PCR reaction mixture contains: TaqMan Universal PCR Master Mix (Applied Biosystems, part no. 4324018), 0.3 µM of each primers, 0.15 µM of TaqMan probe, 5–50 ng DNA, and water in a reaction volume of 25 µl. For optimal results, the reaction mixture should be vortexed and mixed well.

iv) Amplification is performed with the GeneAmp 5700 Sequence Detection System (Applied Biosystems; ABI PRISM 7000, 7300, or 7500 can also be used). The cycling profile is: activation of AmpliTaq Gold for 10 minutes at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute.
v) At the end of reaction, real-time fluorescence measurements will be taken with a built-in charge-coupled device (CCD) camera. A threshold will be set to be above the baseline that begins to detect the increase in signal associated with an exponential increase of PCR product. Samples will be defined as negative if the Ct (threshold cycle) values exceed 40 cycles. Samples with Ct value lower than 40 cycles are considered to be positive. To confirm the real-time PCR results, an aliquot of PCR product can be subjected to electrophoresis on a 4% ethidium bromide-agarose gel and photographed. An 81-bp DNA fragment can be visualised in the samples that are positive for IHHNV.

vi) It is necessary to include a ‘no template’ control in each reaction run. This is to rule out the presence of fluorescence contaminants in the reaction mixture or in the heat block of thermal cycler. A positive control should also be included, they can be a plasmid containing the target sequence, or purified virions, or DNA from IHHNV-infected tissue.

4. RATING OF TESTS AGAINST PURPOSE OF USE

The methods currently available for surveillance, detection, and diagnosis of IHHNV are listed in Table 2. The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = the method is a standard method with good diagnostic sensitivity and specificity; C = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and D = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category A or B have undergone formal standardisation and validation (see Chapter 1.1.2), their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 2. IHHNV surveillance, detection and diagnostic methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance</th>
<th>Presumptive</th>
<th>Confirmatory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Larvae</td>
<td>PLs</td>
<td>Juveniles</td>
</tr>
<tr>
<td>Gross signs</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Bioassay</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Direct LM</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Histopathology</td>
<td>D</td>
<td>D</td>
<td>C</td>
</tr>
<tr>
<td>TEM</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>Antibody-based assays</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td>DNA Probes in situ</td>
<td>D</td>
<td>D</td>
<td>B</td>
</tr>
<tr>
<td>PCR</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Sequence</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
</tbody>
</table>

PLs = postlarvae; LM = light microscopy; TEM = Transmission electron microscopy; PCR = polymerase chain reaction

5. CORROBORATIVE DIAGNOSTIC CRITERIA

a) Definition of suspect case

Poor hatching success of eggs and poor survival and culture performance of the larval and PL stages (43) when broodstock are used from wild or farmed stocks where IHHNV is enzootic.

In farmed stocks of L. stylirostris, juveniles, subadults and adults may show persistently high mortality rates. In L. vannamii, L. stylirostris, and possibly P. monodon IHHNV infected stocks may
show poor and highly disparate growth, poor overall culture performance, and cuticular deformities, including especially bent rostrums and deformed 6th abdominal segments.

Demonstration of eosinophilic to pale basophilic intranuclear inclusion bodies in the typical target tissues for IHHNV. Because IHHNV intranuclear inclusion bodies are nearly identical in appearance to those occurring in the early stages of WSSV infections, their presence in tissue sections should be considered as a presumptive diagnosis of IHHNV until confirmed with a second test method such as dot-blot or ISH with IHHNV specific DNA probes or positive PCR test results for IHHNV.

b) Definition of confirmed case

Any combination of at least two of the following three methods (with positive results):

Positive dot-blot hybridisation test results for IHHNV.

ISH positive histological signal to IHHNV-type lesions.

PCR positive results for IHHNV.

6. DIAGNOSTIC/DETECTION METHODS TO DECLARE FREEDOM

Two years of history of negative test results for IHHNV using PCR performed on samples of the appropriate type and sample size.

