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PROFICIENCY TEST 2018

Detection of Taura Syndrome Virus (TSV) and Yellow Head Virus (YHV) in Shrimp Pleopods and Lenticules

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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 Taura Syndrome Virus (TSV) and Yellow Head Virus (YHV) in accordance with EC Directive 2006/88. The invitation to participate in this year's proficiency test was sent to 26 NRL's in 24 Member States. Samples were sent to 14 NRL's in 13 Member States, 12 NRL's declined to take part in this trial.

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

Sample Preparation

Viral inoculates of TSV and YHV were originally obtained from the OIE reference laboratory at the University of Arizona, USA. The OIE isolate of TSV (UAZ 00-273) was generated in *P. vannamei* from an original outbreak in *P. vannamei* in Hawaii in 1994. The OIE isolate of YHV (UAZ 99-294) was generated in *P. vannamei* from an original outbreak in *P. vannamei* in Thailand in 1992. Subsequent passages of these isolates into naïve *P. vannamei* held at the Cefas Weymouth laboratory have demonstrated continued infectivity of these isolates.

There are currently no crustacean cell lines available; TSV and YHV infected shrimp carcasses were prepared by direct intramuscular injection of TSV or YHV inoculum into specific pathogen free (SPF) *P. 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 RNA Later for molecular analysis, two pleopods per tube, 5 tubes per shrimp. SPF shrimp provided tissues for TSV and YHV 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 either TSV positive or YHV positive and TSV and YHV negative by nested PCR techniques. The shrimp were homogenised and the homogenate used to inoculate the lenticulating fluid (400µl of homogenate added to 1600µl of lenticulating fluid). 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 positive tissues, confirmation of negative testing for negative tissues and homogeneity of content of sample.

Methods

Extraction of RNA from Pleopods

Ten to twenty milligrams of tissue was placed in a FastPrep lysing matrix A tube (MP Biomedicals) and diluted 1/40 with RNeasy Lysis Buffer (RLT) buffer (Qiagen). Samples were homogenised at 5 m/s for 1 minute and the homogenised tissue was centrifuged at 10,000xg for 2 minutes. 300 µl supernatant was transferred to a sample tube and RNA was extracted by the EZ1 Advanced XL BioRobot® (Qiagen) using the tissue protocol and eluted into 50 µl. Extracts were measured on a Nanodrop 1000 to check RNA quality.

Extraction of RNA from TSV and YHV Virus Inoculums

50 µl of virus inoculum was added to 250 µl RLT buffer in an extraction tube and RNA was extracted by the EZ1 Advanced XL BioRobot® (Qiagen) using the tissue protocol and eluted into 50 µl.

Extraction of RNA from TSV and YHV Lenticules

Each lenticule was dissolved in 250 µl molecular grade water. 50 µl was added to 250 µl RLT buffer in an extraction tube and RNA was extracted by the EZ1 Advanced XL BioRobot® (Qiagen) using the tissue protocol and eluted into 50 µl.

Reverse Transcription

Prior to PCR, RNA extractions were reverse transcribed. For reverse transcription, 4 µl template RNA was added to: 1 µl RNasin (Promega), 1 µl dTNPs (25 µM), 1 µl random primers (Promega), 1 µl M-MLV reverse transcriptase, 4 µl 5X reaction buffer (Promega) and 8 µl molecular grade water. Samples were incubated at 37ºC for 1 hours.

TSV PCR (Nunan *et al.,* 2008)

TSV is a single round RT-PCR.

PCR reaction:

1.25 µl template cDNA was added to a PCR tube containing: 5 µl 5X Flexi buffer (Promega), 2.5 µl $MgCl_2$, 0.25 µl forward primer (10 µM), 0.25 µl reverse primer (10 µM), 0.25 µl dNTPs (25 µM), 0.125 µl *Taq* polymerase and 15.375 µl molecular grade water.

The primer set is 9992F (5'-AAGTAGACAGCCGCGCTT-3') and 9195R (5'-TCAATGAGAGCTTGGTCC-3').

The PCR profile is 40 cycles of 94°C for 45 seconds and 60°C for 45 seconds, and a final 7-minute extension at 60°C.

YHV PCR (Mohr *et al.,* 2015)

YHV is a nested multiplex RT-PCR (protocol three in the OIE diagnostic manual) to detect all currently characterised genotypes in the Yellow Head complex YHV1 to YHV7.

First-Step PCR Reaction:

1 µl template cDNA was added to a PCR tube containing: 5 µl 5X Flexi buffer (Promega), 2.5 µl $MgCl_2$, 1 µl of each primer (10 µM), 0.25 µl dNTPs (25 µM), 0.125 µl *Taq* polymerase and 12.125 µl molecular grade water.

