European Union Reference
Laboratory for Crustacean Diseases
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PROFICIENCY TEST 2017

Detection of White Spot Syndrome Virus in Shrimp Pleopods and Lenticules

EURL Ring Trial Reference Number: EURL17001

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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 NRL's in 23 Member States. Samples were sent to 22 NRL's in 20 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.

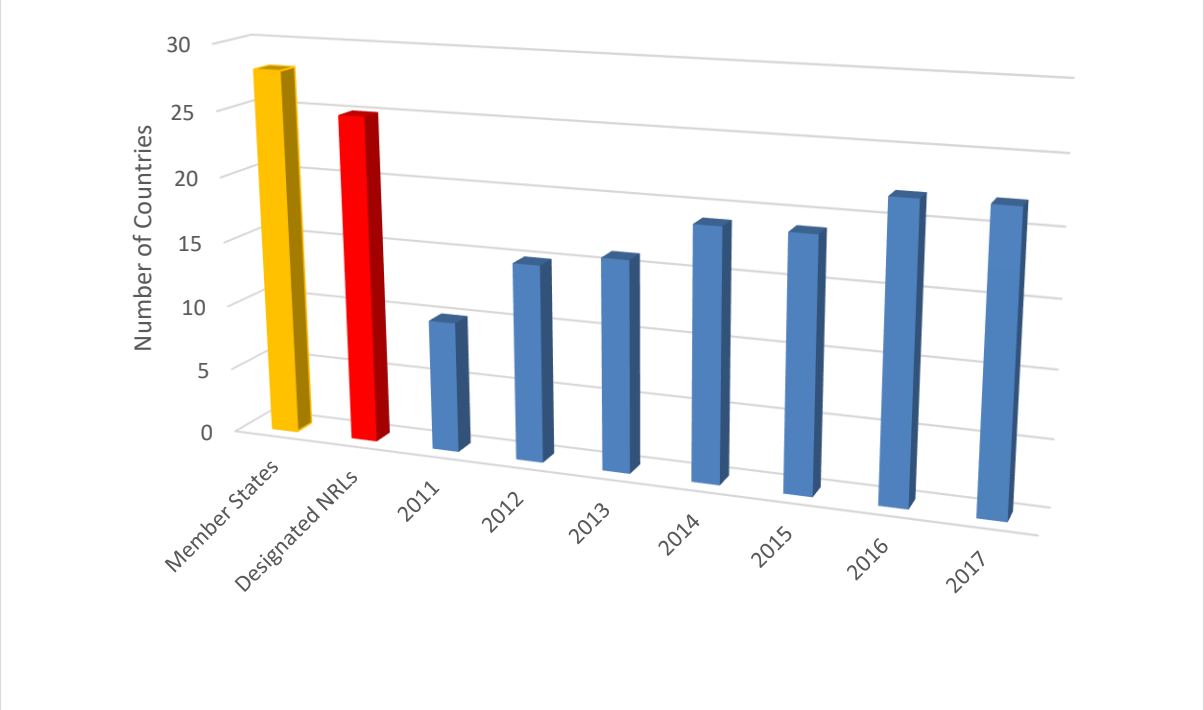

Figure 1. Total number of Member States within the EU, Number of labs with designated NRL for Crustacean Disease and Participation of these labs in the WSSV Proficiency test over recent years.

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.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.

Multiple NRLs received pleopods from the same shrimp.

Lenticules

Shrimps were confirmed as WSSV positive and WSSV negative (SPF) by nested PCR techniques. The shrimp were homogenised and the homogenate used to inoculate a lenticulating fluid at various dilutions. This fluid was then aliquoted into 25 µl 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.

Methods

The desiccation 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 140 µl G2 buffer and 10 µl proteinase K. 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.*, 1996a and b) Table 1. Reactions were performed in 25 µl reaction mix consisting of 1 X Green Go Taq buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.4 mM each of the forward and reverse primer, 0.625 units Go Taq Flexi (Promega), 1 µl extracted nucleic acid and nuclease-free water up to 25 µl. 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 1 cycle of 94 ^{o}C x 2 minutes, 55 ^{o}C x 1 minute and 72^{o}C x 2 minutes, followed by 39 cycles of 94 ^{o}C x 1 minutes, 55 ^{o}C x 1 minute and 72 ^{o}C x 2 minutes, followed by 72^{o}C x 7 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 1 % 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

The three 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 100 nM of WSSV probe, 300 nM of WSSV qPCR forward primer, 300 nM of WSSV qPCR reverse primer (Table 2), 10 µl of Taqman master mix (Applied Biosystems) and 2.5 µl of each sample and the total volume of 20 µl was made up with molecular grade water.

To make the standard curve, 5 microliters of 4 x 10^7 copies/µl were 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 x 10^6 to 4 x 10^{-1} copies/µl. Each of the tenfold serial dilutions (4 x 10^7 to 4 x 10^{-1} copies/µl) were vortexed and 2.5 µl of each dilution was added in triplicate to the QPCR plate containing 17.5 µl of the master mix to give a total volume of 20 µl. This gave final concentrations in the standard curve of: 1 x 10^8 to 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	GCTGCCTTGCCGGAAATTA

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

50^{o}C for 2 minutes
95^{o}C for 10 minutes
Followed by 40 cycles of
95^{o}C for 15 seconds
60^{o}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 22 NRL's 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.

