



**European Union Reference
Laboratory for Crustacean Diseases**

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WSSV PROFICIENCY TEST 2015

Detection of White Spot Syndrome Virus (WSSV) in Shrimp Pleopods and Lenticules

EURL Ring Trial Reference Number: EURL15005

Distribution Date:	29/08/15
Report Date:	15/01/16
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Introduction

This scheme is intended to provide proficiency testing samples for National Reference Laboratories (NRLs) undertaking examination of crustacean tissues for the presence/absence of White Spot Syndrome Virus (WSSV) in accordance with EC Directive 2006/88. The invitation to participate in this year's proficiency test was sent to 23 NRL's in 21 Member States. Samples were sent to 19 NRL's in 17 Member States, 3 NRL's declined to take part in this trial. Figure 1 shows how many labs have participated in this annual testing since it began in 2011.

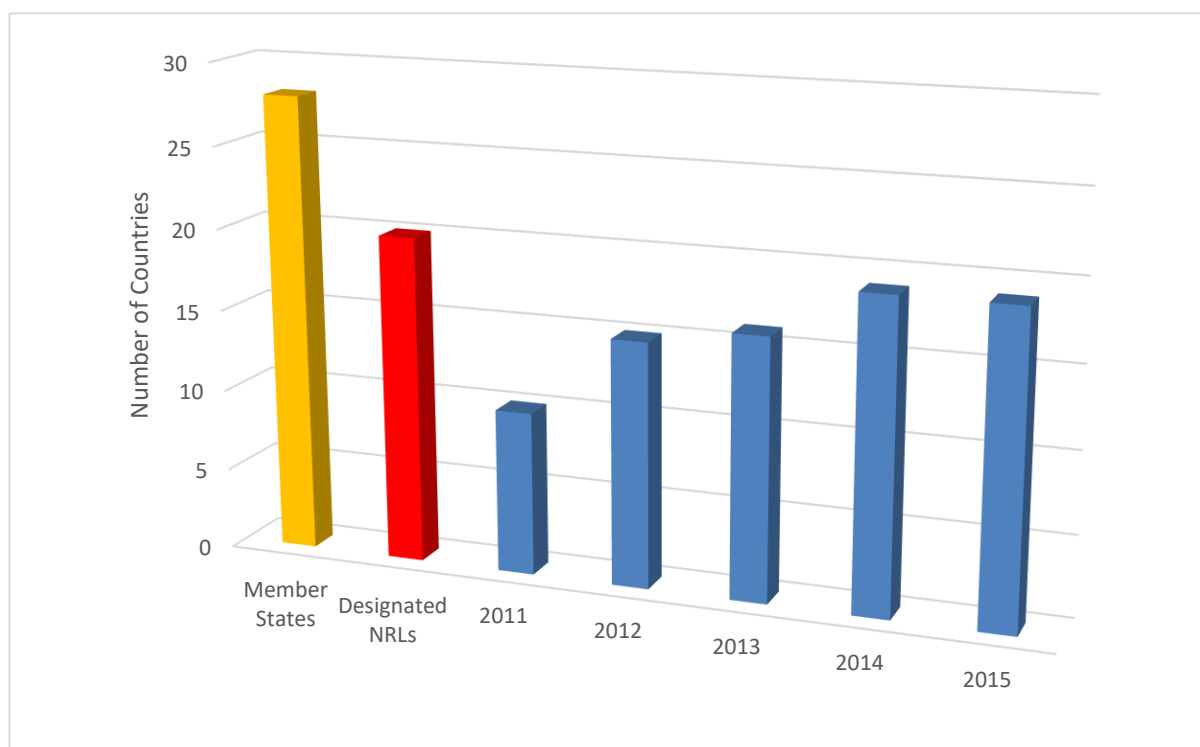


Figure 1. Total number of Member States within the EU, Number of labs with designated NRL for Crustacean Disease and Participation of these labs in the WSSV Proficiency test over recent years.

This Proficiency test was organised by the European Union Reference laboratory (EURL) for Crustacean Diseases.

Further information can be obtained via the EURL website (www.crustaceanrcl.eu)

Sample Preparation

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

There are currently no crustacean cell lines available; WSSV infected shrimp carcasses were prepared by direct intramuscular injection of WSSV inoculum into

specific pathogen free (SPF) *L. vannamei* at a rate of 10 µl g¹ shrimp weight. Water temperature was held constant at 24°C. Shrimp were monitored throughout the day for five days, dead and moribund shrimp were removed from the experimental tanks.

Pleopods

Pleopods were fixed in ethanol for molecular analysis, two pleopods per tube, 5 tubes per shrimp. SPF shrimp provided tissues for WSSV negative samples. Prior to distribution the EURL tested one set of pleopods from each individual shrimp to ensure a satisfactory titre in the tissue and homogeneity of content of sample.

Multiple NRLs received pleopods from the same shrimp.

