European Union Reference Laboratory for Fish and Crustacean Diseases
NATIONAL INSTITUTE OF AQUATIC RESOURCES, TECHNICAL UNIVERSITY OF DENMARK

EURL for Crustacean Diseases

Report of the Inter-laboratory proficiency test 2024 for identification of White Spot Syndrome Virus (WSSV), Taura Syndrome Virus (TSV) and Yellow Head Virus 1 (YHV1)

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Introduction

A comparative test of diagnostic procedures for the detection of White Spot Syndrome Virus (WSSV), Taura Syndrome Virus (TSV), and Yellow Head Virus 1 (YHV1) was provided by the European Union Reference Laboratory (EURL) for Fish and Crustacean Diseases at DTU AQUA in accordance with EU Regulation (EU) 2017/625 § 94. The invitation to participate in this year's proficiency test was sent to 24 laboratories including 20 NRLs of EU Member States. All laboratories send the results.

Each laboratory was given a code number to ensure the anonymity of this report.

A coded version of the report is provided to all participants along with individual certificates where the code number of each participant is supplied. In this way each participating laboratory can compare its diagnostic performances with the overall performances. In the certificates, comments related to underperformances are included, if present.

An un-coded version of the report is sent to the European Commission.

Sample Preparation

Viral inoculates of WSSV (Isolate UAZ 00-173B), TSV (isolate UAZ 00-273), and YHV1 (isolate UAZ 99-294) were obtained from the Cefas laboratory in Weymouth, UK. These isolates were originally obtained from the WOAH reference laboratory at the University of Arizona, USA. Subsequent passages of this isolate into naïve *P. vannamei* held at DTU AQUA have demonstrated continued infectivity of these isolates.

Organ homogenate from infected *Penaeus vannamei* was tested by qPCR and RT-PCR (depending on the virus) and diluted in media culture (MEM).

To produce the FTA card test material, 65 µl of the diluted shrimp homogenate was applied to each circular FTA card area (QIAcard FTA Classic [Qiagen WB120305]), upon which the cards were dried for three days at room temperature. Subsequently, the adsorbed cards were tested to ensure the expected pathogen was found in each batch. Five replicates of each sample were tested, and the batch was accepted only if cards gave consistent results for the viral species and the viral load, assessed by Ct values.

The samples were re-tested after the deadline by qPCR to ensure the stability of the material. All tests yielded the expected results.

Diagnostic methods

Extraction of nucleic acid (DNA and RNA) from FTA cards

A portion of FTA card with approximate dimensions of 5 x 5 mm was cut. The sample was incubated in an Eppendorf tube with TE buffer (250 µl) for 30 min at room temperature on an orbital shaker (600 rpm). 200 µl of the sample was used for automated DNA and RNA using the Indimag Pathogen kit (Indical Bioscience) following manufacturer's instructions on an Indimag 48s extraction robot.

TSV real-time PCR

Based on Tang et al. (2004).
5 µl template RNA was added to a PCR tube containing: 5 µl TaqPath™ 1-Step RT-qPCR Master Mix, CG, 0.8 µl forward primer (10 µM), 0.8 µl reverse primer (10 µM), 0.4 µl hydrolysis Probe (10 µM) and 8 µl molecular grade water. The PCR profile was one cycle of 50°C for 15 minutes and 95°C for 2 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Primer sequences were TSV1004F: 5'-TTG-GGC-ACC-AAA-CGA-CAT-T-3', TSV1075R: 5'-GGG-AGC-TT A-AAC-TGG-ACA-CAC-TGT-3', hydrolysis Probe TSV-P1: 5'-CAG-CAC-TGA-CGC-ACA-ATA-TTC-GAG-CAT-C-3' with fluorescent dyes 6-Carboxyfluorescein (6-FAM) on the 5' end and Black Hole Quencher (BHQ) on the 3' end.