REFERENCES


51. ROCHE APPLIED SCIENCE (2006). DIG Nonradioactive Labeling and Detection Product Selection Guide. Catalog Number 03 908 089 001. Roche Diagnostics, Indianapolis, USA.


* * *

**NB:** There is an OIE Reference Laboratory for Infectious hypodermal and haematopoietic necrosis (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: [www.oie.int](http://www.oie.int)).
CHAPTER 2.3.7.

CRAYFISH PLAGUE
(Aphanomyces astaci)

1. CASE DEFINITION

For the purpose of this chapter, crayfish plague is considered to be infection of crayfish with Aphanomyces astaci Schikora. Aphanomyces astaci is a member of a group of organisms commonly known as the water moulds, as is Saprolegnia parasitica. Although long regarded to be fungi, this group, the Oomycetida, are not members of the Eumycota and are now classified with diatoms and brown algae in a group called the Stramenopiles or Chromista.

2. INFORMATION FOR THE DESIGN OF SURVEILLANCE PROGRAMMES

a) Agent factors

There is evidence that established older A. astaci strains that have been present in Europe for many years are less aggressive than are newer strains introduced with crayfish imports from North America since the 1960s. Some isolates from warmer waters in Spain have temperature/growth curves with higher optimum temperatures compared with those from northern Europe (4).

Although A. astaci is not an obligate parasite and will grow well under laboratory conditions on artificial media, in the natural environment it does not survive well for long periods in the absence of a suitable host.

Aphanomyces astaci, both in culture and in infected crayfish, is killed by a short exposure to temperatures of 60°C or to temperatures of −20°C or less for 48 hours or more (7). Sodium hypochlorite and iodophores are effective for disinfection of contaminated equipment. Thorough drying of equipment (>24 hours) is also effective as Aphanomyces is not resistant to desiccation.

The life cycle is simple with vegetative hyphae invading and ramifying through host tissues, eventually producing extramatrical sporangia that release amoeboid primary spores, each of which then germinates as a biflagellate zoospore. Biflagellate zoospores swim in the water column and, on encountering a susceptible host, attach and germinate to produce invasive vegetative hyphae. Zoospores are capable of repeated encystment and re-emergence, extending the period of their infective viability.

There is good field and experimental evidence that movements of fish from areas in which crayfish plague is active can transmit infection from one watershed to another (3, 7). Aphanomyces astaci can also be spread by contaminated equipment (nets, boots clothing etc).

b) Host factors

All stages of all populations of European crayfish, including the Noble crayfish (Astacus astacus) of north-west Europe, the white claw crayfish (Austropotamobius pallipes) of south-west and west Europe, the related Austropotamobius torrentium (mountain streams of south-west Europe) and the slender clawed or Turkish crayfish (Astacus leptodactylus) of eastern Europe and Asia Minor are highly susceptible. Laboratory challenges have demonstrated that Australian species of crayfish are also highly susceptible. The only other crustacean known to be capable of infection by
Chapter 2.3.7. - Crayfish plague (*Aphanomyces astaci*)

*A. astaci* is the Chinese mitten crab (*Eriocheir sinensis*) and this only under laboratory conditions. North American crayfish such as the signal crayfish (*Pacifastacus leniusculus*), Louisiana swamp crayfish (*Procambarus clarkii*) and *Orconectes* spp. are infected by *A. astaci*, but under normal conditions are able to control the infection. All North American crayfish are therefore actual or potential lifelong carriers of crayfish plague.

In susceptible species, all tissues can be infected, but in earlier stages of infection, once the cuticle has been penetrated, *A. astaci* primarily spreads through connective tissues and blood vessels, although eventually all tissues are invaded.

In North American crayfish, infection is usually restricted to the cuticle.

c) **Disease pattern**

The primary transmission mechanism is the zoospore of *A. astaci*, which swims actively in the water column and has been demonstrated to show positive chemotaxis toward crayfish.

