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

Inter-laboratory proficiency test 2020

Detection of Taura Syndrome Virus (TSV) and Yellow Head Virus 1 (YHV1) in Shrimp Pleopods

Organised by the
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Introduction

A comparative test of diagnostic procedures for the detection of Taura Syndrome Virus (TSV) and Yellow Head Virus 1 (YHV1) in shrimp was provided by the European Union Reference Laboratory (EURL) for Fish and Crustacean Diseases at DTU AQUA in accordance with EC Directive 2006/88. The invitation to participate in this year's proficiency test was sent to 27 laboratories including 20 NRLs of EU Member States. 16 laboratories including 12 NRLs of EU Member States accepted the invitation to participate and send in their test results.

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

TSV and YHV1 inoculates were prepared by grinding half of a shrimp carcass infected with TSV or YHV1 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 inoculum was then centrifuged at 3000 g for 30 minutes and the supernatant frozen at -80°C in 2 ml aliquots. Before use, the inoculum was diluted 1:20 with PBS and sterile filtered through a 0.22 µm sterile filter mounted on a syringe. Infected shrimp carcasses were prepared by direct intramuscular injection of 100 µl inoculum into specific pathogen free (SPF) *P. vannamei*. During the following days, dead and moribund shrimp infected with YHV1 were collected from the experimental tanks. As the shrimp used for virus propagation have high resistance towards TSV, shrimp injected with TSV were collected after 7 days regardless if they showed symptoms or not. The shrimp were kept in a flow-through system with artificial sea water with a salinity of ca. 20 ppt and a temperature of ca. 26˚C.

All 10 pleopods were removed from newly dead animals and fixed in RNAlater for molecular analysis, with each matching set of pleopods stored in the same tube (i.e. 5 tubes per shrimp). Pleopods from SPF shrimp served as TSV and YHV1 negative samples. Prior to distribution the EURL tested one set of pleopods from each individual shrimp to ensure that infection had resulted in a satisfactory titre that was measurable with standard PCR based methods.

Multiple NRLs received pleopods from the same shrimp.

Shrimp were confirmed as TSV or YHV positive or negative by PCR using the following procedures.

Diagnostic methods

Extraction of RNA from Pleopods

RNA was extracted using an Indimag Pathogen kit (Indical Bioscience) on an Indimag 48s extraction machine. Half of a pleopod was homogenized using bead beating with a 5 mm metal bead in 200 µl

PBS in a TissueLyser II (QIAGEN) for 2 x 2 min. The DNA was then purified using the manual enclosed in the kit.

TSV real-time PCR

Based on Tang et al. (2004).
2 µl template RNA was added to a PCR tube containing: 12.5 µl Quantitect Probe RT-PCR kit master mix (Qiagen), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 0.5 µl Taqman Probe (10 µM), 0.25 µl Quantitect RT mix and 7.75 µl molecular grade water. The PCR profile is one cycle of 50°C for 30 minutes and 95°C for 15 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 EURL Fish WorkshopA-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 gBlocks gene fragment representing the TSV PCR amplicon.

YHV nested PCR

Based on Mohr et al. (2015).
First round RT-PCR: 2 µl template RNA was added to a PCR tube containing: 5 µl Qiagen OneStep RT-PCR kit buffer (Qiagen), 0.75 µl of each forward primer (10 µM), 0.75 µl of each reverse primer (10 µM), 1 µl of dNTP (10 mM each), 1 µl of Enzyme Mix and 13 µl molecular grade water. The PCR profile is one cycle of 50°C for 30 minutes and 95°C for 15 minutes, followed by 35 cycles of 94°C for 45 seconds, 60°C for 45 seconds and 68°C for 45 seconds, followed by one cycle of 68°C for 7 minutes. Second round PCR: 1 µl first round PCR product was added to a PCR tube containing: 5 µl 5X Green GoTaq Flexi buffer (Promega Biotech AB), 1 µl of each forward primer (10 µM), 1 µl of each reverse primer (10 µM), 0.25 µl of dNTP (25 mM each), 2.5 µl of $MgCl_2$ (25 mM), 0.125 µl of GoTaq G2 Flexi DNA Polymerase and 12.125 µl molecular grade water. The PCR profile is one cycle of 95°C for 15 minutes, followed by 35 cycles of 94°C for 45 seconds, 60°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 for first PCR were: YC-F1a: 5'-ATC-GTC-GTC-AGC-TAC-CGC-AAT-ACT-GC-3', YC-F1b: 5'-ATC-GTC-GTC-AGY-TAY-CGT-AAC-ACC-GC-3', YC-R1a: 5'-TCT-TCR-CGT-GTG-AAC-ACY-TTC-TTR-GC-3', YC-R1b: 5'-TCT-GCG-TGG-GTG-AAC-ACC-TTC-TTG-GC-3'. Primer sequences for second PCR were: YC-F2a: 5'-CGC-TTC-CAA-TGT-ATC-TGY-ATG-CAC-CA-3', YC-F2b: 5'-CGC-TTY-CAR-TGT-ATC-TGC-ATG-CAC-CA-3', YC-R2a: 5'-RTC-DGT-GTA-CAT-GTT-TGA-GAG-TTT-GTT-3', YC-R2b: 5'-GTC-AGT-GTA-CAT-ATT-GGA-GAG-TTT-RTT-3'. The primers were manufactured by Integrated DNA Technologies.