The two outer primer sets are YC-F1a (5'-ATCGTCGTCAGCTACCGCAATACTGC-3') and YC-F1b (5'-ATCGTCGTCAGYTAYCGTAACACCGC-3'), and YC-R1a (5'-TCTTCRCGTGTGAACACYTTCTTRGC-3') and YC-R1b (5'-TCTGCGTGGGTGAACACCTTCTTGGC-3').

The PCR profile is one cycle of 94°C for 2 minutes, followed by 30 cycles of 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 45 seconds and a final 7-minute extension at 72°C.

Second-Step Reaction:

1 µl of the first-step PCR reaction product was added to a PCR tube containing: 5 µl 5X Flexi buffer (Promega), 2.5 µl $MgCl_2$, 1 µl of each primer (10 µM), 0.25 µl dNTPs (25 µM), 0.125 µl *Taq* polymerase and 12.125 µl molecular grade water.

The second (inner) primer pair sets are
YC-F2a (5'-CGCTTCCAATGTATCTGYATGCACCA-3') and YC-F2b (5'-CGCTTYCARTGTATCTGCATGCACCA-3'), and YC-R2a (5'-RTCDGTGTACATGTTTGAGAGTTTGTT-3') and YC-R2b (5'-GTCAGTGTACATATTGGAGAGTTTRTT-3').

The PCR profile is one cycle of 94°C for 15 minutes, followed by 35 cycles of 94°C for 30 seconds, 66°C for 30 seconds, and 72°C for 45 seconds and a final 7-minute extension at 72°C.

Gel Electrophoresis

To visualise, PCR products were run on a 2% (w/v) agarose gel stained with Ethidium Bromide.

Quality Control

The replicate lenticules of each batch (negative control and TSV positive and YHV positive) produced consistent results in PCR assays.

Distribution

Samples were sent to 14 NRL's in 13 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 1. Expected results of the Proficiency Test

Sample ID	Sample Type	TSV Results[1]	YHV Results[2]
RA18006 - 1	Shrimp Pleopods	TSV Positive	Negative
RA18006 - 2	Shrimp Pleopods	Negative	Negative
RA18006 - 3	Shrimp Pleopods	Negative	Negative
RA18006 - 4	Shrimp Pleopods	Negative	YHV Positive
RA18006 - 5	Lenticule disc	Negative	Negative
RA18006 - 6	Lenticule disc	TSV Positive	Negative
RA18006 – 7	Lenticule disc	Negative	YHV Positive
RA18006 – 8	Lenticule disc	Negative	Negative

[1] Nunan et al., 1998

[2] Mohr et al., 2015

Actual Results

Results were received from 13 laboratories, Table 2 highlights the results received from each individual laboratory.

- 1 laboratory did not test the samples which were received.
- 8 laboratories correctly diagnosed all samples, 8/8 (100%).
- 4 laboratories correctly diagnosed 7/8 samples (88%);
    - 1 laboratory had samples in the wrong order, error was corrected when double checking results.

    - Fresh tissue samples were supplied to 3 of the labs, after analysing the fresh tissues 1 laboratory correctly diagnosed 6/8 samples (75%). Upon re-analysing the second sample set the laboratory correctly diagnosed 7/8 (88%).
    - 2 laboratories did not complete the re testing within the period of this ring trial.
- 1 laboratory correctly diagnosed 6/8 samples (75%); fresh tissue samples were supplied to this laboratory, after analysing the fresh tissues the laboratory correctly diagnosed all samples (100%). The laboratory reported problems with contamination and unspecific PCR amplification in the initial YHV testing.

