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 specific but not necessarily sensitive 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.

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.

C. 2003 – 2004 Position Statements

1. **Revise LMBV PCR protocol needs to be altered to match the new primer sets. Should the protocol exactly match that of John Grizzle's 2003 paper?**

   a. The published procedure incorporated the use of uracil to substitute for thymine in the master mix due to a potential contamination issue in that lab.

   b. The Oversight Committee noted that this substitution would unnecessarily complicate lab supplies/tracking/labeling in most PCR capable laboratories. A modified procedure that uses thymine (as all other PCR procedures in the Manual) has also been tested by Jason Woodland Becky Lasee, Patricia Varner, and others and found to work. This also was discussed with John Grizzle, Andy Goodwin and others who have the opinion that the modification shouldn't cause any problems with the LMBV-PCR procedure as published when doing confirmations from viruses isolated on tissue culture.

   c. Although the intent of the editors and contributors to the Manual is to stick with procedures that are published and therefore "scientifically defensible", it was quickly realized by the Oversight Committee that the modified procedure would probably be the best one to adopt. However, there is some hesitation due to the lack of a definitive "bench mark test" that has been published.

   d. The virology sub-committee recommends leaving the uracil technique out of the LMBV PCR and including instead as a general option in the PCR section. This justified by the following.

      I. The results of current users show the procedure using thymine is robust and accurate for detecting LMBV.
      II. This will improve the LMBV section for practicing labs that run inspections by standardizing the components of the master mixes used for all PCR confirmations III. Standardizing the components will reduce QA/QC concerns and increase efficiency in the PCR lab.
      III. We are not ignoring the value or the inclusion of the uracil procedure in the Manual, but it is best put into the general PCR section to help labs deal with contamination issues as they arise in all PCR procedures.
      IV. The writers and editors of the Manual are considered the "experts" and should know the best course of action on any modification.

2. **Should the SVCV PCR protocol be updated to utilize the new OIEprimers?**

   a. The subcommittee discussed conversations with Jim Winton regarding the current protocols not working on certain Asian strains of this virus. OIE has apparently accepted primer sets identified by Stone et al. 2002 that will work on all known strains

of SVCV.

b. Although there is some controversy regarding the taxonomic placement of some "cross reacting" Pike Fry Rhabdovirus isolates, the subcommittee discussed the desirability of using the OIE (Stone) protocols at least until the International Convention on Taxonomy of Viruses sort out the taxonomy.

c. The subcommittee voted to recommend that the Oversight Committee adopt the OIE protocols for SVCV PCR confirmation.

3. Can we remove the blind pass from virology procedures?

a. The subcommittee discussed and reviewed the data submitted by Susan Gutenberger, USFWS. We also discussed the OIE requirements and the lack of data to support the proposition that population certifications would likely stay the same if blind passes were dropped.

b. The subcommittee members were sympathetic about the expense, time and labor expended in doing blind passes, but scientifically we could not support dropping blind passes at this time. The biggest questions are reported cases of significant changes in detection of European VHSV and LMBV through the use of blind passes. We suggested that the USFWS or the AFS-FHS attempt to query other agencies regarding data on blind passes.

c. The subcommittee voted to recommend to the Oversight Committee that the blind pass requirement be kept in the Inspection Manual at this time.

4. Add IHN-IHC (immuno-histochemical) confirmation protocol for IHNV

a. The subcommittee reviewed and discussed the protocol as submitted by Ken Nichols and Scott Foot, USFWS. We noted that although several confirmations already exist, the protocols as submitted are rigorous, referenced, and utilized defined reagents as required by the procedures of the Manual.

b. The subcommittee voted to recommend to the Oversight Committee that this protocol be added to the IHNV confirmation procedures in the Manual.