Distribution

Each laboratory participating in the proficiency test received a pair of pleopods from each of two shrimp infected with TSV, two shrimp infected with YHV1 and two non-infected animals. Multiple NRLs received pleopods from the same shrimp. 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.

Expected results

Participants were asked to identify the infection status of the content of each of the six received tubes by the method used in their laboratory. The infection status of the tube contents is shown in Table 1.

Table 1. Expected results of the proficiency test.

Sample ID	Sample type	WSSV infection status
Sample XX-001	*P. vannamei* pleopods in RNAlater	Positive TSV (UAZ 00-273)
Sample XX-002	*P. vannamei* pleopods in RNAlater	Positive TSV (UAZ 00-273)
Sample XX-003	*P. vannamei* pleopods in RNAlater	Positive YHV1 (UAZ 99-294)
Sample XX-004	*P. vannamei* pleopods in RNAlater	Negative
Sample XX-005	*P. vannamei* pleopods in RNAlater	Negative
Sample XX-006	*P. vannamei* pleopods in RNAlater	Positive YHV1 (UAZ 99-294)

Actual results

Results were received from all 16 participating laboratories.
• 11 laboratories correctly diagnosed all samples, 6/6 (100 %).
• 5 laboratories correctly diagnosed 5/6 samples (83.3 %).

The following methods were used by the participants to diagnose TSV:
• 8 laboratories used real time PCR
• 8 laboratories used single PCR

The following methods were used by the participants to diagnose YHV:
• 7 laboratories used nested PCR
• 7 laboratories used single PCR
• 2 laboratories used real time PCR

3 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.

Table 2. Proficiency test results submitted by the individual laboratories. Reported cycle thresholds for qPCR is shown in brackets (for the EURL this is based on an average of all samples used for the test). Samples diagnosed as negative for both TSV and YHV are marked as –ve while samples diagnosed as positive for TSV are marked as TSV and samples positive for YHV are marked as YHV. Diagnoses that did not match the expectations are shown in red.

Laboratory Code	Method TSV	Method YHV	XX-001	XX-002	XX-003	XX-004	XX-005	XX-006	Score
EURL	qPCR	Nested PCR	TSV (27.7)	TSV (27.7)	YHV	-ve	-ve	YHV	
1	This NRL did not participate in the TSV/YHV1 proficiency test								
2	This NRL did not participate in the TSV/YHV1 proficiency test								
3	qPCR	Nested PCR	TSV (31.4)	TSV (24.9)	YHV	-ve	-ve	YHV	6/6
4	This NRL has outsourced its crustacean diagnostics to FLI in Germany								
5	This NRL did not participate in the TSV/YHV1 proficiency test								
6	This NRL did not participate in the TSV/YHV1 proficiency test								
7	qPCR	Nested PCR	TSV (38.08)	TSV (38.34)	YHV	-ve	-ve	YHV	6/6
8	This NRL did not participate in the TSV/YHV1 proficiency test								
9	PCR	PCR	TSV	-ve	YHV	-ve	-ve	YHV	5/6
10	This NRL did not participate in the TSV/YHV1 proficiency test								
11	PCR	Nested PCR	TSV	TSV	YHV	-ve	-ve	YHV	6/6
12	PCR	Nested PCR	TSV	TSV, YHV	YHV	-ve	-ve	YHV	5/6
13	qPCR	qPCR	-ve	TSV (33.0)	YHV (16.3)	-ve	-ve	YHV (18.7)	5/6
14	qPCR	PCR	TSV (35.1)	TSV (26.2)	YHV	-ve	-ve	YHV	6/6
15	This NRL did not participate in the TSV/YHV1 proficiency test								
16	This NRL did not participate in the TSV/YHV1 proficiency test								
17	This NRL has outsourced its crustacean diagnostics to The Netherlands								
18	PCR	PCR	-ve	TSV	YHV	-ve	-ve	YHV	5/6
19	PCR	PCR	TSV	TSV	YHV	-ve	YHV	YHV	5/6
20	PCR	PCR	TSV	TSV	YHV	-ve	-ve	YHV	6/6
21	qPCR	PCR	TSV (24)	TSV (31)	YHV	-ve	-ve	YHV	6/6
22	This NRL did not participate in the TSV/YHV1 proficiency test								
23	qPCR	Nested PCR	TSV (17.5)	TSV (23.2)	YHV	-ve	-ve	YHV	6/6
24	PCR	PCR	TSV	TSV	YHV	-ve	-ve	YHV	6/6
25	This NRL did not participate in the TSV/YHV1 proficiency test								
26	qPCR	Nested PCR	TSV (29.7)	TSV (36.8)	YHV	-ve	-ve	YHV	6/6
27	PCR	Nested PCR	TSV	TSV	YHV	-ve	-ve	YHV	6/6
28	qPCR	qPCR	TSV (21.9)	TSV (29.4)	YHV (13.1)	-ve	-ve	YHV (15.8)	6/6