A positive RT-qPCR control, consisting of a synthesized RNA fragment representing the TSV PCR amplicon was included

YHV real-time PCR

Based on WOAH manual (Moody, 2023)

5 µl template RNA was added to a PCR tube containing: 5 µl TaqPath™ 1-Step RT-qPCR Master Mix, CG, 1.8 µl forward primer (10 µM), 1.8 µl reverse primer (10 µM), 0.4 µl hydrolysis Probe (10 µM) and 6 µl molecular grade water. The PCR profile was one cycle of 25°C for 2 minutes, 50°C for 15 minutes and 95°C for 2 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 30 seconds.

Primer sequences were YHV1-12-qF: 5'-AGT-CTA-CAG-TGC-TCT-GAT-CT-3', YHV1-12-qR: 5'-GAT-TCT-TGA-AGC-GCA-TGA-GT-3', hydrolysis Probe YHV1-12-qPr: FAM-TCT-CAT-GTG/ZEN/TCA-TGATAT-TCT-CAA-GCG-AGT-IABkFQ.

A positive PCR control, consisting of an *in vitro* transcribed RNA fragment representing the YHV RT-q PCR amplicon was included.

YHV conventional PCR

Based on Mohr et al. (2015).
First round RT-PCR: 5 µl template RNA was added to a PCR tube containing: 5 µl Qiagen OneStep RT-PCR kit buffer (Qiagen), 1.5 µl forward primer (10 µM), 1.5 µl reverse primer (10 µM), 1 µl of dNTP (10 mM each), 1 µl of Enzyme Mix and 10 µl molecular grade water. The PCR profile is one cycle of 50°C for 30 minutes and 95°C for 15 minutes, followed by 40 cycles of 94°C for 30 seconds, 58°C for 45 seconds, and 72°C for 45 seconds, followed by one cycle of 72°C for 7 minutes.

RT-PCR products were subsequently run on 2 % e-gels (Invitrogen).

Primer sequences were: 10F: 5'-CCG-CTA-ATT-TCA-AAA-ACT-ACG-3', 144R: 5'--AAG-GTG-TTA-TGT-CGA-GGA-AGT-3'.

WSSV real-time PCR

Based on Durand & Lightner (2002).
5 µl template DNA was added to a PCR tube containing: 10 µl Luna® Universal Probe qPCR Master Mix (New England Biolabs), 0.8 µl forward primer (10 µM), 0.8 µl reverse primer (10 µM), 0.4 µl Taqman Probe (10 µM) and 6 µl molecular grade water. The PCR profile is one cycle of 94°C for 15 minutes, followed by 50 cycles of 94°C for 15 seconds and 60°C for 60 seconds.

Primer sequences were WSS1011F: 5'-TGG-TCC-CGT-CCT-CAT-CTC-AG-3', WSS1079R: 5'-GCT-GCC-TTG-CCG-GAA-ATT-A-3', hydrolysis Probe: 5'-AGC-CAT-GAA-GAA-TGC-CGT-CTA-TCA-CAC-A-3' with fluorescent dyes 6-Carboxyfluorescein (6-FAM) on the 5' end, Iowa Black FQ (IBFQ) on the 3' end and an internal ZEN quencher.

A positive PCR control, consisting of a synthesized gBlocks gene fragment representing the WSSV PCR amplicon was included.

Distribution

Each laboratory participating in the proficiency test received nine FTA cards, including 3 negative cards adsorbed with MEM, 2 cards adsorbed with WSSV at two different concentrations, 2 cards adsorbed with TSV at two different concentrations, and 2 cards adsorbed with YHV-1 at two different concentrations.

The test samples were sent out according to current international regulations for the shipment of diagnostic specimens UN 3373, "Biological substance, Category B". All proficiency tests were shipped by courier.

Proficiency test content and expected results

The proficiency test consisted of nine samples. The contents of the Proficiency Test are shown in Table 1.

Table 1. Expected results of the proficiency test.