Geographical distribution: the disease first occurred in Europe in the third quarter of the 19th century in the Franco–German border region. From there 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 north-west to Finland and finally, in 1907, to Sweden. In the 1960s the first outbreaks in Spain were reported and in the 1980s further extensions of infection to the British Isles, Turkey, Greece and Norway were reported (1). The reservoir of the original infections in the 19th century was never established; *Orconectes* spp. were not introduced until the 1890s, but the post-1960s extensions are largely linked to movements of North American crayfish introduced more recently for purposes of crayfish farming. Escapes of such introduced species were almost impossible to prevent and *Pacifastacus leniusculus* and *Procambarus clarkii* are now widely naturalised in many parts of Europe. Not all such naturalised crayfish populations are infected, but most populations are suspected to be infected.

When the infection first reaches a naive population of susceptible crayfish, rapid mortality occurs so that the bottoms of lakes, rivers and streams are covered with dead and dying crayfish. A band of mortality will spread from the initial outbreak site downstream at the speed of the flow of the river; upstream spread is slower. The virulence of *A. astaci* is such that no survivors remain. Where populations of susceptible crayfish are low, fewer zoospores will be produced, the spread of infection will be slower and evidence of mortality less dramatic. Water temperature has some effect on speed of spread and this is most evident in low-density crayfish populations where animal-to-animal spread takes longer and challenge intensity will be lower. Lower water temperatures and reduced numbers of zoospores are associated with slower mortalities and a greater range of clinical signs in affected animals (3).

Although remnant populations of susceptible crayfish species remain in many European watersheds, the dense populations that existed 150 years ago are now all gone. Characteristically, populations of susceptible crayfish may re-establish, but once population density and geographical distribution is sufficient for susceptible animals to come into contact with infection, new outbreaks of crayfish plague in the form of large-scale mortalities will occur.

d) **Control and prevention**

The only practical means of control of crayfish plague is to prevent its introduction into susceptible stocks. Currently there is no evidence that vaccines offer long-term protection in crustaceans and even if this were not to be the case, vaccination of natural populations of crayfish is impossible. The traditional aquaculture fungicide, malachite green, has been shown to be effective in preventing the transmission of crayfish plague with fish movements, but its use is now no longer acceptable. Pyceze has not been tested against *A. astaci*. Virulence of *A. astaci* towards
susceptible crayfish is such that mortalities that have been investigated have always reached 100%, rendering a search for resistant European crayfish impossible. In the 125 years that crayfish plague has been recognised in Europe, no evidence of resistant populations of European crayfish has been found. The fact that North American crayfish are resistant means that selection for resistance must be possible and laboratory studies using *A. astaci* strains attenuated for virulence might be successful. No such study as yet been undertaken.

3. DIAGNOSTIC METHODS

Until recently the diagnosis of crayfish plague strictly required the isolation and characterisation of the pathogen, *A. astaci*, using simple mycological media fortified with antibiotics to control bacterial contamination (3). 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 that can cause such total mortality 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 lesions is so large that, except for the experienced eye, such clinical signs are of limited diagnostic value. The publication of suitably validated molecular methods has now rendered diagnosis easier and less dependent on experience.

a) Field diagnostic methods

Clinical signs: gross clinical signs are extremely variable and depend on challenge severity and water temperatures. 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 may show evident loss of co-ordination in their movements, and easily fall over on their backs and remain unable to right themselves. Often, however, unless waters are carefully observed, the first sign that there is a problem will be the presence of large numbers of dead crayfish in a river or lake (3). In susceptible species where sufficient numbers of crayfish are present to allow infection to spread rapidly, particularly at summer water temperatures, infection will spread quickly and stretches of over 50 km may lose all their crayfish in less than 21 days from the first observed mortality. Crayfish plague has unparalleled severity of effect, infected susceptible crayfish do not survive – 100% mortality is the norm. It must be emphasised, however, that the presence of large numbers of dead crayfish, even in crayfish plague affected watersheds, is not on its own sufficient for diagnosis. The general condition of other aquatic fauna must be assessed. Mortality or disappearance of other aquatic crustaceans as well as crayfish, even though fish survive, may indicate pollution (e.g. insecticides such as cypermethrin have been associated with initial mis-diagnoses).

