

A3.1 Position Statements Introduction

A. 2000 – 2002 Position Statements

1. Initial Position Statement

This chapter sums up why and how we have assembled this document. We have tried to explain the purpose and make-up of the document so there is as little confusion as possible. We had to recognize there was no way to please everyone, when so many people with differing needs and wants will use the document. We have done our best to provide what we believe is the best possible document at this time. We have adhered very closely to OIE guidelines to make this document as useful as possible for international trade inspections. We are hopeful this document will continue to grow and evolve. Individual jurisdictions are likely to require different criteria for an aquatic animal health inspection and those criteria shall supercede the recommendations set forth in this chapter.

B. 2002 – 2003 Position Statements

No changes or reviews requested.

A3.2 Sampling Position Statements

A. 2000 – 2002 Position Statements

1. Initial Position Statement

This chapter was by far the most difficult to develop, due to the vast nature of situations and scenarios an inspector might come across. It is impossible to cover all scenarios and situations; therefore, while we have done our best to cover as much as we can, it will remain incumbent on the inspector to determine how best to sample in any given situation. Individual jurisdictions are likely to require different criteria and those criteria shall supercede the recommendations set forth in this chapter.

B. 2002 – 2003 Position Statements

1. Should a sampling exemption be recommended for facilities on SPF water, no fish introductions, and a history of negative testing?

a. All members of the committee recognized the importance of limiting the destruction of valuable brood stock but felt strongly that annual surveillance of all lots must be mandatory. The committee compared the lot definitions and sampling requirements of this handbook with the OIE and Title 50. The OIE does allow reduced sample sizes after two years of negative results but no exemptions are given. In conclusion, the committee does not agree that any exemption would be appropriate, but did agree to discuss, over the next year, whether sampling requirements could be modified to spare valuable broodfish while still maintaining sufficiently stringent disease surveillance.

2. Changes made as a result of the inclusion of *Piscirickettsia salmonis*.

- a. *Piscirickettsia salmonis* added to Table 2.1.
- b. Tissue collection procedures added to 2.2.E.3.

A3.3 Bacteriology Position Statements

A. 2000 – 2002 Position Statements

1. Initial Position Statement

The pathogens selected were those the committee felt were of the greatest regulatory importance at the time the handbook was being developed. The four bacterial fish pathogens considered in this chapter represent etiological agents which are known to exist in carrier states, but which have the potential for generating severe epizootics of clinical disease under the appropriate conditions. The methods are described for detection and identification of each pathogen in the absence of clinical signs. While other bacterial pathogens exist which can cause serious disease in fish, they are often widely distributed and cannot be controlled through transfer restrictions due to their ubiquitous nature (such as the pathogenic *Flavobacterium* species), and therefore, are not the focus of these inspection procedures.

The accurate identification of a bacterial species is based upon patterns of characteristics observed when live, pure bacterial isolates are cultured under a variety of environmental and biochemical conditions. All four bacterial fish pathogens considered for identification during a fish health inspection are culturable. All have been exhaustively characterized in a variety of widely recognized bacteriological manuals (Bergey's 1984; MacFaddin's 1980 and 2000; Austin and Austin 1987). The extensive characterization of these species has led to the establishment of simple testing schemes for presumptive identification of bacteria isolated from fish tissues as described in these protocols. *Renibacterium salmoninarum*, however, is relatively fastidious and difficult to culture and characterize phenotypically in the period of time desired to accomplish the completion of a fish health inspection. Serological techniques are also considered to be rapid, highly specific means for achieving presumptive identification of bacteria. Because of its fastidious nature, the fluorescent antibody technique has been long developed as a presumptive screening tool for the detection of *R. salmoninarum* in fish tissues.

It is generally agreed that identification of a bacterial isolate based on phenotypic or serological characteristics alone poses the possibility that a population of fish be inaccurately labeled as diseased on a fish health inspection report. Although either method of identification is acceptable as a screen for pathogens in fish, neither technique alone is precise enough to distinguish between some similar organisms. For these reasons, it is always necessary to apply a second testing regime, referred to here as “confirmatory,” to establish the accuracy of the screening test. The protocols described in this document are presented in such a manner. In past decades, studies with nucleic acids and genetic methods have furthered the accuracy in the classification and identification of bacterial species. These tools, however, were limited to research because of the difficulty in applying them accurately under clinical situations. The more recent developments in polymerase chain technology, however, have revolutionized the use of molecular biology in pathogen detection in clinical laboratories. PCR is a practical, sensitive, and accurate means to confirm the presumptive identification of a bacterial pathogen by the isolation and amplification of segments of DNA existing within fish tissues. It is presented in these protocols as an alternative to time consuming selective culture for confirmation of positive *R. salmoninarum* FAT results.