Sample ID	Virus
Sample 01	WSSV UAZ 00-173B, low concentration dilution factor 1:300
Sample 02	YHV UAZ 99-294, high concentration dilution 1:10
Sample 03	Negative (cell culture supernatant)
Sample 04	TSV UAZ 00-273, low concentration dilution factor 1:300
Sample 05	WSSV UAZ 00-173B, high concentration dilution factor 1:10
Sample 06	Negative (cell culture supernatant)
Sample 07	YHV UAZ 99-294, low concentration, dilution factor 1:100
Sample 08	TSV UAZ 00-273, high concentration, dilution factor 1:10
Sample 09	Negative (cell culture supernatant)

Results

Participants were asked to identify the content of each of the nine received FTA cards by the method used in their laboratory. Each correct answer accounted for two points, for a total of 18 points. Some laboratories are testing only for WSSV, as they have derogated their testing for YHV and TSV. In this case, the maximum score that can be obtained is 10 points.

Results were received from all 25 participating laboratories

- 18 laboratories correctly diagnosed all samples (100 %)
- 1 laboratory did not confirm the TSV in sample IV, concluded TSV/undetermined and got minor underperformance 17/18
- 2 laboratories did not identify YHV in sample VII and got one underperformance (16/18)
- 2 laboratories did not identify 2 samples and got 2 underperformance each (14/18)
- 1 laboratory had 3 underperformances (12/18)
- 1 laboratory had 5 underperformances (8/18)
- 3 out of 25 laboratories tested only for WSSV, and therefore could obtain a maximum score of 10

A detailed overview of the results is shown in Table 2.

Evaluation of results

The error rate of the results received in 2024 was comparable to previous proficiency tests from 2022 and 2023, but higher than that of 2021, in which 100% of the samples were correctly identified. All the erroneous results consist of false negative tests.

The laboratory with the lowest performance may have cross-contaminated samples during or after nucleic acid purification. A new batch of the ILPT will be offered to this participant so that they can reassess their procedures.

Sample VII, containing TSV at low concentration has given some challenges to some participants prompting a re-assessment of the sensitivity of the current procedures applied. Most laboratories that do not detect TSV in ampoule VII use an end-point PCR assay, possibly having a lower sensitivity than the RT-qPCR assay (Tang et al.,2004).

During the Annual Workshop, when the content of the ampoule was disclosed, there was some discussion about the lack of detection of the host DNA in the samples, as some laboratories include an endogenous internal control in their standard procedures. The EURL has explained that the starting material for the proficiency test was diluted several orders of magnitude, and further dilutions were done during preparation for inoculation in FTA cards. This may have caused a dilution of the host DNA to a point beyond the limit of detection of the assays used. Although the EURL does not evaluate the detection of the internal control, only the correct detection of the target pathogen, and the use of internal control will be considered during the preparation of the next proficiency test.