5. Change ISAV cell line for screening from SHK-1 to ASKII cell line

a. The subcommittee considered the OIE manual which allowed the use of cell lines other than SHK-1, and also considered the reference in the Vol. 23(2), 2003 Bulletin of the European Association of Fish Pathologists, pp 80-85. "Comparative isolation of infectious salmon anaemia virus (ISAV) from Scotland on TO, SHK-1 and CHSE-214 cells" by R. Grant and D.A. Smail.

b. Both Ray and Scott offered their experiences working with both cell lines in the past and their agreement that ASK cells were far easier to work with than SHK-1 cells. ASKII cells are also readily available from the ATCC collection and well referenced.

c. Based on theses discussions, the subcommittee voted to recommend to the Oversight Committee that these cells be included as acceptable for the screening of ISAV.

D. 2004 – 2005 Position Statements

1. **Change the incubation temperature range from 20-25 C to 25-30 C for LMBV, and shorten the incubation period from 14 days to 7 days before doing a blind pass.**

   Three citations were provided showing 25-30 C is the optimal temperature range for LMBV replication (Piaskoski et al. 1999, Grant et al. 2003 and Mc Clenahan et al. 2005). At this higher temperature, CPE will occur faster and there is no need to incubate for 14 days. Seven days is sufficient.

   Based on these citations, the Oversight Committee was unanimous in its vote to accept these changes.

2. **Eliminate the need for blind passage for general viral isolation.**

   Supportive documentation was not provided with this suggested change. The same suggestion was made in 2003-2004 and was addressed above. Since no new information was provided in support of this change, the Oversight Committee voted to make no changes regarding the need for blind passage.

E. 2006 – 2007 Position Statements

1. **Review and update the Viral Hemorrhagic Septicemia Virus (VHSV) protocols to reflect current knowledge in light of its recent emergence in the Great Lakes.**

   a. The list of susceptible species now includes those from the Great Lakes.
   b. BF-2 and FHM cell lines were added to the EPC cell line as appropriate cell lines for testing. c. RNA extraction method changed to either phase-separation method or RNA affinity spin column instead of the Heat RNA Release Method to increase efficiency.
   c. PCR primers were changed to match OIE primers, and second round PCR was eliminated as it was viewed as unnecessary for a confirmatory assay.
   d. Information on obtaining antisera for serum neutralization test was added.

2. **Update the Infectious Hematopoeitic Necrosis Virus Primers.**

   a. RNA extraction method changed to either phase- separation method or RNA affinity spin column instead of the Heat RNA Release Method to increase efficiency.

   b. PCR primers were changed to target the central portion of the G gene, instead of the previously published primers which targeted the N gene. The G gene primer set has been tested extensively (Emmenegger et al., 2000, Kurath et al., 2003) and avoids using a series of six consecutive 'G's' that were less than optimal for PCR in the N gene forward primer. Additionally, second round PCR was eliminated as it was viewed as unnecessary for a confirmatory assay.

F. 2008 – 2010 Position Statements

No changes or reviews requested.

G. 2011 – 2012 Position Statements

a. An annual review of the currently available information on blind passage was initiated by the 2002-2003 Review and Oversight Committee (A3.4B2a). During the 2011 - 2012 review, the committee agreed that the data available clearly demonstrates that blind passages increase the sensitivity of virus detection using cell culture. Given this finding, and continued OIE requirement for blind passage, this committee recommends suspending the annual review of blind passage data requirements, until published information becomes available refuting the benefit of blind passage, or a more sensitive assay procedure is developed.

H. 2013 – 2014 Position Statements

1. **Amend the infectious salmon anemia virus (ISAV) methods in the Inspection Manual to remove the indirect fluorescent antibody test (IFAT) and add the real-time reverse transcriptase PCR (Snow et al. 2006).**

   The full committee felt it was premature to remove the IFAT from the list of acceptable confirmatory tests. An *ad hoc* subcommittee was formed to investigate adding the real-time reverse transcriptase PCR as a confirmatory test to identify ISAV isolated in cell culture. The subcommittee did not have any major issues with adding the real-time PCR as one of the possible confirmatory tests for ISAV. However, the full committee debated whether this method is appropriate as a confirmatory test because the assay is primarily used as a screening method. Furthermore, a conventional reverse-transcriptase PCR that produced a PCR amplicon for sequencing would be a better choice for confirming ISAV grown in cell culture. The committee recognized that the ISAV diagnostic and inspection chapters are out of date. The committee opted not to take a final vote on this amendment and conduct a more thorough review of the ISAV chapters in the 2014 – 2015 cycle.