b) Clinical methods

• Gross pathology

Depending on a range of factors, foci of infection in crayfish may be easily seen by the naked eye or may not be discernable despite careful examination. Such foci can best be seen under a low power stereo microscope and are most commonly recognisable by localised whitening of the muscle beneath the cuticle. In some cases a brown colouration of cuticle and muscle may occur, and in others, hyphae are visible in infected cuticles in the form of fine brown (melanised) tracks in the cuticle itself. Sites for particular examination include the intersternal soft ventral cuticle of the abdomen and tail, the cuticle of the perianal region, the cuticle between the carapace and tail, the joints of the pereiopods (walking legs), particularly the proximal joint and finally the gills.
Chapter 2.3.7. - Crayfish plague (*Aphanomyces astaci*)

- **Microscopic pathology**
  - **Wet mounts**

Small pieces of soft cuticle excised from the regions mentioned above and examined under a compound microscope using low to medium power will confirm the presence of aseptate fungal hyphae 7–9 µm wide. The presence of host haemocytes and melanisation closely associated with and encapsulated in the hyphae give good presumptive evidence that the hyphae represent a pathogen rather than a secondary opportunist invader. In some cases, examination of the surface of such mounted cuticles will demonstrate the presence of characteristic *A. astaci* sporangia with clusters of encysted primary spores (see below).

- **Fixed sections**

Unless the selection of tissue for fixation has been well chosen, *A. astaci* hyphae can be difficult to find in stained preparations. Additionally, such material does not prove that any hyphae observed are those of the primary pathogen. A histological staining technique is the Grocott silver stain, counterstained with conventional haematoxylin and eosin.

c) **Agent detection and identification methods**

- **Direct detection methods**
  - **Microscopic methods**
    - **Wet mounts**

As indicated above, presumptive identification of *A. astaci* may be made from the presence of hyphae and sporangia of the correct morphological types (see below) on the surface of crayfish exoskeletons.

  - **Agent isolation and identification**
    - **Isolation and culture**

Isolation methods are as described by Alderman & Polglase (2). An agar medium (isolation medium) is used that contains yeast extract and glucose in river water with antimicrobial agents (penicillin G and oxolinic acid) to prevent the growth of most bacteria and enable easy and rapid isolation of the pathogen.

*Isolation medium (IM)*: 12.0 g agar; 1.0 g yeast extract; 5.0 g glucose; 10 mg oxolinic acid; 1000 ml river water; and 1.0 g penicillin G (sterile) added after autoclaving and cooling to 40°C. River water = any natural river or lake water as opposed to demineralised water.

Simple aseptic excision of infected tissues, which are then placed as small pieces (1–2 mm³) on the surface of isolation medium plates, will normally result in successful isolation of *A. astaci* from moribund or recently dead (<24 hours) animals. Depending on a range of factors, foci of infection in crayfish may be easily seen by the naked eye or may not be discernable despite careful examination. Such foci can best be seen under a low power stereo microscope and are most commonly recognisable by localised whitening of the muscle beneath the cuticle. In some cases a brown colouration of cuticle and muscle may occur, and in others, hyphae are visible in infected cuticles in the form of fine brown (melanised) tracks in the cuticle itself. Sites for particular examination include the intersternal soft ventral cuticle of the abdomen and tail, the cuticle of the perianal region, the cuticle between the carapace and tail, the joints of the pereiopods (walking legs), particularly the proximal joint and finally the gills.
Provided that care is taken in excising infected tissues for isolation, contaminants need not present significant problems. Small pieces of cuticle and muscle may be transferred to a Petri dish of sterile distilled water and there further cut into small pieces with sterile instruments for transfer to IM isolation medium. Suitable instruments for such work are cataract knives and fine electron microscope or instrument-grade forceps and scissors.

