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NATIONAL INSTITUTE OF AQUATIC RESOURCES, TECHNICAL UNIVERSITY OF DENMARK

DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND CONFIRMATION OF INFECTION WITH YELLOW HEAD VIRUS (YHV1)

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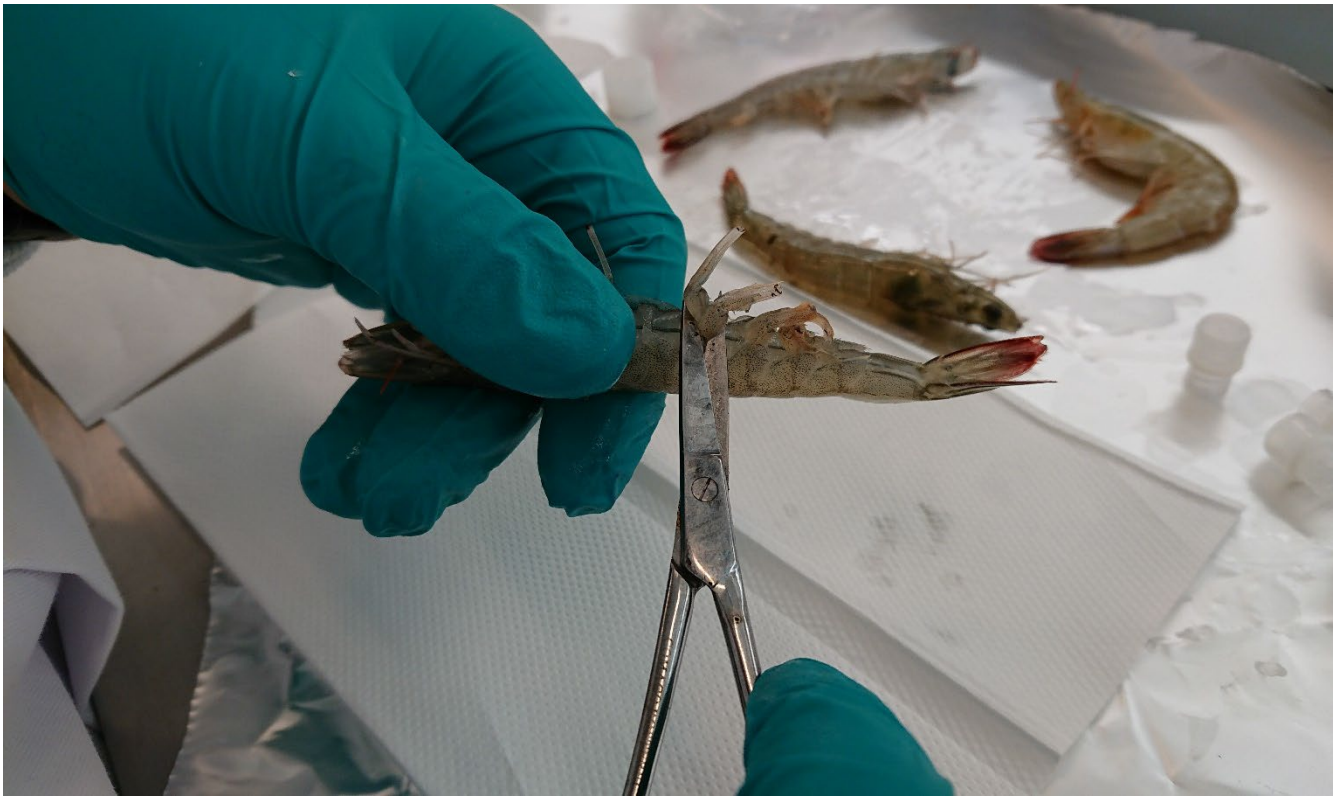

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DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE AND DETECTION OF INFECTION WITH YELLOW HEAD VIRUS (YHV1)

1. DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEILLANCE OF INFECTION WITH YHV1

1.1. Definitions.

The *Okavirus* genus so far comprises eight viral genotypes, named YHV1 – YHV8. Only YHV1 (also referred to as YHV) is known to cause serious disease in shrimp, and is the genotype subjected to regulations as set out in REGULATION (EU) 2016/429 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL ('Animal Health Law') and in associated delegated acts.

