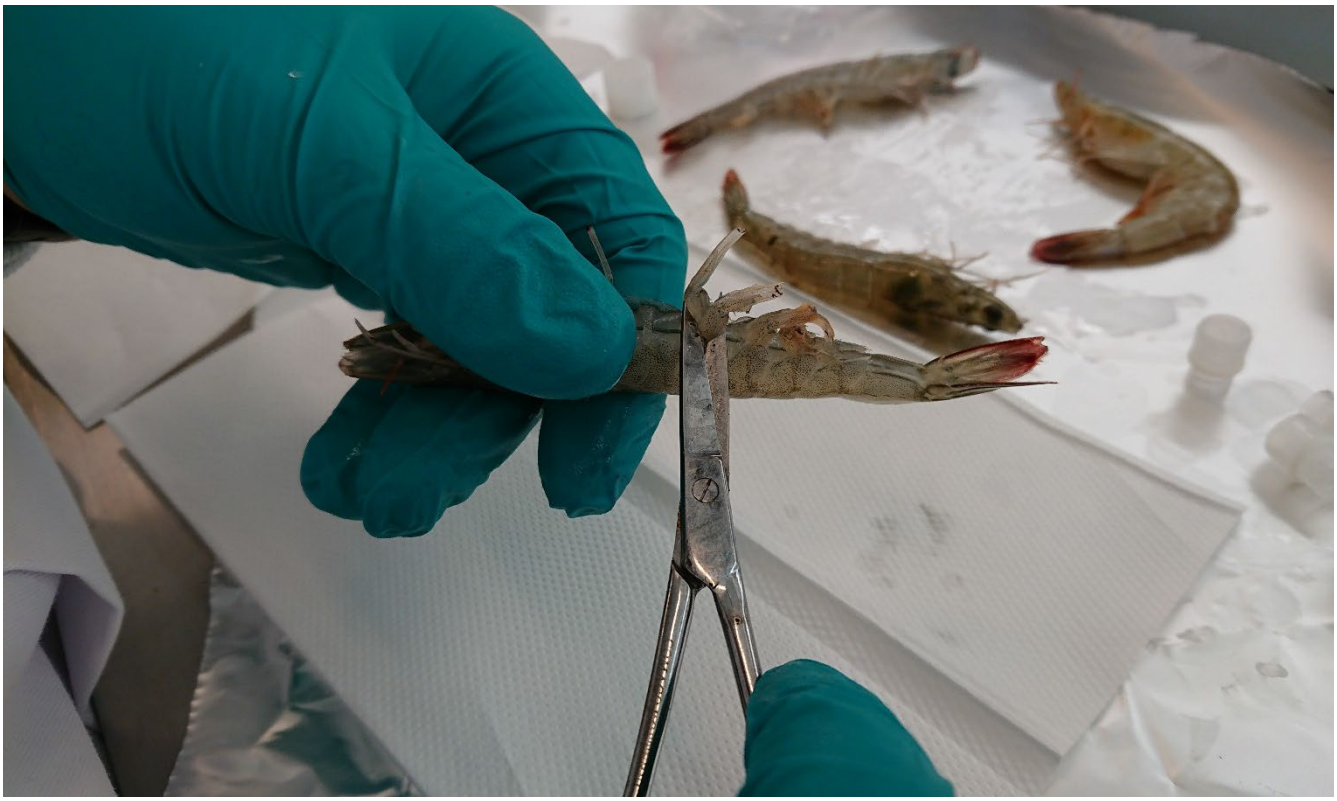
EURL FOR FISH AND CRUSTACEAN DISEASES

# DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND DETECTION OF INFECTION WITH WHITE SPOT SYNDROME VIRUS (WSSV)



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1. Diagnostic procedures for detection of WSSV.

   When sampling and laboratory examination for the purpose of the surveillance and eradication programmes set out in Part II chapter 6 section 2 of **COMMISSION DELEGATED REGULATION (EU) 2020/689** are carried out, using the diagnostic methods set out in Part II chapter 6 Section 5 of **(EU) 2020/689**, the following detailed methods and procedures which have been approved by the EURL for Crustacean Diseases must be followed.

   The methods and procedures described in this chapter on White spot syndrome virus (WSSV) are adapted from the ISO 17025 accredited test applied at the European Union Reference Laboratory for Crustacean Diseases. Alternative approaches, using equivalent conditions or kits produced by different manufacturers, but which offer proven equivalent sensitivity and specificity to those described in this Part may be applied. In all instances, a PCR amplified product shall be sequenced to confirm identity as WSSV (Claydon et al. 2004).