B. 2002 – 2003 Position Statements

1. Should *Piscirickettsia salmonis* be added to the handbook?

- a. The bacteriology subcommittee agreed that *Piscirickettsia salmonis* inspection procedures should be included in the bacteriology chapter at this time because the pathogen has the potential for causing severe epizootics and it is of regulatory concern.
- b. Two screening methods were selected: (1) isolation in tissue cell culture without antibiotics and/or (2) identification of characteristic cells in stained tissue impressions. Inoculation of susceptible cells is the most sensitive method for detecting *P. salmonis*. However, because *P. salmonis* is sensitive to low levels of antibiotics typically used in cell culture, all cultures must be free of antibiotics. Samples collected aseptically in the field may easily become contaminated by other bacteria. For this reason, an additional screening method (Giemsa stained tissue impressions) was included.
- c. For confirmation of *P. salmonis*, serological (immunofluorescence or immunohistochemistry) and PCR procedures were included. Screening and confirmatory methods are consistent with those in the OIE Diagnostic Manual for Aquatic Animal Diseases.
- d. It was also recognized that the screening methods may not detect covert infections; however, these methods represent the best available at this time. A statement addressing limitations of the screening assays was included.
- e. The incubation times selected for tissue culture were selected based on published peer-reviewed journal articles and the OIE Manual. The committee expressed concern the combined 42-day incubation was too long, but at this time there is no available information to make a change in the incubation time.

A3.4 Virology Position Statements

A. 2000 – 2002 Position Statements

1. Initial Position Statement

The eight viral pathogens considered in this chapter represent agents that may exist in a carrier state, have the potential for causing severe epizootics, and/or are currently of regulatory concern. This list will likely change as these concerns vary and new control measures are developed. Techniques provided for screening and confirmation are considered to be sensitive, practical, and efficient, and applicable to the large numbers of samples necessary to detect viral pathogens in carrier states. The potential variety of techniques is limited to cell culture for screening and serum neutralization and/or PCR for confirmation to simplify the writing of this initial handbook. Other serological methods such as immunoblot and fluorescent antibody tests are available for some of these viruses and applications may be made to add these to later versions.

Cell culture is the screening method used and broad spectrum cell lines have been chosen whenever possible to aid the testing laboratory in getting the most information from the samples.

Blind passage of samples has been included to determine if it will significantly increase the ability of the laboratories to detect carrier stages of these viruses using these methods.

Since cell culture amplifies the virus, it allows for the use of a highly sensitive but not necessarily specific confirmation method (see Chapter 1). The utility of serum neutralization tests for the confirmation of IHNV, IPNV, SVCV, and VHSV has been shown with years of use and for that purpose it is included here, however, the reagents are not available for all of the viruses in this handbook. PCR is a newer technique that is also highly specific but much more rapid than serum neutralization and the detailed methods for using it to confirm IHNV, ISAV, LMBV, and VHSV are also included. PCR techniques are being developed for IPNV, OMV, and WSHV and applications may be made to include them in future version as the methods and reagents become available.

B. 2002 – 2003 Position Statements

1. Include WSIV and CCV.

- a. The committee has determined that the current cell culture technique for WSIV does not have adequate sensitivity to use as a screening method and, although the CCV cell culture technique will detect overt infections, it does not detect covert infections. These limitations may lead to a false negative status for the population being inspected by these procedures. Therefore, the committee members agreed that at this time the inspection criteria for these two viruses are better handled by regional policy or on a case-by-case basis.

2. Remove the requirement for blind passage.

- a. The current literature and procedures in the OIE Diagnostic Manual suggest that blind passage will increase detection of some viral agents in fish populations. With the cooperation of laboratories using the procedures in this manual, information will be gathered to determine how frequently a change in the classification of the inspected populations occurs due to the use of blind passage. The committee members agreed that the data collected from laboratories using this procedure should be reviewed each year until enough information has been gathered to support or refute the benefit of blind passage.