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]
RA17001 - 1	Lenticule disc	Positive	Positive CT 24
RA17001 - 2	Lenticule disc	Negative	Negative
RA17001 - 3	Lenticule disc	Negative	Negative
RA17001 - 4	Shrimp Pleopods	Positive	Positive
RA17001 - 5	Shrimp Pleopods	Positive	Positive
RA17001 - 6	Shrimp Pleopods	Negative	Negative

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

[2] Durand and Lightner, 2002

Actual Results

Results were received from all 22 laboratories

- 18 laboratories correctly diagnosed all samples, 6/6 (100%).
- 1 laboratory correctly diagnosed 5/6 samples (83%); fresh tissue samples were supplied to the lab, after analysing the fresh tissues the laboratory correctly diagnosed all samples (100%).
- 3 laboratories correctly diagnosed 4/6 samples (66%); fresh tissue samples were supplied to two of these laboratories, after analysing the fresh tissues both laboratories correctly diagnosed all samples (100%). One laboratory declined the offer of sending fresh materials.

The following methods were used by the participants:

- 12 laboratories used Nested methods
- 7 laboratories used real time PCR
- 3 laboratories used multiple methods

Table 4. Proficiency test results submitted by the individual laboratories

Laboratory Code	RA17001-1	RA17001-2	RA17001-3	RA17001-4	RA17001-5	RA17001-6
EURL	+ve	-ve	-ve	+ve	+ve	-ve
1	+ve	-ve	-ve	+ve	+ve	-ve
2	-ve	+ve	-ve	+ve	+ve	-ve
3	+ve	-ve	-ve	-ve	+ve	+ve
Retest	+ve	-ve	-ve	+ve	+ve	-ve
4	+ve	-ve	-ve	+ve	+ve	-ve
5	+ve	-ve	-ve	+ve	+ve	-ve
6	+ve	-ve	-ve	+ve	+ve	-ve
7	+ve	-ve	-ve	+ve	+ve	-ve
8	+ve	-ve	-ve	+ve	+ve	-ve
9						
10						
11	+ve	-ve	-ve	+ve	+ve	-ve
12	+ve	-ve	-ve	+ve	+ve	-ve
13	+ve	-ve	-ve	+ve	+ve	+ve
Retest	+ve	-ve	-ve	+ve	+ve	-ve
14	+ve	-ve	-ve	+ve	+ve	-ve
15	+ve	-ve	-ve	+ve	+ve	-ve
16	+ve	-ve	-ve	+ve	+ve	-ve
17	+ve	-ve	-ve	+ve	+ve	-ve
18	+ve	+ve	-ve	+ve	+ve	+ve
Retest	+ve	-ve	-ve	+ve	+ve	-ve
19	+ve	-ve	-ve	+ve	+ve	-ve
20	+ve	-ve	-ve	+ve	+ve	-ve
21	+ve	-ve	-ve	+ve	+ve	-ve
22	+ve	-ve	-ve	+ve	+ve	-ve
23	+ve	-ve	-ve	+ve	+ve	-ve
24	+ve	-ve	-ve	+ve	+ve	-ve
25						

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

Investigation

As mentioned previously all laboratories received the same batch of lenticules and multiple laboratories had received pleopods from the same shrimp, each sample set of pleopods had been recorded so that results from the different labs could be compared (see Table 5).

Table 5. 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 4	Tube 5	Tube 6	Laboratory Code	Result
1	EURL16004 – 21c	EURL16004 – 29b	EURL16003 - 16b	20	100%
2	EURL16004 – 21d	EURL16004 – 29c	EURL16003 – 16c	24	100%
3	EURL16004 -22b	EURL16004 – 29d	EURL16003 – 16d	1	100%
4	EURL16004 – 22c	EURL16004 – 29e	EURL16003 – 16e	22	100%
5	EURL16004 – 22d	EURL16004 – 30b	EURL16003 – 17b	21	100%
6	EURL16004 – 22e	EURL16004 – 30c	EURL16003 – 17c	6	100%
7	EURL16004 – 23b	EURL16004 – 30d	EURL16003 – 17d	3	66%
8	EURL16004 – 23c	EURL16004 – 30e	EURL16003 – 17e	14	100%
9	EURL16004 – 23d	EURL16004 – 31b	EURL16003 – 18b	11	100%
10	EURL16004 – 23e	EURL16004 – 31c	EURL16003 – 18c	7	100%
11	EURL16004 – 24b	EURL16004 – 31d	EURL16003 – 18d	13	83%
12	EURL16004 – 24c	EURL16004 – 31e	EURL16003 – 18e	19	100%
13	EURL16004 – 24d	EURL16004 – 32b	EURL16003 – 19b	18	66%
14	EURL16004 – 24e	EURL16004 – 32c	EURL16003 – 19c	8	100%
15	EURL16004 – 25b	EURL16004 – 32d	EURL16003 – 19d	23	100%
16	EURL16004 – 25c	EURL16004 – 32e	EURL16003 – 19e	5	100%

Table 5 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. One laboratory incorrectly diagnosed the lenticule samples. From this analysis 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 results received in 2017 are very similar to those submitted in 2016 and have highlighted an issue with the processing of whole shrimp tissues as opposed to the lenticule system. It appears that the NRL testing facilities experience issues when processing shrimp pleopods, the problems likely occurred during the processing of samples at each NRL. The shrimp pleopods originate from a passage of WSSV

infection in a biosecure experimental facility, as such it is likely the shrimp tissues will have a high viral loading. Following the results of this ring trial the EURL have highlighted the potential for cross contamination between samples, and suggested that laboratories review procedures to limit this risk.

General Comments

The results presented in this report were discussed at the 9th Annual Meeting of National Reference Laboratories for Crustacean Diseases, 26th – 27th October 2017 in Venice, Italy.

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