European Union Reference Laboratory
for Fish and Crustacean Diseases

NATIONAL INSTITUTE OF AQUATIC RESOURCES, TECHNICAL UNIVERSITY OF DENMARK

DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND CONFIRMATION OF INFECTION WITH TAURA SYNDROME VIRUS (TSV)

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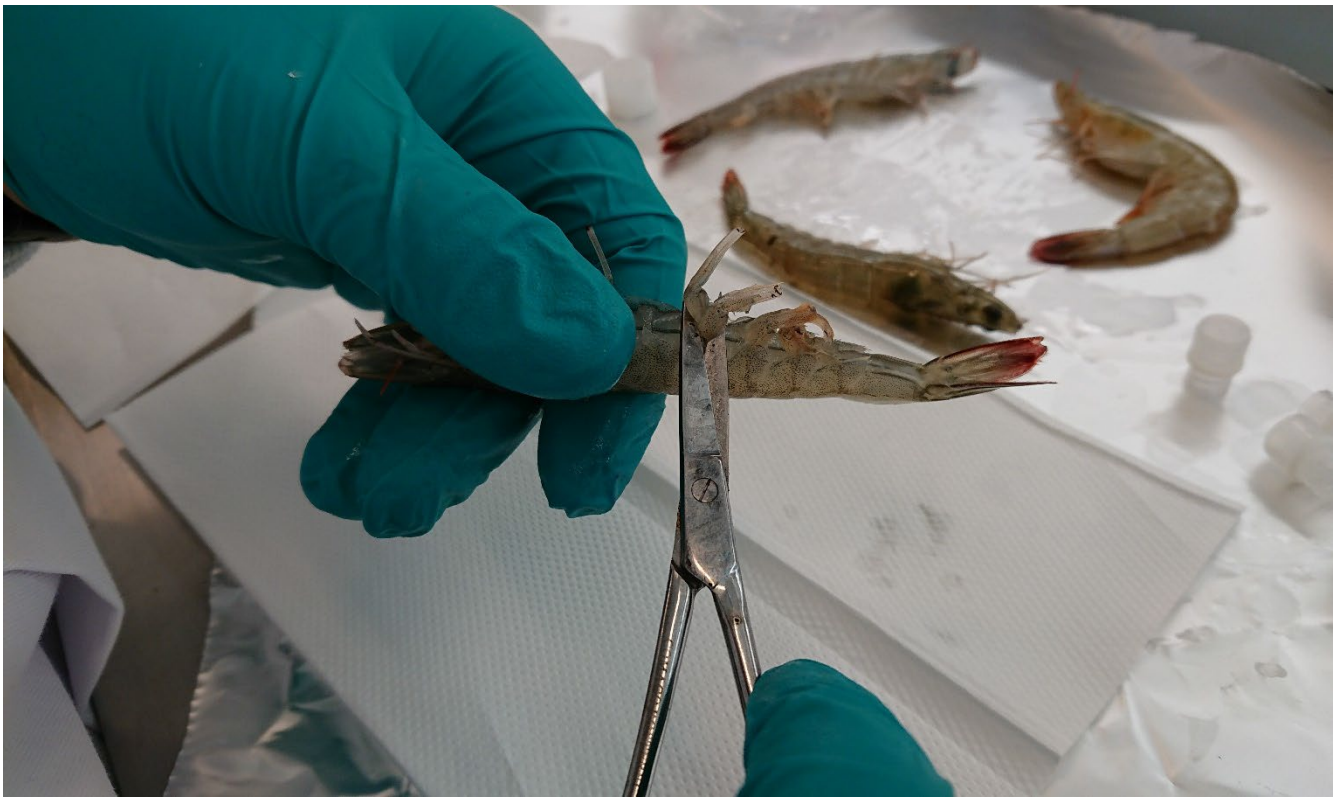

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We highly acknowledge the very valuable inputs and support of colleagues from:

- **IZSVe (Tobia Pretto)**
- **CEFAS (Kelly Bateman)**
- **CSIRO ACDP (Nick Moody and Peter Mohr)**

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1. DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE OF INFECTION WITH TSV

1.1. Diagnostic procedures for detection of TSV.

When sampling and laboratory examination for the purpose of confirming or ruling out a suspect of TSV as set out in ANNEX XII of **COMMISSION DELEGATED REGULATION (EU) 2020/687,** as well as for conducting surveillance in surveillance zone as set out in Annex XV of **COMMISSION DELEGATED REGULATION (EU) 2020/687** is carried out, the following detailed methods and procedures, which have been approved by the EURL for Crustacean Diseases, must be followed. 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.