Table 2. Proficiency test results submitted by the individual laboratories

Laboratory Code	RA18006-1	RA18006-2	RA18006-3	RA18006-4	RA18006-5	RA18006-6	RA18006-7	RA18006 -8
EURL	TSV +ve Pleopods	-ve Pleopods	-ve Pleopods	YHV +ve Pleopods	-ve Lenticule	TSV +ve Lenticule	YHV +ve Lenticule	-ve Lenticule
1								
2								
3	TSV +ve	-ve	YHV +ve	YHV +ve	-ve	TSV +ve	YHV +ve	-ve
Re test	TSV +ve	-ve	-ve	YHV +ve	-ve	TSV +ve	YHV +ve	-ve
4	TSV +ve	-ve	-ve	YHV +ve	-ve	TSV +ve	YHV +ve	-ve
5								
6								
7	TSV +ve	-ve	-ve	YHV +ve	-ve	TSV +ve	YHV +ve	-ve
8								
9								
10								
11	TSV +ve	-ve	-ve	YHV +ve	-ve	TSV +ve	YHV +ve	-ve
12	TSV +ve	-ve	-ve	YHV +ve	-ve	TSV +ve	YHV +ve	-ve
13	-ve	-ve	-ve	YHV +ve	-ve	TSV +ve	YHV +ve	-ve
Re test								
14	TSV and YHV +ve	-ve	-ve	YHV +ve	YHV +ve	TSV +ve	YHV +ve	-ve
Re test	TSV +ve	-ve	-ve	YHV +ve	-ve	TSV +ve	YHV +ve	-ve
15								
16								
17	-ve	-ve	-ve	YHV +ve	-ve	TSV +ve	YHV +ve	-ve
Re test								
18	TSV +ve	-ve	-ve	YHV +ve	-ve	TSV +ve	YHV +ve	TSV +ve
Re test	TSV +ve	-ve	-ve	YHV +ve	-ve	TSV +ve	TSV and YHV +ve	TSV +ve
Re-test 2	TSV +ve	-ve	-ve	YHV +ve	-ve	TSV +ve	YHV +ve	TSV +ve
19	TSV +ve	-ve	-ve	YHV +ve	-ve	TSV +ve	YHV +ve	-ve
20	TSV +ve	-ve	-ve	YHV +ve	-ve	TSV +ve	YHV +ve	-ve
21	TSV +ve	-ve	-ve	YHV +ve	-ve	TSV +ve	YHV +ve	-ve
22								
23	TSV +ve	-ve	-ve	YHV +ve	-ve	TSV +ve	YHV +ve	-ve
24								
25								
26								

Red text highlights where diagnosis was incorrect
Orange boxes highlight where Re testing was not completed during the period of this ring trial
Grey boxes indicate that the lab did not take part in the testing

Investigation

Pleopods

As mentioned previously multiple laboratories 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 3).

Table 3. Sample reference numbers were recorded for each tube in the sample sets which were sent to various labs. Grey and white 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	Laboratory Code	Result
5	EURL18003 - 14b	EURL18001 - 2b	EURL18001 - 7b	EURL18004 - 3b	14	100%
6	EURL18003 - 14c	EURL18001 - 2c	EURL18001 - 7c	EURL18004 - 3c	13	83%
7	EURL18003 - 14d	EURL18001 - 2d	EURL18001 - 7d	EURL18004 - 3d	7	100%
8	EURL18003 - 14e	EURL18001 - 2e	EURL18001 - 7e	EURL18004 - 3e	24	Not completed
9	EURL18003 - 17b	EURL18001 - 3b	EURL18001 - 8b	EURL18004 - 4b	11	100%
10	EURL18003 - 17c	EURL18001 - 3c	EURL18001 - 8c	EURL18004 - 4c	12	100%
11	EURL18003 - 17d	EURL18001 - 3d	EURL18001 - 8d	EURL18004 - 4d	21	83%
12	EURL18003 - 17e	EURL18001 - 3e	EURL18001 - 8e	EURL18004 - 4e	3	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 two labs experienced a few problems with Tube 1 there were no consistencies between the incorrect results, each lab experiencing a slightly different problem. 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.

Lenticules

Two laboratories incorrectly diagnosed the lenticule samples, each lab providing a false positive response to one or more of the lenticule samples. One laboratory reporting problems with contamination and unspecific PCR amplification in the initial YHV testing. As mentioned previously each lab received lenticules from positive and negative batches of materials. The EURL randomly tested 10% of positive and negative lenticule batches to ensure consistency and accuracy of results, as such we are confident that the samples initially sent and received by each lab were negative for TSV and YHV.

General Comments

Nearly half the laboratories which took part in this testing experienced problems highlighting that further work is needed to improve the diagnostic testing of both TSV and YHV within the NRL network. One lab experienced further issues when re-testing fresh samples and it is suggested that this lab undergo a full evaluation of their working practices. The results highlight an issue with potential contamination issues during the processing of samples. Following the results of this ring trial the EURL would like to again highlight the potential for cross contamination between samples and suggest that laboratories review all laboratory procedures to limit this risk.

References

Mohr, P. G., et al., 2015. New yellow head virus genotype (YHV7) in giant tiger shrimp *Penaeus monodon* indigenous to northern Australia. Diseases of Aquatic Organisms. 115**,** 263-268.

Nunan, L. M., et al., 1998. Reverse transcription polymerase chain reaction (RT-PCR) used for the detection of Taura Syndrome Virus (TSV) in experimentally infected shrimp. Diseases of Aquatic Organisms. 34**,** 87-91.