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

Inter-laboratory proficiency test for Crustacean diseases 2023

Detection of Taura Syndrome Virus (TSV), Yellow Head Virus 1 (YHV1) and White Spot Syndrome Virus on FTA cards

Organised by the
European Union Reference Laboratory for Fish and Crustacean Diseases,
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Introduction

A comparative test of diagnostic procedures for the detection of Taura Syndrome Virus (TSV), Yellow Head Virus 1 (YHV1) and White Spot Syndrome Virus (WSSV) 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 26 laboratories including 19 NRLs of EU Member States. 25 laboratories including 18 NRLs of EU Member States accepted the invitation to participate and send in their test results for diagnostic assays not derogated to other laboratories.

Each laboratory was given a code number to ensure discretion. The code number of each participant is supplied to the respective laboratories with this report. Furthermore, the EURL-team has included comments to the participants if relevant. An un-coded version of the report is sent to the European Commission.

Sample Preparation

Viral inoculates of TSV, YHV1 and WSSV were obtained from the Cefas laboratory in Weymouth, UK who originally obtained them from the WOAH reference laboratory at the University of Arizona, USA. The WOAH isolate of TSV (UAZ 00-273) was generated in *Penaeus vannamei* from an original outbreak in *P. vannamei* in Hawaii in 1994. The WOAH isolate of YHV1 (UAZ 99-294) was generated in *P. vannamei* from an original outbreak in *P. vannamei* in Thailand in 1992. The WOAH isolate of WSSV (UAZ 00-173B) was generated in *P. vannamei* from an original outbreak in *Fenneropenaeus chinensis* in China in 1995. Subsequent passages of this isolate into naïve *P. vannamei* held at Cefas have demonstrated continued infectivity of these isolates.

Test material was prepared by grinding half of a shrimp carcass infected with either TSV, YHV1 or WSSV or being specific pathogen free (SPF) in a mortar with a small amount of sand and 4 ml PBS. PBS was added to a total volume of 4 ml per gram of shrimp tissue and the homogenate was then centrifuged at 3000 x *g* for 30 minutes and the supernatant frozen at -80°C in 2 ml aliquots.

To produce the FTA card test material, 65 µl of shrimp homogenate was applied to each circular FTA card area (QIAcard FTA Micro [Qiagen WB120210]), upon which the cards were dried for one day at room temperature and stored at 5 °C. For each of the three viruses, two kinds of FTA cards were produced: one with moderate virus concentration (C_T around 25) and one with low virus concentration (C_T around 30). Each participating laboratory was send eight FTA cards. Prior to distribution, the EURL tested three FTA cards of each category to ensure that the FTA cards worked satisfactorily, using the methods described below.

Diagnostic methods

Extraction of RNA from FTA cards

A ca. 5 x 5 mm piece of an FTA card was cut out with a sterile scissor and incubated in PBS (250 µl) for 30 min with occasional vortexing. The liquid was collected into a clean tube, and the tube containing the strip was centrifuged 5 min at 10.000 rpm. The remainder of the liquid was collected and added to the first collection. RNA was extracted from the liquid using an Indimag Pathogen kit (Indical Bioscience) on an Indimag 48s extraction machine according to the manual enclosed in the kit.

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 Taqman 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', Taqman 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. The primers were manufactured by Integrated DNA Technologies and the probe by TAG Copenhagen A/S.

A positive PCR control was included, which consisted of a synthesized RNA fragment representing the TSV PCR amplicon.

YHV conventional PCR

Based on Mohr et al. (2015).
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.

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'. The primers were manufactured by Integrated DNA Technologies.

A positive PCR control was included, which consisted of an in-vitro transcribed RNA fragment representing the YHV PCR amplicon.

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', Taqman 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 between the 9th and the 10th base. All primers were manufactured by Integrated DNA Technologies.

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

Distribution

Each laboratory participating in the proficiency test received eight FTA cards, of which two were infused with TSV material, two with YHV1 material, two with WSSV and two with SPF material. The test samples were sent out according to current international regulations for shipment of diagnostic

specimens UN 3373, “Biological substance, Category B”. All proficiency tests were delivered by courier.

Table 1. Expected results of the proficiency test.

Sample ID	Sample type	Infection status
Sample 01	FTA card infused with shrimp homogenate	TSV, low concentration
Sample 02	FTA card infused with shrimp homogenate	Negative
Sample 03	FTA card infused with shrimp homogenate	Negative
Sample 04	FTA card infused with shrimp homogenate	WSSV, high concentration
Sample 05	FTA card infused with shrimp homogenate	YHV1, low concentration
Sample 06	FTA card infused with shrimp homogenate	YHV1, high concentration
Sample 07	FTA card infused with shrimp homogenate	WSSV, low concentration
Sample 08	FTA card infused with shrimp homogenate	TSV, high concentration

Expected results

Participants were asked to identify the content of each of the eight FTA cards by the method used in their laboratory. The sample contents are shown in Table 1.