Table 2 – Result overview of the ILPT for crustacean diseases 2024

Participant number	**Sample I WSSV**	**Sample II YHV**	**Sample III Negative**	**Sample IV TSV**	**Sample V WSSV**	**Sample VI Negative**	**Sample VII YHV**	**Sample VIII TSV**	**Sample IX Negative**	**SCORE**
1	WSSV	YHV	Neg	TSV*	WSSV	Neg	YHV	TSV	Neg	17/18
2	WSSV	YHV	Neg	TSV	WSSV	Neg	YHV	TSV	Neg	18/18
3	WSSV	YHV/TSV	YHV	TSV	WSSV	YHV	WSSV	TSV	YHV	8/18
4	WSSV	Neg	YHV	TSV	WSSV	Neg	YHV	TSV	YHV	12/18
5	WSSV	YHV	Neg	TSV	WSSV	Neg	YHV	TSV	Neg	18/18
6	WSSV	Neg WSSV	Neg WSSV	Neg WSSV	WSSV	Neg WSSV	Neg WSSV	Neg WSSV	Neg WSSV	10/10
7	WSSV	YHV	Neg	TSV	WSSV	Neg	YHV	TSV	Neg	18/18
8	WSSV	YHV	Neg	TSV	WSSV	Neg	YHV	TSV	Neg	18/18
9	WSSV	YHV	Neg	TSV	WSSV	Neg	YHV	TSV	Neg	18/18
10	WSSV	Neg	Neg	Neg	WSSV	Neg	Neg	Neg	Neg	10/10
11	WSSV	YHV	Neg	TSV	WSSV	Neg	YHV	TSV	Neg	18/18
12	WSSV	YHV	Neg	TSV	WSSV	Neg	Neg	TSV	Neg	16/18
13	WSSV	YHV	Neg	TSV	WSSV	Neg	YHV	TSV	Neg	18/18
14	WSSV	Neg	Neg	Neg	WSSV	Neg	Neg	Neg	Neg	10/10
15	WSSV	YHV	Neg	TSV	WSSV	Neg	YHV	TSV	Neg	18/18
16	WSSV	YHV	Neg	TSV	WSSV	Neg	Neg	TSV	Neg	16/18
17	WSSV	YHV	Neg	TSV	WSSV	Neg	YHV	TSV	Neg	18/18
18	WSSV	YHV	Neg	TSV	WSSV	Neg	YHV	TSV	Neg	18/18
19	WSSV	YHV	Neg	TSV	WSSV	Neg	YHV	TSV	Neg	18/18
20	WSSV	YHV	Neg	TSV	WSSV	Neg	YHV	TSV	Neg	18/18
21	WSSV	YHV	Neg	TSV	WSSV	Neg	YHV	TSV	Neg	18/18
22	WSSV	YHV	WSSV	TSV	Neg	Neg	YHV	TSV	Neg	14/18
23	WSSV	YHV	Neg	TSV	WSSV	Neg	YHV	TSV	Neg	18/18
24	WSSV	YHV	Neg	Neg	WSSV	Neg	Neg	TSV	Neg	14/18
25	WSSV	YHV	Neg	TSV	WSSV	Neg	YHV	TSV	Neg	18/18
Correct/total	25/25	23/25	22/25	25/25	24/25	24/25	22/25	25/25	23/25	18/25

*the final result was “undetermined”.

Table 3 Molecular methods used by the participant laboratories for pathogen detection in the ILPT for crustacean diseases 2024

Participant number	**Method 1 WSSV**	**Method 2 WSSV**	**Method 1 YHV**	**Method 2 YHV**	**Method 1 TSV**	**Method 2 TSV**	**SCORE**
1	qPCR	PCR	qPCR		PCR	qPCR	17/18
2	qPCR		PCR		qPCR		18/18
3	PCR		PCR		PCR		8/18
4	qPCR		PCR		qPCR		12/18
5	qPCR		qPCR	PCR	qPCR		18/18
6	qPCR		N/A		N/A		10/10
7	Nested PCR	qPCR	Nested PCR		PCR		18/18
8	qPCR	PCR	PCR		PCR		18/18
9	qPCR		PCR		PCR		18/18
10	qPCR		N/A		N/A		10/10
11	Nested PCR		PCR		PCR		18/18
12	qPCR		PCR		qPCR		16/18
13	qPCR		Nested PCR		PCR		18/18
14	PCR		N/A		N/A		10/10
15	qPCR	NestedPCR; PCR	PCR		qCPR		18/18
16	Nested PCR		PCR		PCR		16/18
17	qPCR		PCR		PCR		18/18
18	qPCR		PCR		qPCR		18/18
19	qPCR		qPCR		qPCR		18/18
20	qPCR	PCR	PCR		qPCR		18/18
21	qPCR		PCR		qPCR		18/18
22	qPCR	Nested PCR	PCR		PCR		14/18
23	qPCR		qPCR		qPCR		18/18
24	PCR		PCR		PCR		14/18
25	qPCR		PCR		qPCR		18/18
Correct/total	25/25	23/25	22/25	25/25	24/25	24/25	18/25

The EURL provides the annual proficiency test, collates the data, and processes the figures so that individual laboratories can see how they fare in comparison to the other participants. It is up to the individual laboratories to assess if they perform according to their own expectations and standards. We take the opportunity to provide comments to participants regarding submitted results if relevant. Furthermore, we encourage all participants to contact us with any questions concerning the test or any other diagnostic matters.

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European Union Reference Laboratory for Fish and Crustacean Diseases

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WOAH Manual CHAPTER 2.2.10. INFECTION WITH YELLOW HEAD VIRUS GENOTYPE 1