2. Sample process.

   For surveillance purposes, 150 animals per farm must be tested per visit. The sampling must be carried out whenever the water temperature is likely to reach its highest annual point. The requirement concerning water temperature must also apply to health visits where these are feasible. If weak or moribund crustaceans are present in the production units, these must primarily be selected. If such crustaceans are not present, those selected must include crustaceans of different size cohorts namely juveniles and adults of the selected susceptible species, proportionally represented in the sample. If more than one water source is utilized for crustacean production, susceptible crustaceans representing all water sources must be included for sampling.

   Samples can be taken from post-larvae, juveniles or adults. Samples should be handled and packaged with great care to minimise the potential for cross contamination among samples. New gloves and plastic sample bags or vials must be used for each sample. Small animals such as shrimp post-larvae may be pooled together in batches of five animals, but otherwise animals should be sampled and tested individually. Live animals may be processed in the field after being iced for 15 – 30 minutes (depending on size) or shipped live to the diagnostic laboratory for testing. Dissected tissues are preserved in 80 – 90% ethanol and transported at ambient temperature. Animals may also be iced or chilled and send to the diagnostic laboratory to be further processed within 24 hours, or they can be frozen and send to the laboratory on dry ice. Finally, small animals (< 3 grams) may be preserved whole in 80 - 90% ethanol and transported at ambient temperature.

   Samples of integumental epidermis, either dissected or contained within pleopods, or gills of the test animal must be fixed in 80 – 90% ethanol prior to the preparation of samples for PCR. Other samples, fixed for histology and transmission electron microscopy may be collected to support diagnostic data arising from PCR. The stages required for the identification of WSSV from tissue samples shall be as follows: Homogenisation of the tissue, extraction of the DNA, specific amplification of WSSV DNA using PCR, visualisation of the amplified product on a gel, purification of the DNA and

sequencing to confirm the identity of the pathogen. For surveillance purposes, quantitative PCR (qPCR) as described in point 5 may be used instead of the conventional PCR described in point 6, but positive results must be confirmed on at least one sample pr sampling site by sequencing of a PCR product.

3. Tissue homogenisation.

   Tissue should be disrupted and homogenized using either a commercial device (e.g. Qiagen TissueLyser, FastPrep24, Precellis or similar) together with a suitable homogenization matrix, or using alternative methods (mortar and pestle, sonicator etc.) with equivalent efficiency. Homogenization should be done in PBS, lysis buffer or liquid nitrogen according to the protocol used for DNA extraction.

4. DNA extraction.

   DNA is most conveniently extracted from the homogenate using a commercial DNA extraction kit based on silica spin columns or magnetic beads using the manufacturer's instructions, but may also be done using a validated manual DNA extraction method. To ensure that the extraction has proceeded successfully, the DNA concentration for all samples and controls may be determined using a NanoDrop spectrofotometer or similar. Alternatively, the DNA quality may be validated using a PCR assay for a host animal gene (see point 8 below). Extracted DNA shall be frozen at -20 °C if not required immediately.

5. WSSV quantitative PCR (qPCR) method.

   The method approved by the EURL is based on the procedure described in Durand and Lightner (2002). The assay uses a TaqMan probe to measure amplification of the 69 bp target region using a qPCR machine.

   The qPCR is performed using the following primers and probe:

   WSS1011F: 5'-TGG-TCC-CGT-CCT-CAT-CTC-AG-3'

   WSS1079R: 5'-GCT-GCC-TTG-CCG-GAA-ATT-A-3'

   Probe: 5'-FAM-AGC-CAT-GAA-GAA-TGC-CGT-CTA-TCA-CAC-A-BHQ1-3'

   The qPCR reaction is set up using a commercial qPCR kit for probes, using the manufacturer's instructions. Some qPCR kits may not be giving satisfying results due to inhibition from compounds in the DNA extract. Before use, it is therefore necessary to test the kit on relevant crustacean tissue known to be WSSV positive. PCR inhibition will be revealed by making a dilution series of extracted DNA, and testing by qPCR that the Ct values plotted against the logarithm of the dilution factor gives a straight line.

   Cycling conditions: 95°C for 10 min; 45 cycles of 95°C for 15 sec and 60°C for 1 min, and fluorescence signal is read at the end of each cycle using the FAM filter (and potentially also the ROX filter if that is used as reference dye). Cycling parameters may differ slightly between different qPCR kits and needs to be validated.