Actual results

Results were received from all 25 participating laboratories.

- 20 laboratories correctly diagnosed all samples, 8/8 (100 %) or 4/4 (100 %).
- 5 laboratories correctly diagnosed five samples, 7/8 (87,5 %).

The following methods were used by the participants to diagnose WSSV:

- 14 laboratories used real time PCR
- 5 laboratories used nested PCR
- 6 laboratories used both real time PCR and nested PCR

The following methods were used by the participants to diagnose TSV:

- 12 laboratories used real time PCR
- 9 laboratories used single PCR
- 1 laboratory used both real time PCR and single PCR

The following methods were used by the participants to diagnose YHV:

- 2 laboratories used real time PCR

- 5 laboratories used nested PCR
- 14 laboratories used single PCR
- 1 laboratory used both real time PCR and single PCR

Three laboratories verified the identity of at least one of the obtained PCR products by sequencing.

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

Evaluation of results

The error rate of the results received in 2023 was comparable to previous proficiency tests. All the erroneous results consist of false negative tests of low concentration samples. Some participants have explained that they actually obtained a very weak signal in the erroneously identified samples but chose to report the samples as negatives. It may be useful for the laboratories in question to evaluate their DNA and RNA extraction and test methods in order to increase the sensitivity of the relevant assays. Alternatively, the criteria for accepting results as positive or negative could be re-evaluated, although this might result in more cases of false positives. Finally, it should be considered to always test samples in duplicates, as this will give more certainty to the test results. At the same time the EURL should strive to not provide proficiency test samples with too low pathogen concentrations, as this may create too many problems for the participants, as there will always be problems doing diagnostics of samples having pathogen concentrations close to the limit of detection.

Table 3 shows the methods used for DNA extraction and PCR amplification. A wide range of methods was used, making it difficult to infer any correlations between methods used and results obtained.

Table 2. Proficiency test results submitted by the individual laboratories. Reported cycle thresholds for qPCR is shown in brackets (if samples were run in replicate, the average C_T value is shown). Samples diagnosed as negative for TSV, YHV and WSSV are marked as -ve while samples diagnosed as positive for one of the viruses are marked accordingly. Diagnostic assays derogated to other laboratories are marked as "Der".