3. In some situations involving the movement of eggs, 28 days is too long to wait for the results of a broodstock inspection since eggs may hatch within that time period.

- a. After much discussion over this concern, the committee members generally agreed to maintain the 28-day total incubation period for the virology samples at this time. This manual is intended to provide laboratories with procedures which will detect several viruses in the same assay and the longer incubation period is necessary for some of the listed viruses, specifically OMV and ISAV. Some of the alternatives discussed were that the laws, policies, and/or regulations of the jurisdictions involved may allow for a statement on the inspection certificate of the procedures used to obtain the results, and methods exist for the handling of the eggs and fry until testing is completed such as chilling the water during egg incubation to slow development and maintaining the eggs or fry in isolation.

4. Review the information available for the optimal cell line to use in screening for ISAV and the choice of primers for the PCR confirmation.

- a. Although at least two cell lines other than SHK-1 have been used to detect ISAV, the current scientific literature does not support a change to either ASK or CHSE-214 cell lines in screening for ISAV. The PCR method included in the manual is capable of detecting both the North American and European strains of ISAV so no change in primers is necessary. The committee members agreed that this section should be reviewed again at a later time when more information is available.

5. Include more serological tests for confirmation of the viruses.

- a. The committee members had many reservations regarding the use of serological confirmation methods that included concerns about both the availability and quality of the necessary antibodies and antisera. However, with the stipulation that appropriate QA/QC procedures are used, the committee agreed to leave serum neutralization as a confirmation method for IHNV, IPNV, SVCV, and VHSV and to add the indirect fluorescent antibody test as a confirmation method for IHNV, IPNV, ISAV, and VHSV.

6. PCR procedures for IPNV, OMV, and SVCV are now available and should be included as confirmatory methods for these viruses.

- a. They have been added to the 2003 edition of the manual.

7. Review the PCR procedure for LMBV with respect to the extraction method and primer sequences.

- a. Some laboratories that currently work with LMBV were contacted in regard to this comment. At least three of them had used one or more of the commercially available DNA extraction kits and had found them to be adequate for identifying the virus from cell culture material. The committee members agreed to remove the extraction method that required the use of chloroform and phenol and replace it with the more worker and environmentally friendly extraction kits. The newer primer sequence was considered to be an improvement in the method, so a change has also been made to that part of the procedure.

A3.5 Parasitology Position Statements

A. 2000 – 2002 Position Statements

1. Initial Position Statement

The pathogens selected were those the committee felt were of the greatest regulatory importance at the time the handbook was being developed. Rationale for selection of the screening and confirmatory assays for each of the fish parasites considered in Chapter 5 Parasitology are detailed below. Confirmatory procedures will only be used if the sample is presumptively positive using the approved screening method.

a. *Myxobolus cerebralis*

i. Screening

The pepsin-trypsin digest procedure was selected as the assay of choice for isolation and concentration of spore stages from fish cartilage. Although it was acknowledged that the plankton centrifuge method offers some advantages in the ease of assay performance, review of the literature and of laboratories performing *M. cerebralis* diagnostics supported selection of the digest assay for reasons of increased sensitivity. The procedure does allow pooling of up to five fish, which is likely to decrease detection sensitivity. However, it was considered that processing of individual fish would constitute a workload beyond the capability of many laboratories, and that in some regions of the country this would be considered unacceptable. The decision was to allow pooling with the realization that in areas most affected by the parasite, there would be requirement by the states to process single fish.

ii. Confirmation

Confirmation is either by identification of spores in histological sections or detection of parasite DNA by polymerase chain reaction (PCR) assay. Detection in histological sections is the current standard. Although the committee felt that it is of lower sensitivity than the PCR assay, it will remain an acceptable confirmatory tool at this time. For DNA detection, the nested PCR assay was selected because it is scientifically acceptable and citable and it is used successfully in a number of laboratories. Because the sampling and preparation procedures described in the original publication were primarily for research purposes, the protocol described here references methods more in line with those required during field collections of fishes of different sizes. These collection and preparation methods are compatible with performing the nested assay.

b. *Ceratomyxa shasta*

i. Screening

Presumptive identification is based on identification of any parasite stages in wet mount scrapings, the procedure currently recommended.