6. WSSV Polymerase Chain Reaction (PCR).

The conventional PCR method for WSSV surveillance and diagnostics approved by the EURL is the nested PCR procedure described in Lo et al. (1996) and optimized following the OIE Aquatic Manual of 2009 paragraph 4.3.1.2.4.1 (https://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/2009/2.2.05_WSD.pdf). The method is also the test recommended in the OIE Aquatic Manual.

The first round PCR is performed using the following primers:

146F1 5'-ACT-ACT-AAC-TTC-AGC-CTA-TCT-AG-3'

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

The second round PCR is performed using the following primers:

146F2 5'-GTA-ACT-GCC-CCT-TCC-ATC-TCC-A-3'

146R2 5'-TAC-GGC-AGC-TGC-TGC-ACC-TTG-T-3'

Cycling conditions: 94°C for 2 min; 30 cycles of 94°C for 30 sec, 62°C for 30 sec and 72°C for 30 sec; one cycle of 72°C for 2 min. The amplified amplicon has a length of 1447 bp, and can be visualized on an agarose gel (1.5 – 2 %).

1 µl of the first round PCR is used as template in the second round PCR using the same cycling conditions. The amplified amplicon has a length of 941 bp, and can be visualized on an agarose gel (1.5 – 2 %) with a suitable marker.

7. Sequencing of PCR products.

   In order to verify the identity of positive PCR products it is important to sequence at least one PCR product per sampling batch. The sequenced PCR product should come from a reaction giving a single band of the correct size on the agarose gel. The PCR reaction is purified using a commercial PCR purification kit, or a manual procedure of similar efficiency. The yield is subsequently measured on a Nanodrop Spectrophotometer. The PCR product can then be send to a sequencing provider with either of the PCR primers used as sequencing primer, or it may be sequenced in house using bigDye termination kits according to the manufacturer's instructions. The obtained sequences should be trimmed for primer sequences and low quality regions. Subsequently they should be used as queries in a BLAST search in Genbank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to verify that the best hit is a WSSV sequence (e.g. MN840357).

8. Control reactions.

   Proper controls need to be run in parallel to the test samples to verify the validity of the obtained results. In order to check the validity of the DNA extractions, at least one sample known to be infected with WSSV and one sample known to be free of WSSV infection should be included in each batch of extractions. Alternatively, extraction efficiency can be checked with PCR using host specific primers. For real-time PCR, Cowley et al. (2018) used the primer/probe set Pmon-

EF1qF1/Pmon-EF1qR1/ Pmon-EF1qPr1 to amplify part of the elongation factor 1 gene in most Penaeid shrimp but not all. The PCR setup and cycling parameters are similar to the ones used for the WSSV real time PCR assay. The EURL successfully use this procedure to check the efficiency of DNA extraction from *P. vannamei* pleopods. For conventional PCR, Lo et al. (1996) used the primer set 143F and 145R that amplifies a 848 bp fragment of the 18s gene in *P. monodon*, which will also work for most other decapods. The PCR setup and cycling parameters are similar to the ones used for the first round of the nested conventional PCR procedure.

For both assays (qPCR and PCR), two additional controls have to be included. A negative control in which water is used instead of template, and a positive control in which the template is DNA corresponding to the amplicon sequence. The positive control can either be DNA previously tested WSSV positive by PCR, or it can be a plasmid or a synthesized DNA fragment containing the amplicon sequence. The EURL can provide a positive PCR control if needed.

REFERENCES:

Claydon, K., Cullen, B. and Owens, L. (2004) OIE white spot syndrome virus PCR gives false-positive results in *Cherax quadricarinatus*. *Diseases of Aquatic Organisms*, 62, 265-268.

Cowley, J. A., Rao, M. and Coman, G. J. (2018) 'Real-time PCR tests to specifically detect IHHNV lineages and an IHHNV EVE integrated in the genome of Penaeus monodon', *Diseases of Aquatic Organisms*, 129, 145-158. doi: 10.3354/dao03243.

Durand, S. V. and Lightner, D. V. (2002) 'Quantitative real time PCR for the measurement of white spot syndrome virus in shrimp', *Journal of Fish Diseases*, 5, 381-389. doi: 10.1046/j.1365-2761.2002.00367.x.

Lo, C. F., Leu, J. H., Ho, C. H., Chen, C. H., Peng, S. E., Chen, Y. T., Chou, C. M., Yeh, P. Y., Huang, C. J., Chou, H. Y., Wang, C. H. and Kou, G. H. (1996) 'Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction', *Diseases of Aquatic Organisms*. 25, 133–141. doi: 10.3354/dao025133.

World Organisation for Animal Health (OIE) (2019) Manual of Diagnostic Tests for Aquatic Animals. https://www.oie.int/en/standard-setting/aquatic-manual/