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

Inter-laboratory proficiency test 2019

Detection of White Spot Syndrome Virus in Shrimp Pleopods

Organised by the
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Introduction

A comparative test of diagnostic procedures for the detection of White Spot Syndrome Virus (WSSV) 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 22 NRL's in 21 Member States.

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 have 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 WSSV were obtained from the Cefas Weymouth laboratory who originally obtained them from the OIE reference laboratory at the University of Arizona, USA. The OIE isolate of WSSV (UAZ 00-173B) was generated in *Litopenaeus vannamei* from an original outbreak in *Fenneropenaeus chinensis* in China in 1995. Subsequent passages of this isolate into naïve *L. vannamei* held at the Cefas have demonstrated continued infectivity of this isolate.

A WSSV inoculum was prepared by grinding half of a shrimp carcass infected with WSSV 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. WSSV infected shrimp carcasses were prepared by direct intramuscular injection of 100 µl WSSV inoculum into specific pathogen free (SPF) *L. vannamei*. Water temperature was held constant at 26˚C. During the following days, dead and moribund shrimp were removed from the experimental tanks.

All 10 pleopods were removed from newly dead animals and fixed in 70 % ethanol 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 WSSV 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 amount of virus that was measurable with standard PCR based methods.

Three to four NRLs received pleopods from the same shrimp.

Shrimp were confirmed as WSSV positive and WSSV negative by real-time PCR using the following procedure.

Diagnostic method

Extraction of DNA from Pleopods

DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN). Half of a pleopod was homogenized using bead beating with a 5 mm metal bead in 80 µl PBS in a TissueLyser II (QIAGEN) for 1 min. 100 µl of buffer ATL and 20 µl of proteinase K was added and the samples were incubated at 56 °C over night. The DNA was then purified using the manual enclosed in the kit.

WSSV real-time PCR

Based on Durand & Lightner (2002).
1.5 µl template DNA was added to a PCR tube containing: 5 µl TaqPath 1 Step RTqPCR Master Mix (Life Technologies), 0.8 µl forward primer (10 µM), 0.8 µl reverse primer (10 µM), 0.4 µl Taqman Probe (10 µM)

and 11.5 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 a pair of pleopods from each of three infected and three 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 and when possible participants were provided with a tracking number so they were able follow the shipment.

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 19-001	*L. vannamei* pleopods in EtOH	Negative
Sample 19-002	*L. vannamei* pleopods in EtOH	Positive (UAZ 00-173B)
Sample 19-003	*L. vannamei* pleopods in EtOH	Positive (UAZ 00-173B)
Sample 19-004	*L. vannamei* pleopods in EtOH	Negative
Sample 19-005	*L. vannamei* pleopods in EtOH	Positive (UAZ 00-173B)
Sample 19-006	*L. vannamei* pleopods in EtOH	Negative

Actual results

Results were received from all 23 participating laboratories.
- 22 laboratories correctly diagnosed all samples, 6/6 (100%).
- 1 laboratory correctly diagnosed 4/6 samples (66%).

The following methods were used by the participants:
- 13 laboratories used nested PCR methods (Lo et al. 1996)
- 8 laboratories used real time PCR (Durand & Lightner 2002)

• 2 laboratories used both methods
• 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. Samples diagnosed as negative for WSSV are marked as –ve while samples diagnosed as positive for WSSV are marked as +ve. Diagnoses that did not match the expectations are shown in red.