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 and (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, leaving 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 river water (natural water with available cations encourages sporulation better than does distilled water), sporangia form readily in 20–30 hours at 16°C and 12–15 hours at 20°C. Thalli transferred from broth culture may be washed with sterile river water in a sterile stainless steel sieve, before transfer into fresh sterile river water for induction of sporulation. Thalli in agar should be transferred by cutting out a thin surface sliver of agar containing the fungus so that a minimum amount of nutrient containing agar is transferred. Always use a large volume of sterile river water relative to the amount of fungus being transferred (100:1). Sporangia are myceloid, terminal or intercalary, developing from undifferentiated vegetative hyphae. The sporangial form is variable: terminal sporangia are simple, developing from new extramatrical hyphae, while 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 × 8 µm) that 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 achlyoid, that is, the first spore stage is an aplanospore that encysts at the sporangial orifice and probably represents the suppressed saprolegniaceous primary zoospore. No evidence has been found 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 minutes) and the individual primary spores (=cytoplasmic units) pass through the tip of the sporangium and accumulate around the sporangial orifice. The 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. The duration from release to encystment is 2–5 minutes. 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 spores remain together as a coherent...
Encysted primary spores are spherical, (8)9–11(15) µm in diameter, and are relatively few in number, (8)15–30(40) µm per sporangium in comparison with other Aphanomyces spp. Spores remain encysted for 8–12 hours. Optimum temperatures for sporangial formation and discharge for the majority of European isolates of A. astaci are between 16 and 24°C (2). For some isolates, particularly from Spanish waters, slightly higher temperature optima may prevail (4). 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. Aphanomyces astaci does not survive at –5°C and below for more than 24 hours in culture, although –20°C for >48 hours may be required in infected crayfish tissues, nor does it remain viable in crayfish tissues that have been subject to normal cooking procedures.

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. This 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 a flagellar extrusion begins and the spore shape changes gradually from spherical to reniform. Flagellar attachment is lateral; zoospores are typical saprolegniaceous secondary zoospores measuring 8 × 12 µm. Active motility takes some 5–20 minutes 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 hours.

**Molecular techniques:** polymerase chain reaction (PCR)

The method described follows the method of Oidtmann et al. (6). Amplification of a 569 bp fragment of A. astaci DNA is performed using primers targeting the ITS region (internal transcribed spacer) of the protist: 5'-GCT-TGT-GCT-GAG-GAT-GTT-CT-3' (primer 42) and 5'-CTA-TCC-GAC-TCC-GCA-TTC-TG-3' (primer 640).

**Animals:**

Whenever possible, moribund or recently dead (<24 hours) crayfish should be used for analysis using the PCR method. Live crayfish have to be killed, which can be done using chloroform. When only animals that have died a few days prior to DNA extraction are available, they can be tested, but as degradation of DNA may have occurred, a negative result must be interpreted with caution. If circumstances prevent delivery of crayfish to the specialist laboratory within 2 days, fixation in 70% ethanol (5/1 ethanol to crayfish tissue) is possible, but may result in a reduction of the DNA yield.

**DNA extraction from crayfish soft abdominal cuticle:**

Any superficial contamination should first be removed from the soft intersternal abdominal cuticle by thoroughly wiping the soft abdominal cuticle with a wet (using autoclaved H₂O) clean disposable paper towel. It is then excised and 30–50 mg ground in liquid nitrogen to a fine powder using a pestle and mortar (alternative grinding
techniques may be used, but should be compared with the liquid nitrogen method before routine use). DNA is then extracted from the ground soft abdominal cuticle using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions for insect tissue. The DNA is eluted using 100 µl elution buffer provided with the kit. Alternatively an equivalent proteinase K-based DNA extraction method can be used.

