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

Inter-laboratory proficiency test 2021

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

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) 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 28 laboratories including 20 NRLs of EU Member States. Seventeen 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 test material was prepared by grinding half of a shrimp carcass infected with either TSV or YHV1 or being specific pathogen free 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, upon which the cards were dried for three days at room temperature. Subsequently the cards were cut into strips such that each circular area gave three strips. Each participating laboratory was send six tubes, each containing three strips infused with the same homogenate. Prior to distribution the EURL tested four strips of each category to ensure that the FTA cards worked satisfactorily, using the methods described below.

Diagnostic methods

Extraction of RNA from FTA cards

One third of an FTA card strip (QIAcard FTA Classic [Qiagen WB120305]) was incubated in TE buffer (200 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 3 seconds and 60°C for 30 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: 5 µl template RNA was added to a PCR tube containing: 5 µl Qiagen OneStep RT-PCR kit buffer (Qiagen), 0.75 µl of each of the two forward primers (10 µM), 0.75 µl of each of the two reverse primers (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 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. 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 of the two forward primers (10 µM), 1 µl of each of the two reverse primers (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 six tubes that each contained three FTA card strips of the same category. Two tubes contained FTA cards infused with TSV material, two tubes contained material infused with YHV1 material, and two tubes contained material infused 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.

Expected results

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

Table 1. Expected results of the proficiency test.

Sample ID	Sample type	Infection status
Sample XX-006	FTA card infused with shrimp homogenate	Negative
Sample XX-007	FTA card infused with shrimp homogenate	Positive YHV1 (UAZ 99-294)
Sample XX-008	FTA card infused with shrimp homogenate	Negative
Sample XX-009	FTA card infused with shrimp homogenate	Positive TSV (UAZ 00-273)
Sample XX-010	FTA card infused with shrimp homogenate	Positive TSV (UAZ 00-273)
Sample XX-011	FTA card infused with shrimp homogenate	Positive YHV1 (UAZ 99-294)

Actual results

Results were received from all 17 participating laboratories.
• 17 laboratories correctly diagnosed all samples, 6/6 (100 %).

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

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

2 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 results received in 2021 are better than those from previous proficiency tests. Of 102 samples tested, all were diagnosed correctly (100 %). One explanation for this may be the use of FTA cards instead of shrimp pleopods. The viral load can easily be controlled on FTA cards in contrast to pleopods originating from infected shrimp, which tends to have very high titers of YHV1 and rather low titers of TSV, resulting in cross contamination from YHV1 positive samples to YHV1 negative samples, and in falsely diagnosing TSV positive samples as TSV negative samples. It thus appears as the use of FTA cards has improved the test results, although the use of pleopods would more accurately mimic the normal diagnostic procedure done on shrimp from aquaculture.

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.

Laboratory Code	Method TSV	Method YHV	XX-001	XX-002	XX-003	XX-004	XX-005	XX-006	Score
EURL	qPCR	Nested PCR	-ve	YHV	-ve	TSV (22.9)	TSV (22.9)	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	-ve	YHV	-ve	TSV (19.9)	TSV (20.5)	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	PCR + qPCR	PCR	-ve	YHV	-ve	TSV (25.2)	TSV (25.2)	YHV	6/6
8	This NRL did not participate in the TSV/YHV1 proficiency test								
9	qPCR	PCR	-ve	YHV	-ve	TSV (24.9)	TSV (24.8)	YHV	6/6
10	This NRL did not participate in the TSV/YHV1 proficiency test								
11	PCR + qPCR	Nested PCR	-ve	YHV	-ve	TSV (23.6)	TSV (24.0)	YHV	6/6
12	This NRL did not participate in the TSV/YHV1 proficiency test								
13	qPCR	qPCR	-ve	YHV (32.4)	-ve	TSV (30.4)	TSV (29.5)	YHV (31.5)	6/6
14	qPCR	PCR	-ve	YHV	-ve	TSV (24.8)	TSV (25.5)	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	YHV	-ve	TSV	TSV	YHV	6/6
19	PCR	PCR	-ve	YHV	-ve	TSV	TSV	YHV	6/6
20	PCR	PCR	-ve	YHV	-ve	TSV	TSV	YHV	6/6
21	qPCR	PCR	-ve	YHV	-ve	TSV (27)	TSV (27)	YHV	6/6
22	This NRL did not participate in the TSV/YHV1 proficiency test								
23	qPCR	Nested PCR	-ve	YHV	-ve	TSV (22.7)	TSV (22.3)	YHV	6/6
24	PCR	PCR	-ve	YHV	-ve	TSV	TSV	YHV	6/6
25	PCR	PCR	-ve	YHV	-ve	TSV	TSV	YHV	6/6
26	qPCR	Nested PCR	-ve	YHV	-ve	TSV (21.2)	TSV (20.7)	YHV	6/6
27	PCR	Nested PCR	-ve	YHV	-ve	TSV	TSV	YHV	6/6
28	qPCR	qPCR	-ve	YHV (31.8)	-ve	TSV (24.9)	TSV (25.7)	YHV (32.5)	6/6
29	PCR	PCR	-ve	YHV	-ve	TSV	TSV	YHV	6/6

Table 3. DNA extraction and PCR methods used by the participating laboratories. Numbers refer to codes of participating laboratories.

Laboratory Code	DNA Extraction Method	PCR Kit
EURL	IndiMag Pathogen Kit with INDIMAG robot	TaqPath™ 1-Step RT-qPCR Master Mix; Qiagen OneStep 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
7	RNA isolation kit from A&A Biotechnology; Qiagen RNeasy Mini Kit	Super-Script One-Step RT-PCR with Platinium Taq
9	NucleoSpin RNA Virus	One-Step Probe PCR Mix
11	QiaAmp Viral RNA Mini Kit	One Step RT-PCR Kit; Platinum PCR SuperMix Kit; QuantiNova Pathogen + IC Kit
13	Biomerieux NucliSENS® easyMAG®	Taqman® reverse transcription reagent kit; SYBR.GR Master mix
14	RNeasy Protect Mini Kit	SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase
18	RNeasy Mini Kit	Qiagen One Step RT-PCR Kit
19	RNeasy Mini Kit	Qiagen OneStep RT-PCR Enzyme Mix
20	NucleoSpin RNA Kit	QIAGEN OneStep RT-PCR Kit; Supersript III Platinum Taq Polymerase
21	RNeasy Mini Kit using QIAcube platform	QuantiTect Probe RT-PCR OneStep kit; Qiagen OneStep RT-PCR Kit
23	QIAamp Viral RNA Mini Kit	TaqPath 1-step RT-qPCR Master Mix; Qiagen OneStep RT-PCR Kit; GoTaq G2 Flexi DNA polymerase
24	Jena Bioscience Total RNA Purification Kit	SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase
25	RNeasy Mini Kit from Qiagen	QIAGEN OneStep RT-PCR Kit
26	Indical IndiMag Pathogen kit with KingFisher Flex system	ThermoFisher AgPath-ID One-Step RT-PCR; NZYTaq II Green Mix
27	QIAamp Viral RNA Kit	Superscript lll RT/Platinum Taq mix; HotStarTaq Master Mix
28	QIAamp Viral RNA Mini Kit	AgPath-ID™ One-Step RT-PCR Reagents
29	UltraClean Tissue and Cells RNA isolation Kit	OneTaq One-Step RT-PCR Kit

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

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 13th Annual Workshop of the National Reference Laboratories for Crustacean Diseases to be held 1st of June 2022 as a combination of virtual and physical meeting.

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