- ii. Confirmation
Because of the distinctive morphology of the *C. shasta* spore, its identification is sufficient for confirmation. If spores are not identified, a presumptive positive can be confirmed by detection of the parasite DNA by PCR. The protocol described is published and has been developed for diagnosis in field situations. Other confirmatory procedures requiring monoclonal antibodies were not considered because these reagents are not commercially available.
- c. *Tetracapsula bryosalmonae*
 - i. Screening
Presumptive identification is made by identifying any parasite in stained imprints or using lectins. These two methods were proposed because identification of the parasite is difficult without practice, and the lectin has been shown to increase detection.
 - ii. Confirmation
At this time, confirmation is by identification of any parasite stages in histological sections. Although this method is not highly sensitive and requires a trained eye, it was agreed that scientific review of other methods made them unfeasible at this time. The lectin stain has been demonstrated to cross-react with other myxozoans and there is also question about the specificity of published PCR assays. The committee felt that this protocol would probably be updated in the near future as a demonstrated specific PCR assay becomes available.
- d. *Bothriocephalus acheilognathi*
 - i. Screening
Presumptive identification is by identification of basic characteristics of the cestode.
 - ii. Confirmation
Presumptive cestodes are confirmed by identification of key morphological characteristics. These visual identification methods are accepted in the scientific literature and are the current Blue Book standard.

B. 2002 – 2003 Position Statements

1. Review use of digest material for PCR confirmation of *Myxobolus cerebralis*.

- a. Adoption of the nested PCR technique on digest material for confirmation of the presence of *Myxobolus cerebralis* can be scientifically defended at this time. Baldwin and Myklebust's work statistically determined sensitivity of single round PCR from pooled digest material from infected and non-infected reference animals, and added additional information regarding specificity. Though statistically significant, the number of samples examined was quite low, and although not determined, the confidence limits for sensitivity and specificity would likely be quite large. Qureshi et al. examined a large number of clinical samples (580 fish) using nested PCR on the digest product and compared results with the current gold standard, histologic examination, as well as with the tissue digest. Testing of additional animals should be done and levels of sensitivity need to be determined for the nested procedure applied to

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digest material, but there is already more information available on this assay than for almost any other test.

2. Review histological confirmation of *Myxobolus cerebralis*.

- a. The committee recommended that the criteria for determining a sample negative by histology be made more stringent. Wording will be changed to include serial step sections in samples where the initial sections examined were negative, and inability to detect any spores in tissue will no longer be considered sufficient to certify a lot of fish as negative.

3. Review protocols allowing freezing of samples for *Myxobolus cerebralis* spore recovery.

- a. The committee recommended that no change be made to the current procedure, which allows PTD processing of frozen samples with modifications of enzyme concentrations. There is insufficient peer-reviewed scientific data to prove and quantify the effects of freezing on spore recovery and requiring processing of fresh heads would present a problem for many laboratories.

4. Review of PCR diagnosis of *Tetracapsula bryosalmonae*.

- a. The committee agreed that recent publications on this assay demonstrate that it is a valid confirmation test and this will be added as an alternative to histology. Concerns about this and other PCR assays continue to be QA/QC issues like availability of positive control tissues.

A3.6 Polymerase Chain Reaction (PCR) Position Statements

A. 2000 – 2002 Position Statements

1. Initial Position Statement

This chapter was designed to supplement references to molecular techniques referenced in earlier chapters. Included are general considerations for insuring that contamination does not occur and to insure the integrity of the assay. These general protocols that can be found in many general primers for PCR and are intended to provide background information for laboratories that are just setting up PCR diagnostics.

B. 2002 – 2003 Position Statements

No changes or reviews requested.

A3.7 Appendix 1 The Handbook and Oversight Committee Position Statements

A. 2000 – 2002 Position Statements

1. Initial Position Statement

This appendix is truly the heart of this document. It lays out the structure of the handbook and the revision and oversight committee. It explains how the handbook will be maintained and by who. Most importantly, however, this appendix details the manner in which this handbook shall be reviewed and revised. This detailed procedure is what gives this document its advantage over previous documents of its kind. Additionally, these reviews are mandatory on an annual basis, which means the document can be kept current from a technique and pathogen standpoint, such that in the future there should be no need to create any new handbooks or manuals.

B. 2002 – 2003 Position Statements

1. Inserted months into locations that make the timeline for review easier to comprehend.
2. Removed printed document as a means of dissemination.