**PCR**

The PCR is carried out in a 50 µl reaction volume containing 1 × PCR buffer (75 mM Tris/HCl, pH 8.8, 20 mM [NH₄]₂SO₄, 0.01% (v/v) Tween 20), 1.5 mM MgCl₂, 0.2 mM each of dATP, dCTP, dTTP, and dGTP, 0.5 µM of each primer, and 1.25 units of Thermoprime Plus DNA Polymerase (AB Gene, Epsom, UK) or equivalent Taq polymerase and 2 µl DNA template. The mixture is denatured at 96°C for 5 minutes, followed by 50 amplification cycles of: 1 minute at 96°C, 1 minute at 59°C and 1 minute at 72°C followed by a final extension step of 7 minutes at 72°C. Amplified DNA is analysed by agarose gel electrophoresis. Confirmation of the identity of the PCR product by sequencing is recommended.

**PCR method for detection of carriers**

For carrier identification, 30–50 mg tissue from each soft abdominal cuticle and Telson are sampled, DNA is extracted and submitted to PCR separately. The PCR protocol described for susceptible crayfish species is used.

- **Indirect detection methods**

  None available.

**4. RATING OF TESTS AGAINST PURPOSE OF USE**

The methods currently available for surveillance, detection, and diagnosis of crayfish plague are listed in Table 1. The designations used in the Table indicate: A = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; B = requires experience and diagnostic expertise that may not be readily available; C = not recommended and lacks diagnostic sensitivity and specificity; D = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility.

**Table 1. Crayfish plague surveillance, detection and diagnostic methods**

<table>
<thead>
<tr>
<th>Method</th>
<th>Surveillance of susceptible species</th>
<th>Surveillance of resistant species</th>
<th>Presumptive</th>
<th>Confirmatory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross and microscopic signs</td>
<td>B</td>
<td>C</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Isolation and culture</td>
<td>A</td>
<td>C</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>PCR</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Histopathology</td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
</tbody>
</table>

PCR = polymerase chain reaction.
5. CORROBORATIVE DIAGNOSTIC CRITERIA

a) Definition of suspect case

Any extensive mortality solely of susceptible species of freshwater crayfish, where all other aspects of the flora and fauna, particularly other aquatic crustaceans are normal and healthy.

b) Definition of confirmed case

Confirmation of presence of *Aphanomyces astaci* by isolation and culture or by PCR.

6. DIAGNOSTIC/DETECTION METHODS TO DECLARE FREEDOM

**Susceptible species:** Crayfish farms keeping susceptible crayfish would need to be inspected at a frequency outlined in Chapter I.3. A history of no relevant mortalities occurring within the population over a period of at least 12 months combined with absence of clinical signs, as well as gross and microscopic pathology at the time of inspection are suitable methods for this purpose. Surveillance of wild crayfish stocks presents greater problems, especially where the species concerned is endangered. As movements of fish stocks from infected waters present a risk of disease transmission, monitoring the status of crayfish populations to confirm that they remain healthy, is necessary.

**North American crayfish species:** In North American crayfish species, animals would need to be sampled and analysed using the PCR assay described above. This applies to both farmed and naturalised stocks, and surveillance programmes need to take into account the risks of indirect transmission by movements of fish.

REFERENCES


* *

**NB:** There is an OIE Reference Laboratory for Crayfish plague (*Aphanomyces astaci*) (see Table at the end of this Aquatic Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).
NB: This disease is no longer listed in chapter 1.2.3 of the Aquatic Code. This chapter has not been updated since 2003

CHAPTER 2.3.8.

SPAWNER-ISOLATED MORTALITY VIRUS DISEASE

SUMMARY

Disease due to infection by spawner-isolated mortality virus (SMV) was first recognised in captive spawners of Penaeus monodon at a research station in northern Queensland, Australia.