Country/ Laboratory	Method	19-001	19-002	19-003	19-004	19-005	19-006	Score
EURL	qPCR	-ve	+ve (12.36-19.19)	+ve (11.3-15.19)	-ve	+ve (13.36-20.35)	-ve	
1	Nested PCR	-ve	+ve	+ve	-ve	+ve	-ve	6/6
2	Nested PCR	-ve	+ve	+ve	-ve	+ve	-ve	6/6
3	Nested PCR	-ve	+ve	+ve	-ve	+ve	-ve	6/6
4								
5	Nested PCR	-ve	+ve	+ve	-ve	+ve	-ve	6/6
6	qPCR	-ve	+ve (20.52)	+ve (18.33)	-ve	+ve (21.20)	-ve	6/6
7	Nested PCR and qPCR	-ve	+ve (17.22)	+ve (15.30)	-ve	+ve (18.63)	-ve	6/6
8	Nested PCR	-ve	+ve	+ve	-ve	+ve	-ve	6/6
9	Nested PCR	-ve	+ve	+ve	-ve	+ve	-ve	6/6
10								
11	Nested PCR	-ve	+ve	+ve	-ve	+ve	-ve	6/6
12	Nested PCR	-ve	+ve	+ve	-ve	+ve	-ve	6/6
13	qPCR	-ve	+ve (19.91)	+ve (16.37)	-ve	+ve (17.00)	-ve	6/6
14	Nested PCR	-ve	+ve	+ve	-ve	+ve	-ve	6/6
15	qPCR	-ve	+ve (25.18, 25.07)	+ve (17.93, 17.97)	-ve	+ve (23.94, 23.70)	-ve	6/6
16	Nested PCR	+ve	+ve	+ve	+ve	+ve	-ve	4/6
17								
18	Nested PCR	-ve	+ve	+ve	-ve	+ve	-ve	6/6
19	Nested PCR	-ve	+ve	+ve	-ve	+ve	-ve	6/6
20	Nested PCR and qPCR	-ve	+ve (18.61)	+ve (18.14)	-ve	+ve (17.50)	-ve	6/6
21	qPCR	-ve	+ve (19)	+ve (16)	-ve	+ve (15)	-ve	6/6
22	qPCR	-ve	+ve (17.05)	+ve (14.62)	-ve	+ve (14.35)	-ve	6/6
23	qPCR	-ve	+ve (15.41)	+ve (11.29)	-ve	+ve (10.89)	-ve	6/6
24	qPCR	-ve	+ve (16.69)	+ve (12.66)	-ve	+ve (13.78)	-ve	6/6
25								
26	Nested PCR	-ve	+ve	+ve	-ve	+ve	-ve	6/6
27	qPCR	-ve	+ve (25.97)	+ve (25.93)	-ve	+ve (25.94)	-ve	6/6

Evaluation of results

The results received in 2019 are similar to those from previous proficiency tests. Of 138 samples tested, two were not diagnosed correctly. 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 WSSV are marked in grey, and samples diagnosed as positive for WSSV are marked in yellow.

		Pleopod ID				
	Shrimp ID	A	B	C	D	E
Non-inoculated Shrimp	19-5656-1	EURL	1	2	3	5
	19-5656-2	EURL		6	7	8
	19-5656-3	EURL	9	11	12	13
	19-5656-4	EURL	14	15	16	18
	19-5656-5	EURL	19	20	21	22
	19-5656-6	EURL	23	24	26	27
	19-5656-8	EURL	1	2	3	5
	19-5656-9	EURL		6	7	8
	19-5656-10	EURL	9	11	12	13
	19-5656-11	EURL	14	15	16	18
	19-5656-12	EURL	19	20	21	22
	19-5656-13	EURL	23	24	26	27
	19-5656-15	EURL	1	2	3	5
	19-5656-16	EURL		6	7	8
	19-5656-17	EURL	9	11	12	13
	19-5656-18	EURL	14	15	16	18
	19-5656-19	EURL	19	20	21	22
	19-5656-20	EURL	23	24	26	27
Shrimp inoculated with WSSV	19-5656-49	EURL	1	2	3	5
	19-5656-50	EURL		6	7	8
	19-5656-51	EURL	9	11	12	13
	19-5656-52	EURL	14	15	16	18
	19-5656-53	EURL	19	20	21	22
	19-5656-54	EURL	23	24	26	27
	19-5656-56	EURL	1	2	3	5
	19-5656-57	EURL		6	7	8
	19-5656-58	EURL	9	11	12	13
	19-5656-59	EURL	14	15	16	18
	19-5656-60	EURL	19	20	21	22
	19-5656-61	EURL	23	24	26	27
	19-5656-63	EURL	1	2	3	5
	19-5656-64	EURL		6	7	8
	19-5656-65	EURL	9	11	12	13
	19-5656-66	EURL	14	15	16	18
	19-5656-67	EURL	19	20	21	22
	19-5656-68	EURL	23	24	26	27

Table 3 highlights that in both cases negative samples were diagnosed as positives, and that the pleopods in question originated from different shrimp, for which the remaining four pairs of pleopods were tested negative for WSSV by four other laboratories (including the EURL). From this analysis we are confident that the samples sent were diagnosed correctly by the EURL, and find that the most likely reason for the unexpected diagnoses is cross-contamination in the laboratory in question. It is thus worth highlighting that disease agent diagnostics using PCR based methods is very sensitive and highly prone to cross-contamination issues, and consequently it is important that NRLs take all possible measures to avoid this problem.

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 3rd – 4th of June 2020 in Copenhagen, Denmark.

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