Lenticules

Shrimp were confirmed as WSSV positive and WSSV negative (SPF) by nested PCR techniques. These shrimp were homogenised and the homogenate used to inoculate a lenticulating fluid at various dilutions. This fluid was then aliquoted into 25µl drops which formed the lenticule discs. Prior to distribution the EURL tested 10% of lenticule discs produced to ensure a satisfactory titre in the tissue and homogeneity of content of sample.

All pleopod and lenticule samples were tested following PCR protocols accredited under ISO 17025 standards.

Methods

The dessication filter was removed from the tubes containing the Lenticules and 1ml of molecular grade water was added and left for 5 minutes at room temperature. The sample was vortexed until the lenticule was fully dissolved and 50µl of this was added to 140µl G2 buffer and 10µl proteinase K. The samples were incubated for 4 hrs at 56°C. Total DNA was extracted from the samples using an EZ1 DNA tissue kit and EZ1 Advanced XL BioRobot® (Qiagen) following manufactures' instructions.

Separate first and second round (Nested) PCR reactions were performed on each DNA extract using the OIE recommended WSSV primer sets (Lo *et al.* 1996) Table 1. Reactions were performed in 50µl reaction mix consisting of 1 X Green Go Taq buffer, 2.5mM MgCl₂, 0.25mM dNTPs, 100 pmol each of the forward and reverse primer, 0.25 units Go Taq Flexi (Promega), and 2.5µl extracted nucleic acid. Amplifications were performed using the following WSSV thermal cycler program on a Peltier PTC-225 thermal cycler: 94°C x 2 minutes followed by 30 cycles of 94°C x 30 seconds, 62°C x 30 seconds and 72°C x 30 seconds, followed by 72°C x 2 minutes and held at 4°C. A 1447bp product should be seen for positive samples in the first round PCR and a 941bp product in the second round.

Amplification products were resolved on 2% agarose gels stained with ethidium bromide and visualised using a UV illuminator.

Table 1. WSSV PCR primers (Lo *et al.*, 1996)

Primer name	Sequence
WSSV 146 F1	ACTACTAACTTCAGCCTATCTAG
WSSV 146 R1	TAATGCGGGTGTAAATGTTCTTACGA
WSSV 146 F2	GTAAGTGGCCCTTCCATCTCCA
WSSV 146 R2	TACGGCAGCTGCTGCACCTTGT

A nested PCR reaction was not completed for the neat samples as these produced a bright product of the correct size in the 1st round PCR.

The five batches of 20 samples were also tested using the OIE recommended QPCR protocol for the detection of WSSV (Durand *et al.* 2002). Each QPCR reaction contained 100nM of WSSV probe, 300nM of WSSV QPCR forward primer, 300nM of WSSV QPCR reverse primer (Table 2), 10µl of Taqman master mix (Applied Biosystems) and 2.5µl of each sample and the total volume of 20µl was made up with molecular grade water.

To make the standard curve 4µl of WSSV plasmid (1.6×10^9 copies/µl) was added to 156µl of molecular grade water and vortexed to give 4×10^7 copies/µl. Five microlitres of this was added to a microfuge tube containing 45µl molecular grade water vortexed to mix and subsequent serial tenfold dilutions were made to give concentrations from 4×10^6 to 4×10^1 copies/µl. Each of the tenfold serial dilutions (4×10^7 to 4×10^1 copies/µl) were vortexed and 2.5µl of each dilution was added in triplicate to the QPCR plate containing 17.5µl of the master mix to give a total volume of 20µl. This gave final concentrations in the standard curve of: 1×10^8 to 1×10^1 copies.

Table 2. WSSV qPCR primers and probes (Durand & Lightner, 2002)

Primer and probe name	Sequence
WSSV probe	6FAM AGCCATGAAGAATGCCGTCTATCACACA Tamra
WSSV forward primer 1011F	TGGTCCCGTCCTCATCTCAG
WSSV reverse primer 1079R	GCTGCCTTGCCGGAATTA

Three qPCR plates were run using the Applied Biosystems Stepone Plus Real Time qPCR machine using the following program parameters:

50°C for 2 minutes
 95°C for 10 minutes
 Followed by 40 cycles of
 95°C for 15 seconds
 60°C for 1 minute

Quality Control

The replicate lenticules of each batch (negative control and dilutions of WSSV) produced consistent results in both the PCR, where products amplified were of similar brightness, and the qPCR, where CT values between replicates of the same dilution were no more than a log different from each other.

Distribution

Samples were sent to 20 NRL's in 17 Member States. The test was sent out according to current international regulations for shipment of diagnostic specimens UN 3373, "Biological substance, Category B". All proficiency tests were handled by courier and were delivered to all participants within three days.

Multiple NRLs received pleopods from the same shrimp.

All NRLs received lenticule discs from the same batch.

Expected Results

Participants were asked to identify the content of each tube by the method used in their laboratory.

