European Union Reference
Laboratory for Crustacean Diseases
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WSSV PROFICIENCY TEST 2016

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

EURL Ring Trial Reference Number: EURL16005

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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 25 laboratories in 23 Member States. Samples were sent to 22 laboratories in 20 Member States, 3 laboratories declined to take part in this trial. Figure 1 shows how many labs have participated in this annual testing since it began in 2011.

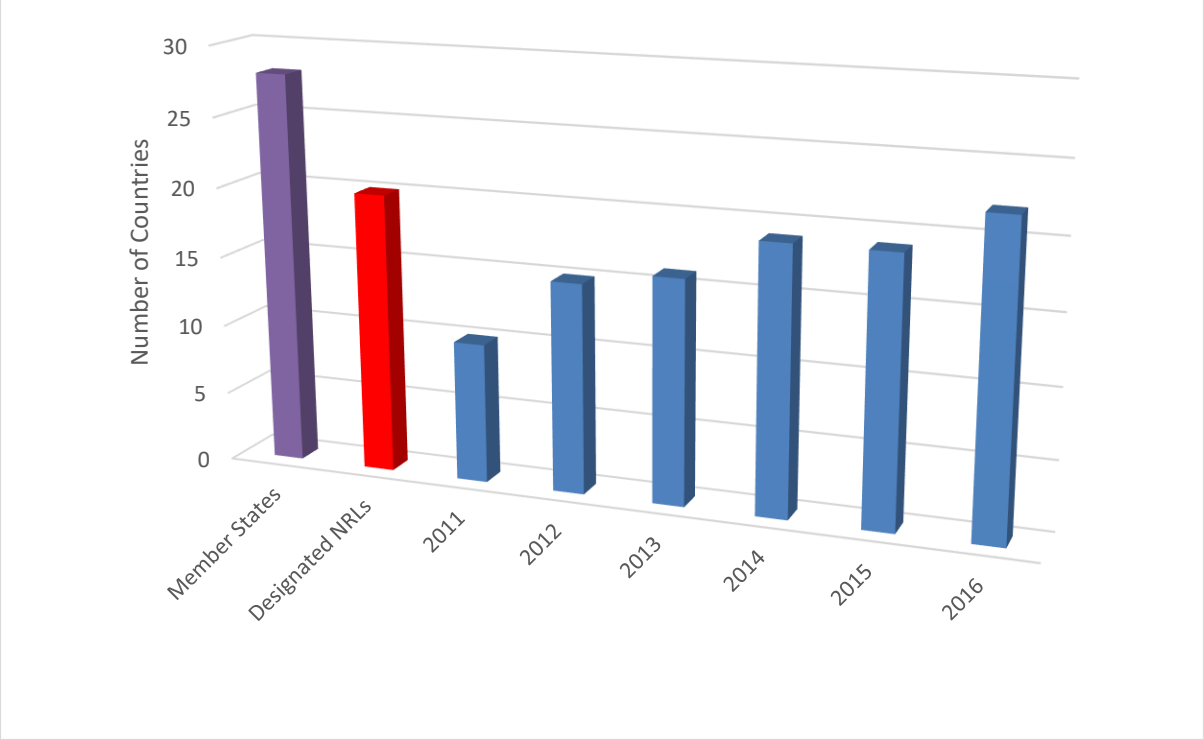

Figure 1. Total number of Member States within the EU, Number of labs with designated NRL for Crustacean Disease and Participation of these labs in the WSSV Proficiency test over recent years.

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.crustaceancrl.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 g^{1} 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. All pleopod samples were tested following PCR protocols accredited under ISO 17025 standards.

Multiple NRLs received pleopods from the same shrimp.

Methods

Pleopod tissue was weighed and diluted 1:10 in G2 buffer (Qiagen, West Sussex, UK) and 10 µl Proteinase K. Samples were homogenised using a Fast prep FP120 machine (MP Biomedical, UK) at the highest setting for 2 min. The samples were incubated for 4 hrs at 56^{o}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 $MgCl_2$, 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^{o}C x 2 minutes followed by 30 cycles of 94^{o}C x 30 seconds, 62^{o}C x 30 seconds and 72^{o}C x 30 seconds, followed by 72^{o}C x 2 minutes and held at 4^{o}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	TAATGCGGGTGTAATGTTCTTACGA
WSSV 146 F2	GTAACTGCCCCTTCCATCTCCA
WSSV 146 R2	TACGGCAGCTGCTGCACCTTGT

Quality Control

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

Multiple NRLs received pleopods from the same shrimp.

Distribution

Samples were sent to 22 laboratories in 20 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.

Expected Results

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

Table 2. Expected results of the Proficiency Test

Sample ID	Sample Type	Nested Results[1]
RA16005-1	Shrimp pleopods	Positive
RA16005-2	Shrimp pleopods	Negative
RA16005-3	Lenticule disc	Positive
RA16005-4	Lenticule disc	Positive
RA16005-5	Lenticule disc	Negative

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

Actual Results

Results were received from all 22 laboratories (Table 3).