Laboratory No.	S-01	S-02	S-03	S-04	S-05	S-06	S-07	S-08	Score
EURL	TSV (28.7)	-ve	-ve	WSSV (20.0)	YHV1	YHV1	WSSV (28.9)	TSV (23.0)	
1	Der	-ve	-ve	WSSV	Der	Der	WSSV	Der	4/4
2	TSV	-ve	-ve	WSSV	YHV1	YHV1	-ve	TSV	7/8
3	TSV (27.5)	-ve	-ve	WSSV (16.8)	YHV1	YHV1	WSSV (26.7)	TSV (21.2)	8/8
4	This NRL has derogated its crustacean diagnostics to FLI in Germany								
5	Der	-ve	-ve	WSSV (21.16)	Der	Der	WSSV (31.84)	Der	4/4
6	TSV (27.25)	-ve	-ve	WSSV (24.9)	YHV1	YHV1	WSSV (35.05)	TSV (22.76)	8/8
7	TSV (28.4, 31.8)	-ve	-ve	WSSV (26.9, 27.3)	YHV1	YHV1	WSSV (34.13, 35.3)	TSV (24.2, 28.0)	8/8
8	TSV	-ve	-ve	WSSV (22.5)	YHV1	YHV1	WSSV (32.67)	TSV	8/8
9	TSV (28.86)	-ve	-ve	WSSV (21.15)	-ve	YHV1	WSSV (34.5)	TSV (22.81)	7/8
10	Did not participate in 2023								
11	TSV (29.12)	-ve	-ve	WSSV (23.14)	YHV1	YHV1	WSSV (32.67)	TSV (23.82)	8/8
12	TSV	-ve	-ve	WSSV	YHV1	YHV1	WSSV	TSV	8/8
13	TSV (29.3)	-ve	-ve	WSSV (21, 20.4)	-ve	YHV1 (32.9)	WSSV (32.9, 32.4)	TSV (24.1)	7/8
14	TSV (31.10)	-ve	-ve	WSSV (22.78)	YHV1	YHV1	WSSV (31.48)	TSV (25.99)	8/8
15	Der	-ve	-ve	WSSV (22.84, 22.89, 22.95)	Der	Der	WSSV (31.94, 31.9, 31.99)	Der	4/4
16	TSV (32.69)	-ve	-ve	WSSV (22.02)	YHV1	YHV1	WSSV (35.05)	TSV (34.51)	8/8
17	This NRL has derogated its crustacean diagnostics to The Netherlands								
18	TSV	-ve	-ve	WSSV (23.16)	YHV1	YHV1	WSSV (33.26)	TSV	8/8
19	TSV	-ve	-ve	WSSV	YHV1	YHV1	WSSV	TSV	8/8
20	TSV	-ve	-ve	WSSV (24.92)	YHV1	YHV1	WSSV (33.81)	TSV	8/8
21	TSV (37.5)	-ve	-ve	WSSV (23)	-ve	YHV1	WSSV (31.5)	TSV (29)	7/8
22	TSV (28.80)	-ve	-ve	WSSV (20.54)	YHV1	YHV1	WSSV (30.45)	TSV (25.21)	8/8
23	TSV (28.3)	-ve	-ve	WSSV (18.9)	YHV1 (30.7)	YHV1 (25.6)	WSSV (28.6)	TSV (22.3)	8/8
24	TSV	-ve	-ve	WSSV (22.32)	YHV1	YHV1	-ve	TSV	7/8
25	TSV	-ve	-ve	WSSV (21.6)	YHV1	YHV1	WSSV (32.29)	TSV	8/8
26	TSV (27.99)	-ve	-ve	WSSV	YHV1	YHV1	WSSV	TSV (28.07)	8/8
27	TSV	-ve	-ve	WSSV (20.23)	YHV1	YHV1	WSSV (33.95)	TSV	8/8
28	TSV (31.8)	-ve	-ve	WSSV (25.3)	YHV1 (35.8)	YHV1 (32.3)	WSSV (38.5)	TSV (24.3)	8/8
30	This NRL has derogated its crustacean diagnostics to Denmark								

Table 3. Diagnostic assays used by participants. Diagnostic assays derogated to other laboratories are marked as "Der".

Laboratory Code	TSV	YHV	WSSV
EURL	RT-qPCR + RT-PCR	Single RT-PCR	qPCR + PCR
1	Der	Der	Nested PCR
2	Single RT-PCR	Single RT-PCR	Nested PCR
3	RT-qPCR	Nested RT-PCR	qPCR + PCR
5	Der	Der	qPCR
6	RT-qPCR	Single RT-PCR	qPCR
7	Single RT-PCR + RT-qPCR	Single RT-PCR	Nested PCR + qPCR
8	Single RT-PCR	Single RT-PCR	qPCR + PCR
9	RT-qPCR	Single RT-PCR	qPCR
11	RT-qPCR	Nested RT-PCR	qPCR
12	Single RT-PCR	Nested RT-PCR	Nested PCR
13	RT-qPCR	RT-qPCR	qPCR
14	RT-qPCR	Single RT-PCR	qPCR + PCR
15	Der	Der	qPCR
16	RT-qPCR	Single RT-PCR	qPCR
18	Single RT-PCR	Single RT-PCR	qPCR
19	Single RT-PCR	Single RT-PCR	Nested PCR
20	Single RT-PCR	Nested RT-PCR	qPCR + PCR
21	RT-qPCR	Single RT-PCR	qPCR
22	RT-qPCR	Single RT-PCR	qPCR
23	RT-qPCR	RT-qPCR + Single RT-PCR	qPCR
24	Single RT-PCR	Single RT-PCR	qPCR
25	Single RT-PCR	Single RT-PCR	Nested PCR + qPCR
26	RT-qPCR	Single RT-PCR	Nested PCR
27	Single RT-PCR	Nested RT-PCR	qPCR
28	RT-qPCR	RT-qPCR	qPCR

The EURL provides the annual proficiency test, collates the data and process the figures so that individual laboratories can see how they fare in relation to the other participants. It is up to the individual laboratory 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 contacts us with any questions concerning the test or any other diagnostic matters.

The results given in this report were presented and discussed at the 14th Annual Workshop of the National Reference Laboratories for Crustacean Diseases on June 1st 2023, with participation of representatives from the EURL and NRLs.

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Table 4. DNA/RNA extraction and PCR methods used by the participating laboratories. Numbers refer to codes of participating laboratories.

