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 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 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. Twenty six laboratories including 18 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 WSSV 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 WSSV (UAZ 00-173B) was generated in *Penaeus 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 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 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) *P. vannamei*. During the following days, dead and moribund shrimp were removed from the experimental tanks. 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 70 % ethanol for molecular analysis, with each matching set of pleopods stored in the same tube (i.e. 5 tubes per shrimp). Pleopods from specific pathogen free 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 titre that was measurable with standard PCR based methods.

Multiple 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 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.

WSSV real-time PCR

Based on Durand & Lightner (2002).
2 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 a pair of pleopods from each of two 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.

Expected results

Participants were asked to identify the infection status of the pleopods in 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 EtOH	Negative
Sample XX-002	*P. vannamei* pleopods in EtOH	Positive (UAZ 00-173B)
Sample XX-003	*P. vannamei* pleopods in EtOH	Negative
Sample XX-004	*P. vannamei* pleopods in EtOH	Negative
Sample XX-005	*P. vannamei* pleopods in EtOH	Positive (UAZ 00-173B)

Actual results

Results were received from all 26 participating laboratories.
- 23 laboratories correctly diagnosed all samples, 5/5 (100 %).
- 2 laboratories correctly diagnosed 4/5 samples (80 %).
- 1 laboratory correctly diagnosed 3/5 samples (60 %).

The following methods were used by the participants:
• 9 laboratories used nested PCR methods (Lo et al. 1996)
• 13 laboratories used real time PCR (Durand & Lightner 2002)
• 4 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 (for the EURL this is based on an average of all samples used for the test). 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.

Laboratory Code	Method	XX-001	XX-002	XX-003	XX-004	XX-005	Score
EURL	qPCR	-ve	+ve (16.9)	-ve	-ve	+ve (16.9)	
1	Nested PCR	-ve	+ve	-ve	-ve	+ve	5/5
2	Nested PCR	-ve	+ve	-ve	-ve	+ve	5/5
3	Nested PCR	-ve	+ve	-ve	-ve	+ve	5/5
4	This NRL has outsourced its crustacean diagnostics to FLI in Germany						
5	Nested PCR	(+ve)	+ve	-ve	-ve	+ve	4/5
6	qPCR	-ve	+ve (20.4)	-ve	-ve	+ve (21.0)	5/5
7	Nested PCR + qPCR	-ve	+ve (19.5)	-ve	-ve	+ve (20.0)	5/5
8	qPCR	-ve	+ve (20.5, 21.0, 20.0)	-ve	-ve	+ve (21.3, 21.7, 22.2)	5/5
9	qPCR	-ve	+ve (19.9)	-ve	-ve	+ve (21.9)	5/5
10	This NRL did not participate in the WSSV proficiency test						
11	Nested PCR + qPCR	-ve	+ve (20.5)	-ve	-ve	+ve (20.2)	5/5
12	Nested PCR	-ve	+ve	-ve	-ve	+ve	5/5
13	qPCR	-ve	+ve (22.0)	-ve	-ve	+ve (20.5)	5/5
14	Nested PCR	-ve	+ve	-ve	-ve	+ve	5/5
15	qPCR	-ve	+ve (23.5, 23.7)	-ve	-ve	+ve (27.2, 27.1)	5/5
16	qPCR	-ve	+ve (20.1)	-ve	-ve	+ve (25.1)	5/5
17	This NRL has outsourced its crustacean diagnostics to The Netherlands						
18	Nested PCR + qPCR	-ve	+ve (16.3)	-ve	-ve	+ve (17.8)	5/5
19	Nested PCR	-ve	+ve	-ve	-ve	+ve	5/5
20	Nested PCR + qPCR	-ve	+ve (22.2)	-ve	-ve	+ve (17.0)	5/5
21	qPCR	-ve	+ve (21)	-ve	-ve	+ve (17)	5/5
22	qPCR	-ve	+ve (16.8)	+ve (30.2)	(+ve) (34.7)	+ve (11.0)	3/5
23	qPCR	-ve	+ve (18.3)	-ve	-ve	+ve (12.9)	5/5
24	qPCR	-ve	+ve (19.8)	-ve	-ve	+ve (10.9)	5/5
25	qPCR	-ve	+ve (18.0)	-ve	-ve	+ve (14.4)	5/5
26	Nested PCR	-ve	+ve	-ve	-ve	+ve	5/5
27	qPCR	-ve	+ve (16.5)	-ve	-ve	+ve (15.6)	5/5
28	qPCR	-ve	+ve (18.0)	-ve	-ve	+ve (17.0)	5/5
29	Nested PCR	-ve	+ve	-ve	+ve	+ve	4/5

Evaluation of results

The error rate of the results received in 2021 was lower than for most previous proficiency tests. Of 130 samples tested, four were not diagnosed correctly (3.1 %). As multiple laboratories received pleopods from the same shrimp, we can evaluate the likeliness of these unexpected diagnoses (see Table 3).

Table 3 highlights that in all cases of falsely diagnosed samples, negative samples were diagnosed as positives, and that the pleopods in question originated from different shrimp, for which the remaining

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. Samples not used in the test are marked in white.