Evaluation of results

The results received in 2020 are similar to those from previous proficiency tests. Of 96 samples tested, five were not diagnosed correctly (5.2%). As multiple laboratories received pleopods from the same shrimp, we can evaluate the likeliness of these unexpected diagnoses (see Table 3).

Table 3. Diagnoses obtained for each individual pleopod pair. Each shrimp provided five pairs of pleopods labeled A – E. Pleopod pair A was tested by the EURL before sending the samples (B – E). Numbers refer to codes of participating laboratories. Samples diagnosed as negative for both TSV and YHV are marked in grey, samples diagnosed as positive for TSV are marked in yellow and samples diagnosed as positive for YHV are marked in orange.

	Shrimp ID	Pleopod ID				
		A	B	C	D	E
Non-inoculated Shrimp	20-854-51	EURL	3	7	9	11
	20-854-52	EURL	12	13	14	18
	20-854-53	EURL	19	20	21	23
	20-854-54	EURL	24	26	27	28
	20-854-55	EURL	3	7	9	11
	20-854-56	EURL	12	13	14	18
	20-854-57	EURL	19	20	21	23
	20-854-58	EURL	24	26	27	28
Shrimp inoculated with TSV	20-854-102	EURL	3	7	9	11
	20-854-103	EURL	12	13	14	18
	20-854-107	EURL	19	20	21	23
	20-854-111	EURL	24	26	27	28
	20-854-116	EURL	3	7	9	11
	20-854-117	EURL	12	13	14	18
	20-854-123	EURL	19	20	21	23
	20-854-131	EURL	24	26	27	28
Shrimp inoculated with YHV1	20-854-151	EURL	3	7	9	11
	20-854-152	EURL	12	13	14	18
	20-854-153	EURL	19	20	21	23
	20-854-154	EURL	24	26	27	28
	20-854-155	EURL	3	7	9	11
	20-854-156	EURL	12	13	14	18
	20-854-157	EURL	19	20	21	23
	20-854-158	EURL	24	26	27	28

Table 3 shows no clear pattern in the falsely diagnosed samples. Two samples were falsely diagnosed as YHV positive, which probably was caused by contamination from YHV positive samples. This is especially a problem when using nested PCR, as PCR products from the first PCR round very easily can contaminate nearby samples when the PCR tube is opened. Two TSV positive samples were falsely diagnosed as negative samples, both originating from the same shrimp. As three out of five pleopod pairs were tested positive for TSV, we find it unlikely that the shrimp should be TSV negative. However, as most present day shrimp strains used in aquaculture are resistant to TSV, the viral load of the shrimp after inoculation is rather low, which may impose challenges to the detection

of TSV in the pleopod samples. For the shrimp in question, the Ct values were 24.9 for pleopod pair A and 35.1 for pair B, making it likely that a negative test result can be achieved in case the RNA extraction or PCR reaction was sub-optimal. Finally, one YHV sample was tested negative, which most likely was caused by failed RNA extraction or PCR.

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 will be further presented and discussed at the 11th Annual Workshop of the National Reference Laboratories for Crustacean Diseases to be held 4th – 5th of November 2020 as a virtual meeting.

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