Table 3. Expected results of the Proficiency Test

Sample ID	Sample Type	Nested Results ¹	qPCR Results ²
RA15005-1	Shrimp pleopods	Positive	ct 12-19
RA15005-2	Shrimp pleopods	Negative	Negative
RA15005-3	Lenticule disc	Positive	ct 22
RA15005-4	Lenticule disc	Negative	Negative
RA15005-5	Lenticule disc	Positive	ct 28

¹ OIE recommended technique (Lo *et al.*, 1996)

² Durand and Lightner, 2002

Actual Results

Results were received from all 20 laboratories

- 13 laboratories correctly identified all samples, 5/5 (100%).
- 6 laboratories correctly identified 4/5 samples (80%); however after supplying fresh tissue samples to 5 of these labs and requesting these samples to be analysed, all 5 labs correctly identified 100%. One lab declined to receive additional samples.
- 1 laboratory correctly identified 3/5 samples (60%); however after supplying fresh tissue samples and requesting these samples to be analysed this lab correctly identified 100%.

The following methods were used by the participants:

- 13 laboratories used Nested methods
- 5 laboratories used real time PCR
- 2 laboratories used multiple methods

Table 4. Proficiency test results submitted by the individual laboratories

Laboratory Code	RA15005-1	RA15005-2	RA15005-3	RA15005-4	RA15005-5
EURL	+ve	-ve	+ve	-ve	+ve
1	+ve	-ve	+ve	+ve	+ve
2					
3	+ve	-ve	+ve	-ve	+ve
4	+ve	-ve	+ve	-ve	+ve
5	+ve	-ve	+ve	-ve	+ve
6	+ve ct 18.75	-ve	+ve ct 27.45	-ve	+ve ct 35.35
7	+ve	-ve	+ve	-ve	+ve
8 Re-test	+ve	-ve	+ve	+ve	+ve
	+ve	-ve	+ve	-ve	+ve
9 Re-test	+ve	+ve	+ve	-ve	+ve
	+ve	-ve	+ve	-ve	+ve
10					
11	+ve	-ve	+ve	-ve	+ve
12 Re-test	+ve	+ve	+ve	-ve	+ve
	+ve	-ve	+ve	-ve	+ve
13	+ve ct 15.42 ct 15.12	-ve	+ve ct 21.09 ct 21.03	-ve	+ve ct 27.08 ct 26.52
14	+ve	-ve	+ve	-ve	+ve
15 Re-test	+ve ct 17.09 ct 17.13	-ve	+ve ct 22.71 ct 22.75	+ve ct 32.78 ct 32.93	+ve ct 31.93 ct 31.89
	+ve ct 16.72, ct 16.61	-ve	+ve ct 26.43, ct 26.43	-ve	+ve ct 32.05, ct 32.11
16					
17	+ve ct 15.42 ct 15.12	-ve	+ve ct 21.09 ct 21.03	-ve	+ve ct 27.08 ct 26.52
18 1 st Re-test 2 nd Re-test	+ve	+ve	+ve	-ve	+ve
	+ve	+ve	+ve	+ve	+ve
19	+ve	-ve	+ve	-ve	+ve
20	+ve	-ve	+ve	-ve	+ve
21	+ve ct 15.72, ct 15.73, ct 15.80	-ve	+ve ct 23.90, ct 23.70, ct 23.60	-ve	+ve ct 29.71, ct 29.75, ct 29.79
22	+ve	-ve	+ve	-ve	+ve
23	+ve ct 17.11	-ve	+ve ct 22.28	-ve	+ve ct 27.88

24	+ve ct 9.18	+ve ct 24.59	+ve ct 19.96	+ve ct 28.60	+ve ct 26.28
Re-test	+ve 11.95	-ve	+ve 18.14	-ve	+ve 23.58
25					

Red text highlights where diagnosis was incorrect
Grey boxes indicate that the lab did not take part in the testing

General Comments

EURL offered to send fresh ring trial material to the NRL's which did not correctly diagnose all materials; all but one of the NRL's accepted this offer and after analysing the fresh tissues all labs correctly diagnosed the samples. It should be noted that although multiple labs experienced a few problems with the initial analysis there were no consistencies between the incorrect results, each lab experiencing a slightly different problem. From this we are confident that the samples initially sent and received by each lab were the same as those diagnosed and supplied by the EURL. The problems likely occurred during the processing of samples at each NRL.

The results presented in this report were discussed at the 7th Annual Meeting of National Reference Laboratories for Crustacean Diseases, 4th – 5th November 2015 in Weymouth, UK.

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15th January 2016

References

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Lo, C.F., Leu, J.H., Chen, C.H., Peng, S.E., Chen, Y.T., Chou, C.M., Yeh, P.Y., Huang, C.J., Chou, H.Y., Wang, C.H. & Kou, G.H. (1996). Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. *Dis. Aquat. Org.*, **25**, 133–141.