2. **The committee received a proposal to update the confirmation methods for viral hemorrhagic septicemia virus (VHSV) to include two reverse-transcriptase real-time PCR tests for VHSV that appear to be capable of detecting all genotypes of this virus that are currently known. The confirmation methods for this virus also include serum neutralization and the indirect fluorescent antibody test (IFAT). It was proposed that those serum neutralization and IFAT be removed and that molecular methods be made the standard procedure for confirmation.**

   The full committee discussed this amendment and felt that it was premature to remove the serum neutralization and IFAT methods, which are still included in the VHSV OIE manual. If a laboratory is comfortable with these methods, it provides a quick identification of the virus. The committee discussed that definitive confirmation of the virus should include a conventional PCR followed by sequencing of the PCR amplicon. However, the committee recognized that the real time PCR could be another means for rapid identification of the virus grown in cell culture. Particularly since VHSV is reportable and U.S. isolates (derived from regions outside the Pacific Northwest) would likely go to the National Veterinary Services Laboratory (NVSL) for definitive confirmation. The reverse-transcriptase, real-time PCR assays reported by Jonstrup et al. 2012 and Garver et al. 2011 were considered. However, only the assay reported by Jonstrup 2012 is recommended by the OIE manual and the Jonstrup 2012 assay performed the best in the ring tests conducted in the United

States by NVSL. However, the inspection manual was not amended in this cycle because the committee was not able to finalize the methodology. The committee proposes to finalize the VHSV real-time PCR method in the 2014 – 2015 cycle.

I. 2019 – 2020 Position Statements

1. **Should the Viral Hemorrhagic Septicemia Virus (VHSV) quantitative polymerase chain reaction (qPCR) assay (Jonstrup et al. 2013) be adopted into Section 2 (Aquatic Animal Health Inspections) of the American Fisheries Society Fish Health Section Blue Book**. This assay was evaluated for purpose by the USDA-NVSL (Warg et al. 2014a; Warg et al. 2014b).

    a. A VHSV sub-committee was assembled to evaluate the adoption of the real-time PCR assay described above. This subcommittee consisted of Drs. Mohamed Faisal, Gavin Glenney, Rodman Getchell, Nick Phelps, Isaac Standish, and Janet Warg. The sub-committee concluded that the adoption of this validated and widely tested molecular assays into the Fish Health Section Blue Book would be a needed first step to bring the existing document up to date and relevant in the world of aquatic animal molecular diagnostics. The advantages of real-time PCR over conventional PCR are numerous; including quantification in exponential growth phase, reduced contamination (closed-tube, no post-PCR processing), extra level of specificity with probe technology, and increased dynamic range.

    b. The VHSV sub-committee proposed to adopt the VHSV qPCR assay (Jonstrup el al. 2013) into the Blue Book (Section 1. and Section 2.) in a fit for purpose manner (presumptive/screening and confirmatory purposes), thus allowing fish health professionals the versatility needed to detect VHSV with a very specific and sensitive assay under a variety of management purposes. The adoption of the VHSV qPCR assay in this manner would also keep testing done in the U.S.A. and Canada on par and in accordance with the OIE Manual of Diagnostic Tests for Aquatic Animals for this reportable virus.

    c. Standard Operating Procedures (SOPs) for the real-time PCR assay has been written in formal Washington Animal Disease Diagnostic Laboratory (WADDL) SOP format to initiate a consistent new methodology for new molecular assays assimilated into the Blue Book.