		Pleopod ID				
	Shrimp ID	A	B	C	D	E
Non-inoculated Shrimp	20-854-1	EURL	1	2	3	5
	20-854-2	EURL	6	7	8	9
	20-854-3	EURL	11	12	13	14
	20-854-5	EURL	15	16	18	19
	20-854-6	EURL	20	21	22	23
	20-854-7	EURL	24	25	26	27
	20-854-21	EURL	28	1	2	29
	20-854-22	EURL	3	5	6	7
	20-854-24	EURL	8	9	11	12
	19-5656-131	EURL	13	14	15	16
	19-5656-132	EURL	18	19	20	21
	19-5656-133	EURL	22	23	24	25
	19-5656-134	EURL	26	27	28	29
	19-5656-135	EURL	1	2	3	5
	19-5656-136	EURL	6	7	8	9
	19-5656-137	EURL	11	12	13	14
	19-5656-138	EURL	15	16	18	19
	19-5656-139	EURL	20	21	22	23
	19-5656-140	EURL	24	25	26	27
	19-5656-141	EURL	28	29		
Shrimp inoculated with WSSV	19-5656-87	EURL	1	2	3	5
	19-5656-88	EURL	6	7	8	9
	19-5656-89	EURL	11	12	13	14
	19-5656-90	EURL	15	16	18	19
	19-5656-91	EURL	20	21	22	23
	19-5656-92	EURL	24	25	26	27
	19-5656-93	EURL	28	29	1	2
	19-5656-94	EURL	3	5	6	7
	19-5656-95	EURL	8	9	11	12
	19-5656-96	EURL	13	14	15	16
	19-5656-97	EURL	18	19	20	21
	19-5656-98	EURL	22	23	24	25
	19-5656-99	EURL	26	27	28	29

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 also worth noticing that in the two cases were a false positives were obtained by qPCR, the Ct values were quite high, which indicates the presence of only trace amounts of virus DNA, and further corroborating that the false results originated from sample cross-contamination. It is thus worth highlighting that disease agent diagnostics using PCR based methods is very sensitive and highly

Table 4. 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	Luna® Universal Probe qPCR Master Mix (NEB)
1	QIAamp DNA Mini Kit	HotStarTaq DNA Polymerase (Qiagen)
2	QIAamp cador Pathogen Mini Kit with Qiacube robot	Red Master Mix (Bioline)
3	IndiMag Pathogen kit with BioSprint 96 Workstation	GoTaq® Hot Start Green Master Mix (Promega)
5	QIAamp power fecal DNA kit	
6	IndiMag Pathogen kit with Maelstrom-9600 (TANBead) robot	PerfeCTa qPCR ToughMix Low Rox (Quantabio)
7	Qiagen DNA Mini Kit	Platinium Taq DNA Polymerase kit (Invitrogen) & QuantiNova Pathogen + IC Kit (Qiagen)
8	Qiagen DNA Mini Kit	QuantiTect Probe PCR Kit (Qiagen)
9	Qiagen DNEasy Blood & Tissue Kit	5xHOT FIREPol Mix (Solis BioDyne)
11	QiaAmp Viral RNA Mini Kit	Platinum PCR SuperMix Kit (Invitrogen) & QuantiNova Pathogen + IC Kit (Qiagen)
12	Qiagen EZ1 DNA Tissue Kit	GoTaq® G2 Flexi DNA Polymerase (Promega)
13	Biomerieux Easy Mag	TaqMan Fast Universal PCR mix
14	Qiagen DNEasy Blood & Tissue Kit	Taq PCR Master Mix Kit (Qiagen)
15	QIAamp DNA Mini Kit with Qiacube robot	Real Time Taqman® Universal master mix (Life Technologies Ltd)
16	innuPREP AniPath DNA/RNA Kit – IPC16	GoTaq® Probe qPCR Master Mix (Promega)
18	Cador Pathogen for INDIMAG with INDIMAG robot	QuantiTect Probe PCR kit (Qiagen)
19	QIAamp DNA Mini Kit	Platinum Taq DNA Polymerase (Invitrogen)
20	QIAamp DNA Mini-Kit	QIAGEN OneStep RT-PCR Kit
21	Qiagen DNEasy Blood & Tissue Kit with Qiacube	Perfecta qPCR ToughMix UNG ROX (VWR)
22	Qiagen DNEasy Blood & Tissue Kit	Qiagen Multiplex PCR Kit
23	QIAamp DNA Mini Kit	Luna® Universal Probe qPCR Master Mix (NEB)
24	The Real PCR DNA/RNA spin Column Kit" from Idexx	AmpliTaq Gold DNA Polymerase kit (Invitrogen)
25	Qiagen DNEasy Blood & Tissue Kit	GoTaq® Probe qPCR Master Mix (Promega)
26	Indical IndiMag Pathogen kit with Thermo Scientific KingFisher Flex	NZYTaq II Green Mix (NZYTech)
27	Roche High Pure Viral Nucleic Acid Kit	QuantiTect Probe PCR Kit (Qiagen)
28	QIAamp Viral RNA Mini Kit	TaqMan Universal PCR Master Mix (Applied Biosystems).
29	Zymo Research Quick-DNA MiniPrep	"Thermo Scientific Reagents"

prone to cross-contamination issues, and consequently it is important that NRLs take all possible measures to avoid this problem. Compared to 2020, four NRLs changed their methods from nested PCR to qPCR or to a combination of both methods. As nested PCR impose an increased risk for cross contamination compared to qPCR, this change in methods may have contributed to the higher success rate of this year's proficiency test.

Table 4 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 an online meeting to be held after release of the report, and with participation of representatives from the EURL and NRLs.

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References

Durand, S. V. & Lightner, D. V., 2002. Quantitative real time PCR for the measurement of white spot syndrome virus in shrimp. Journal of Fish Diseases, 25, 381-389.

Lo, C. F., et al., 1996. Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimp using polymerase chain reaction. Diseases of Aquatic Organisms, 25, 133-141.