1.2. Diagnostic procedures for detection of YHV1.

When sampling and laboratory examination for the purpose of confirming or ruling out a suspect of YHV1 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 that 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.

1.3. 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 sent 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 YHV1 from tissue samples shall be as follows: Homogenisation of the tissue, extraction of the RNA, RT-PCR amplification of YHV1 RNA using either method A or B in point 1.6 below, visualisation of the amplified product on a gel, purification of the RNA and sequencing to confirm the identity of the pathogen.

1.4. 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.

1.5. 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.

1.6. YHV1 Polymerase Chain Reaction (PCR).

Two conventional RT-PCR methods for YHV1 surveillance and diagnostics are approved by the EURL. The methods corresponds to two of the three conventional PCR assays recommended in the WOAH Aquatic Manual.

Method A is a single PCR method developed by Wongteerasupaya et al. (1997) and modified by Mohr et al. (2015). It is specific for YHV genotype 1.

The RT-PCR is performed using the following primers:

10F 5'-CCG-CTA-ATT-TCA-AAA-ACT-ACG-3'

144R 5'-AAG-GTG-TTA-TGT-CGA-GGA-AGT-3'

Cycling conditions: 50°C for 30 min; 94°C for 15 min; 40 cycles of 94°C for 30 sec, 58°C for 45 sec and 72°C for 45 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 135 bp and can be visualized on an agarose gel (2 %).

Method B is a nested PCR method developed by Wijegoonawardane et al. (2008) and modified by Mohr et al. (2015). This assay will amplify YHV genotypes 1 – 7. In case of a positive PCR, the PCR product thus has to be sequenced in order to reveal the genotype.

The RT-PCR step is performed using the following primers:

YC-F1ab pool: 5'-ATC-GTC-GTC-AGC-TAC-CGC-AAT-ACT-GC-3' and
5'-ATC-GTC-GTC-AGY-TAY-CGT-AAC-ACC-GC-3'

YC-R1ab pool: 5'-TCT-TCR-CGT-GTG-AAC-ACY-TTC-TTR-GC-3' and
5'-TCT-GCG-TGG-GTG-AAC-ACC-TTC-TTG-GC-3'

Each pool consists of two primers in equal concentration, but with small differences in sequence in order to match a wider variety of YHV genotypes.

Cycling conditions: 50°C for 30 min; 95°C for 15 min; then 35 cycles of 94°C for 45 sec, 60°C for 45 sec and 72°C for 45 sec; and 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 358 bp and can be visualized on an agarose gel (1.5 - 2 %).

1 µl of product from the RT-PCR is amplified in a nested PCR using the following primers:

YC-F2ab pool: 5'-CGC-TTC-CAA-TGT-ATC-TGY-ATG-CAC-CA-3' and
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' and
5'-GTC-AGT-GTA-CAT-ATT-GGA-GAG-TTT-RTT-3'

Also in this case each pool consists of two primers in equal concentration, but with small differences in sequence in order to match a wider variety of YHV genotypes.

Cycling conditions: 95° C for 15 min; then 35 cycles of 94°C for 45 sec, 60°C for 45 sec and 72°C for 45 sec; and finally 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 146 bp, and can be visualized on an agarose gel (1.5 - 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 YHV1 and one sample known to be free of YHV1 infection should be included in each batch of extractions.

For the 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 YHV1 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 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 YHV1 IN SUSPECTED OUTBREAKS

When a suspicion of YHV1 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 YHV1 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 sent 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 YHV1 sequence.

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