SMV is one of several viruses associated with mid-crop mortality syndrome (MCMS), which caused significant mortalities among cultured juveniles and subadults of P. monodon cultured in Australia from 1994 to 1996. In the Philippines, P. monodon infected with luminous vibriosis were also found to be infected with SMV (1). Infection and disease due to SMV has only been reported from cultured or captive wild adult P. monodon and cultured Cherax quadricarinatus (4).

SMV has been tentatively classified as a parvovirus (2). Transmission electron microscopy of infected P. monodon showed virus particles that were 20 nm in diameter, hexagonally shaped, and suggestive of an icosahedral symmetry. Accumulations of these 20 nm particles in massive arrays were noted in the cytoplasm of infected gut cells, and the virions appeared to be issuing through pores in the nuclear membrane (2). Partial characterisation of the virus was accomplished by treatment of infected prawn tissue extracts with DNase and RNase. These tests further indicated that SMV is a DNA virus, probably a parvovirus (2).

There are no practical surveillance methods presently available for SMV in penaeid prawns. Confirmatory diagnosis of SMV can be accomplished by transmission electron microscopy of gut tissues.

DIAGNOSTIC PROCEDURES

There are neither pathognomonic clinical signs nor pathognomonic histopathological lesions associated with spawner-isolated mortality virus disease (SMVD). The diagnosis of SMV is based on electron microscopy. Molecular methods that use nonradioactively labelled gene probes are under development for diagnosis of infection by SMV.

The various methods for surveillance, detection, and diagnosis of infections due to SMV are listed in Table 1. The designations used in the Table indicate: − = the method is presently unavailable or unsuitable; + = the method is least suitable; ++ = the method is moderately suitable; +++ = the method is most suitable; and R&D = the method is in development but is not yet available though commercial sources. These are somewhat subjective as suitability involves issues of reliability, sensitivity; and utility.

Table 1. SMV surveillance, detection and diagnostic methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Screening</th>
<th>Presumptive</th>
<th>Confirmatory</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Larvae</td>
<td>PLs</td>
<td>Juveniles</td>
</tr>
<tr>
<td>Gross signs</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Direct BF/LM</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Dark-field LM</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Table 1 continued
### Sampling procedures: see Chapter I.3.

1. **STANDARD SCREENING METHODS FOR SMV**

1.1. **Histological method**

   The histopathological changes associated with SMVD are nonspecific, i.e. SMVD cannot be diagnosed by histopathology. Nonspecific histopathological changes can occur in the hepatopancreas, midgut, and anterior midgut caecum. Juvenile *P. monodon* infected experimentally with tissue extracts from clinically diseased prawns with SMVD displayed haemocytic infiltration, necrosis, and sloughing of cells into the lumens of the midgut and hepatopancreas (2). This, however, is not always seen (figure 2 of ref. 3).

1.2. **Molecular method**

   A nonradioactively labelled DNA probe and PCR test (in kit form) have been developed (3, 4). Commercial availability of the kits is currently being negotiated.

2. **PRESumptIVE DIAGNOSTIC METHODS FOR SMV**

2.1. **Clinical signs**

   There are no specific clinical signs for SMVD. Juvenile prawns in grow-out ponds with clinical viral infections may exhibit signs such as discoloration, lethargy, fouling and anorexia.

2.2. **Molecular method**

   See Section 1.2.
2.3. Bioassay method

This bioassay will confirm the presence of a pathogenic virus, but does not identify the specific virus. The presence of any pathogenic virus may be detected using a relatively simple bioassay in which healthy (specific pathogen free [SPF] if available) juvenile *P. monodon* are exposed to suspect prawns by either feeding them with suspect prawn tissues or by injecting them with cell-free tissue extracts prepared from suspect prawns. Fraser & Owens (2) reported that SMV could be transmitted *per os* by feeding infected carcasses or by injection of cell-free extracts prepared from infected carcasses. With intramuscular injection of cell-free extracts, mortalities began at 14 days post-inoculation and approached 100% by 30 days post-inoculation. Disease development in the bioassay indicator prawns following *per os* exposure required more time, with the first mortalities occurring at ~30 days post-inoculation and reaching 76% mortality by 50 days post-inoculation (2).