1.2. Sample process.

For surveillance purposes, 150 animals per farm must be tested per visit. In case of observation of clinical signs or increased mortality, 10 animals must be tested. 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, such crustaceans 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 plastic sample bags or vials must be used. 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 to be further processed within 24 hours, or they can be frozen and send to the laboratory on dry ice. Dissected tissues are preserved in 80 – 90% ethanol or RNAlater (or similar) and transported at ambient temperature. Small animals (< 3 grams) may be preserved whole in 80 – 90% ethanol or placed in at least five volumes of RNA stabilisation reagent (e.g. RNAlater). Animals may also be iced or chilled and send to the diagnostic laboratory. Not all viruses will be inactivated by RNAlater, so this preservation method may have biosecurity issues that need to be considered when shipping samples.

Samples of integumental epidermis, either dissected or contained within pleopods or gills of the test animal, are the most suitable material for RNA extraction. Other samples, fixed for histology and

transmission electron microscopy may be collected to support diagnostic data arising from PCR. The steps required for the identification of TSV from tissue samples shall be as follows: Homogenisation of the tissue, extraction of the RNA, specific amplification of TSV RNA using RT-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 RT-PCR (qRT-PCR) as described in point 1.5 may be used instead of the conventional RT-PCR described in point 1.6, but positive results must be confirmed by sequencing of a PCR product.

1.3. 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 RNA extraction.

1.4. RNA extraction.

RNA is most conveniently extracted from the homogenate using a commercial RNA extraction kit based on silica spin columns or magnetic beads using the manufacturer's instructions, but may also be done using a validated manual RNA extraction method. To ensure that the extraction has proceeded successfully, the RNA concentration for all samples and controls may be determined using a NanoDrop spectrophotometer or similar. Extracted RNA shall be stored frozen at -80 °C if not tested immediately.

1.5. TSV quantitative PCR (qPCR) method.

The method approved by the EURL is based on the procedure described in Tang et al. (2004). The assay uses a TaqMan probe to measure amplification of the 72 bp target region using a qPCR machine.

The qPCR is performed using the following primers and probe:

TSV1004F: 5'-TTG-GGC-ACC-AAA-CGA-CAT-T-3'

TSV1075R: 5'-GGG-AGC-TTA-AAC-TGG-ACA-CAC-TGT-3'

TSV-P1: 5'-FAM-CAG-CAC-TGA-CGC-ACA-ATA-TTC-GAG-CAT-C-BHQ1-3'

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

Cycling conditions: 50°C for 15 min; 95°C for 2 min; 45 cycles of 95°C for 3 sec and 60°C for 30 sec, 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 qRT-PCR kits and need to be validated.

1.6. TSV Polymerase Chain Reaction (PCR).

The conventional RT-PCR method for TSV surveillance and diagnostics approved by the EURL is the procedure described in Nunan et al. (1998) with some modifications. The method is also one of the two tests recommended in the WOAH Aquatic Manual.

The RT-PCR is performed using the following primers:

9992F 5'-AAG-TAG-ACA-GCC-GCG-CTT-3'

9195R 5'-TCA-ATG-AGA-GCT-TGG-TCC-3'

Cycling conditions: 50°C for 30 min; 95°C for 15 min; 40 cycles of 94°C for 45 sec, 60°C for 45 sec and 72°C for 60 sec; one cycle of 72°C for 7 min. Cycling parameters may differ slightly between different RT-PCR kits and need to be validated. The amplified amplicon has a length of 231 bp, and can be visualized on an agarose gel (2 %).

1.7. 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 RNA extractions, at least one sample known to be infected with TSV and one sample known to be free of TSV infection should be included in each batch of extractions.

For the RT-PCR reactions, 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 RNA corresponding to the amplicon sequence. The positive control can consist of RNA previously tested TSV positive by PCR; it can consist of a synthesized RNA fragment containing the amplicon sequence, or it can consist of RNA *in vitro* transcribed from a PCR product or plasmid containing the amplicon sequence. The EURL can provide a positive PCR control if needed. Alternatively, a plasmid containing the amplicon sequence can be used as positive PCR control, although this will not test the validity of the reverse transcription step.

The qPCR assay is considered valid if the extraction control and PCR control produce amplification curves with sigmoidal shapes and with C_T values below the chosen threshold, whereas the negative extraction control and the negative PCR control produce no amplification curves. The conventional PCR assay is considered valid if a PCR product of the right size is produced in the positive extraction control and positive PCR control, and if no product is produced in the negative extraction control and negative PCR control.

2. DIAGNOSTIC PROCEDURES FOR "THE CONFIRMATION OF" OR "TO RULE OUT" THE SUSPICION OF INFECTION WITH TSV IN SUSPECTED OUTBREAKS

When a suspicion of TSV is required to be confirmed or ruled out, molecular identification of the virus must be performed through DNA sequencing of a PCR amplicon and subsequent matching of the obtained sequence to a known TSV sequence.

2.1. 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 or similar. 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 TSV sequence.

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