- 16 laboratories correctly identified all samples, 5/5 (100%).
- 5 laboratories correctly identified 4/5 samples (80%); two laboratories repeated the testing on the initial tissue samples and either received the same results or experienced further issues (3/5 when samples repeated). All labs were supplied with fresh tissues and all labs then correctly identified 100%.

- 1 laboratory correctly identified 3/5 samples (60%); however, after checking their results they found that they had placed the tubes in the incorrect order, once this was rectified this lab had correctly identified 5/5 (100%).

The following methods were used by the participants:

- 13 laboratories used Nested methods
- 8 laboratories used real time PCR
- 1 laboratory used multiple methods

Table 3. Proficiency test results submitted by the individual laboratories

Laboratory Code	RA16005-1	RA16005-2	RA16005-3	RA16005-4	RA16005-5
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	+ve	+ve	+ve	-ve
Re-Test	+ve	-ve	+ve	+ve	-ve
7	+ve	-ve	+ve	+ve	-ve
8	+ve	-ve	-ve	+ve	-ve
Re-Test	+ve	-ve	+ve	+ve	-ve
9	+ve	+ve	+ve	+ve	-ve
Re-Test	+ve	-ve	+ve	+ve	-ve
10					
11	+ve	-ve	+ve	+ve	-ve
12	+ve	-ve	+ve	+ve	-ve
13	+ve	-ve	+ve	+ve	+ve
Re-Test 1	+ve	+ve	+ve	+ve	+ve
Re-Test 2 (fresh samples)	+ve	-ve	+ve	+ve	-ve
14	+ve	-ve	+ve	+ve	-ve
15	+ve	-ve	+ve	+ve	-ve
16	+ve	+ve	+ve	+ve	-ve
Re-Test 1	+ve	+ve	+ve	+ve	-ve

Re-Test 2	+ve	-ve	+ve	+ve	-ve
17	+ve	-ve	+ve	+ve	-ve
18	+ve	-ve	+ve	+ve	-ve
19	+ve	+ve	+ve	+ve	-ve
Re-Test 1	+ve	-ve	+ve	+ve	-ve
20	+ve	-ve	+ve	+ve	-ve
21	+ve	-ve	+ve	+ve	-ve
22	+ve	-ve	+ve	+ve	-ve
23	+ve	-ve	+ve	+ve	-ve
24	+ve	-ve	+ve	+ve	-ve

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

Investigation

As mentioned previously multiple laboratories had received pleopods from the same shrimp, each sample set had been recorded so that results from the different labs could be compared (see Table 4).

Table 4. Sample reference numbers were recorded for each tube in the sample sets which were sent to various labs. Grey boxes highlight where pleopods were from the same individual shrimp. Laboratory code relates to the lab which received the sample set and the colours indicate the diagnosis, green highlights where samples were correctly diagnosed, red highlights where the lab incorrectly diagnosed the sample.

Sample set	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Laboratory Code	Result
1	EURL16004 - 1b	EURL16003 - 1b	EURL16004 - 6b	EURL16004 - 11b	EURL16003 - 6b	13	4/5
2	EURL16004 - 1c	EURL16003 - 1d	EURL16004 - 6c	EURL16004 - 11c	EURL16003 - 6d	19	4/5
3	EURL16004 - 1d	EURL16003 - 1c	EURL16004 - 6c	EURL16004 - 11d	EURL16003 - 6e	24	100%
13	EURL16004 - 4b	EURL16003 - 4b	EURL16004 - 9b	EURL16004 - 14b	EURL16003 - 9c	9	4/5
14	EURL16004 - 4c	EURL16003 - 4d	EURL16004 - 9c	EURL16004 - 14c	EURL16003 - 9e	12	100%
15	EURL16004 - 4d	EURL16003 - 4c	EURL16004 - 9d	EURL16004 - 14d	EURL16003 - 9b	16	4/5
16	EURL16004 - 4e	EURL16003 - 4e	EURL16004 - 9e	EURL16004 - 14e	EURL16003 - 10b	8	4/5

17	EURL16004 - 5b	EURL16003 - 5e	EURL16004 - 10b	EURL16004 - 15b	EURL16003 - 10d	11	100%
18	EURL16004 - 5c	EURL16003 - 5d	EURL16004 - 10c	EURL16004 - 15c	EURL16003 - 10e	3	100%
5	EURL16004 - 2b	EURL16003 - 2b	EURL16004 - 7b	EURL16004 - 12b	EURL16003 - 7d	6	3/5
6	EURL16004 - 2c	EURL16003 - 2d	EURL16004 - 7c	EURL16004 - 12c	EURL16003 - 7c	21	100%
7	EURL16004 - 2d	EURL16003 - 2e	EURL16004 - 7d	EURL16004 - 12d	EURL16003 - 7b	1	100%

Table 3 highlights where multiple labs received samples from the same shrimp and the variation in results obtained from the different laboratories. 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.

General Comments

The results presented in this report were discussed at the 8th Annual Meeting of National Reference Laboratories for Crustacean Diseases, 19th – 20th October 2016 in Madrid, Spain.

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24th January 2017

References

Durand, S. V. & Lightner, D. V. (2002). Quantitative real time PCR for the measurement of white spot syndrome virus in shrimp. *J. Fish Dis.*, **25**, 381–389.

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.