To perform the bioassay, use the generalised protocol as follows:

i) Prepare a 1:2 or 1:3 ratio (w:v) of SMV-suspect prawn heads or whole prawns with TN buffer (see Chapter 2.3.6. Infectious hypodermal and haematopoietic necrosis virus for the composition of this buffer) or sterile 2% saline prepared with distilled water.

ii) Homogenise the mixture using a tissue grinder or blender. Do not permit the mixture to heat up by excessive homogenisation or grinding. Tissues and resulting homogenate should be kept cool during the entire protocol by maintaining on ice.

iii) Clarify the homogenate by centrifugation at 3000 g for 10 minutes. Decant and save the supernatant. Discard the pellet.

iv) Centrifuge the supernatant fluid at 27,000 g (15,000 rpm) for 20–30 minutes at 4°C. Decant and save the supernatant fluid. Discard the pellet.

v) Dilute the supernatant fluid from step iv to from 1/10 to 1/100 with sterile 2% saline. This solution may now be used as the inoculum to inject indicator prawns (or filter sterilised as described in step vi).

vi) Filter the diluted supernatant from step v using a sterile syringe (size depends on final volume of diluted supernatant) and a sterile 0.45 µm syringe filter. Multiple filters may have to be used as they clog easily. The filtrate should be collected in a sterile test tube or beaker. The solution can now be stored frozen (−20°C for short-term [weeks] storage and −80°C for long-term [months to years] storage) or used immediately to inject indicator prawns.

vii) Indicator prawns should be from stocks of healthy (SPF if available) *P. monodon*.

viii) Inject 0.01 ml per gram of body weight using a 1-ml tuberculin syringe. Indicator prawns should be injected intramuscularly into the third tail segment. If the test prawns begin to die within minutes post-injection, the inoculum contains excessive amounts of proteinaceous material and should be further diluted prior to injecting additional indicator prawns. Sudden death occurring post-injection is referred to as ‘protein shock’, and is the result of systemic clotting of the prawn’s haemolymph in response to the inoculum.

ix) Cell-free extract samples may be diluted (1/10 or 1/20 in TN buffer), filter sterilised (if warranted), and injected into the indicator prawns without further preparation.

x) If a pathogenic virus is present in the inoculum, the indicator prawns should begin to die.

xi) The presence of SMV in the indicator prawns should be confirmed by transmission electron microscopy (and *in situ* hybridisation by gene probe if available).

3. Confirmatory Diagnostic Methods for SMVD

Confirmation of SMV infection in prawns can be achieved by the following method.
3.1. Transmission electron microscopy

The method is essentially that of Fraser and Owens (2). Moribund prawns are sedated in cold water and then the organs of interest are removed into Petri dishes containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.1. The tissues are cut into 1 mm cubes, fixed for 30 minutes at room temperature (24°C) and washed twice for 10 minutes in the 0.1 M cacodylate buffer. The samples are stored in the cacodylate buffer at 4°C. Before embedding, the samples are post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour at 24°C, and then washed twice for 10 minutes with the same buffer. The post-fixed samples are dehydrated and embedded in Spurr’s resin. The sections are stained with 2% uranyl acetate in 50% ethanol acidified with hydrochloric acid for 7 minutes, rinsed four times in distilled water and blotted dry. The sections are then stained with lead acetate for 2 minutes and the rinsing step is repeated. Aggregations of virus-like particles are found associated with the nuclear membrane in the cytoplasm of the gut cells. The virions are approximately 20–25 nm in diameter (3) and hexagonally shaped suggesting icosahedral symmetry. SMV virions are not found in tissues other than the gut.

3.2. Molecular method

See Section 1.2.

REFERENCES


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