Lab Code	DNA/RNA Extraction Method	PCR Kit
EURL	IndiMag Pathogen Kit with INDIMAG robot	TaqPath™ 1-Step RT-qPCR Master Mix; Qiagen OneStep RT-PCR kit
1	MagNA Pure 24 Total NA isolation Kit	Qiagen HotStarTaq DNA Polymerase
2	QIAamp cador pathogen kit	Bioline MyTaq mix; Qiagen One step RT PCR kit
3	IndiMag Pathogen kit with BioSprint 96 Workstation	Go Taq Hot Start Green; MultiScribe Reverse Transcriptase; AgPath-ID One-Step RT-PCR kit
5	QIAamp PowerFecal Pro DNA Kit using QIAcube platform	iTaq Universal Probes mastermix
6	IndiMag Pathogen Kit	PerfeCTa qPCR Toughmix; KiCqStart One-Step RT-PCR Readymix
7	Qiagen DNA Mini Kit; Qiagen RNeasy Mini Kit	Super-Script One-Step RT-PCR with Platinium Taq; QuantiNova Pathogen + IC Kit
8	NucleoMag VET Kit with KingFisher Flex magnetic particle processor	QuantiTect Probe PCR Kit; QIAGEN OneStep RT-PCR Kit
9	DNeasy Blood & Tissue Kit; QiaAmp Viral RNA Mini Kit	One-Step Probe PCR Mix, One-Step RT PCR Mix, 5xHOT FIREPol Mix
11	MagMax CORE Kit with KingFisher Flex magnetic particle processor	One Step RT-PCR Kit; Platinum PCR SuperMix Kit; QuantiNova Pathogen + IC Kit
12	Qiagen DNA Tissue–Biorobot Advanced XL Qiagen RNA Tissue–Biorobot Advanced XL	Promega M-MLV Reverse transcriptase; Promega Go Taq G2 Flexi DNA Polymerase
13	Biomerieux NucliSENS® easyMAG®	Taqman® reverse transcription reagent kit; SYBR.GR Master mix
14	MagMax CORE Kit with KingFisher Flex magnetic particle processor	SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase; Luminaris Probe qPCR Master Mix low ROX; Taq PCR Master Mix Kit
15	QIAamp DNA Mini Kit with Qiacube robot	TaqMan® Universal PCR Master Mix
16	GoTaq® Probe qPCR Master Mix; Qiagen One Step RT-PCR; Amplifyme Probe One Step Universal RTqPCR Mix	innuPREP AniPath DNA/RNA Kit
18	IndiMag Pathogen Kit with INDIMAG robot	QuantiTect Probe PCR Kit; QIAGEN OneStep RT-PCR Kit
19	RNeasy Mini Kit	Platinum Taq DNA Polymerase; Qiagen OneStep RT-PCR Enzyme Mix
20	QIAamp DNA Mini Kit; QIAamp RNA Mini Kit	QIAGEN OneStep RT-PCR Kit; Qiagen QuantiTect Probe RT-PCR one-step kit; DNA QPCR – Perfecta qPCR ToughMix UNG ROX
21	DNEasy kit for Blood and Tissue using QIAcube platform; RNeasy Plus Kit for Tissue using QIAcube platform	QuantiTect Probe RT-PCR OneStep kit; Qiagen OneStep RT-PCR Kit; Perfecta qPCR ToughMix UNG ROX
22	DNeasy Blood&Tissue Kit; RNeasy Mini Kit	Qiagen Multiplex PCR Kit; Qiagen quantitect probe rt-pcr kit; Qiagen one-step RT PCR Kit
23	IndiMag Pathogen Kit with INDIMAG robot	TaqPath 1-step RT-qPCR Mastermix; Qiagen Onestep RT-PCR kit; Luna® Universal Probe qPCR Mastermix
24	Idexx Real PCR DNA/RNA spin Column Kit; Jena Bioscience Total RNA Purification Kit	AmpliTaq Gold DNA Polymerase kit; SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase
25	QIAGEN DNeasy Blood and Tissue Kit; QIAGEN RNeasy Plus Kit	GoTaq® Probe qPCR Master Mix; Qiagen HotStarTaq DNA Polymerase; QIAGEN OneStep RT-PCR Kit
26	IndiMag Pathogen Kit with INDIMAG robot	NZYTaq II Green Mix; Qiagen One-Step RT-PCR kit
27	Roche Viral Nucleic Acid Kit	QuantiTect Probe PCR Kit; QIAGEN OneStep RT-PCR Kit
28	QIAamp Viral RNA Mini Kit	AgPath-ID™ One-Step RT-PCR Reagents TaqMan Universal PCR Master Mix

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