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A comparative analysis of Yellow Head Virus (YHD) diagnostic methods adopted in Sri Lanka to investigate the accuracy and specificity of the virus

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ABSTRACT

Yellow head virus is a common viral pathogen which infects cultured *Penaeus monodon* world-wide, resulting in immature larvae and the growth retardation. Therefore, several detection methods are recommended by the Office des Epizootics (OIE) for use in Asia including Sri Lanka. This study was attempted to evaluate the RT-PCR diagnostic method is the appropriate and accurate YHV detection method which is recommended by the OIE comparing with histological identification and haemolymph Smear method. A total of 150 individuals of *Penaeus monodon*, post larve samples obtained from different shrimp hatcheries located in Northwestern province were screened for the presence of YHV and the results were comparatively analyzed with three different protocols, namely wet mount observation, histopathological observation and RT- PCR detection. Highest prevalence was recorded with wet mount observations (78%) and the lowest was recorded using histopathology (22%). Comparison of PCR tests generated consisted of positive samples 31/51 (2010), 38/65(2011) and 18/34 (2012) respectively. The results obtained with the IQ2000 commercial kit, were the most reliable and it indicated 56.6% average prevalence of YHV.

***Keywords*:** Yellow head virus, Aquaculture, RNA, OIE, *Penaeus monodon*

1. INTRODUCTION

Yellow head virus (YHV) is a common viral pathogen infecting cultured *Penaeus monodon* (Boonyaratpalin *et al.*, 1992).The disease is highly lethal and contagious, killing shrimps rapidly (Flegel *et al.*, 1993).

YHV is a positive sense single stranded RNA virus which is related to corona viruses and arteriviruses (Kasornchandra *et al.*, 1993). It has been reported first in Thailand in 1990. Since its first appearance in Sri Lanka in 1990, several detection methods were recommended by the office Des Epizootics (OIE) to detect the virus and have been used locally. Molecular diagnosis has been applied for shrimp viral disease detection for years (World Organization for Animal Health (OIE) 2009/Yellow Head Diseases/Manual of Diagnostic Tests for Aquatic Animals, Paris, France).

The shrimp industry in Sri Lanka is important and generates a high income for the country (Infante *et al.*, 2005). There are several problems that are related to epizootic diseases caused by viruses which cause loss of quality and quantity outputs to shrimp farmers. The occurrence of disease is also used as a barrier to strictly control the products from Sri Lanka by importing countries. Yellow head disease is caused by YHV bringing serious loses to the shrimp aquaculture business (Flegel *et al.*, 1992). The animal health standards committee deemed it is necessary to establish the Sri Lankan standard diagnostic methods of yellow head diseases in shrimp to use as a guideline for laboratory diagnosis of yellow head disease in shrimp and a reference for the certification of shrimp farms.

The animal health standards establishes details for the diagnosis of yellow head disease in the laboratory using the staining of haemolymph smear method (Spann *et al*., 1992), histopathological method (Spann *et al*., 1992) and reverse transcription polymerase chain reaction method (Kasornchandra *et al*., 1993).

The analysis has been done according to the standards.

- Shrimps are invertebrate animals in the Family *Peneideae*
- Larva is a newly hatched larval shrimp that will undergo metamorphosis into the stages of nauplius, zoea and mysis within approximately 8-11 days.
- Postlarva (PL) is a young shrimp that has the same appendages as adult shrimp, and it is about 5mm long which will grow from mysis stage to juvenile stage in about 25 days and more. The convention is to designate post larva shrimp with the abbreviation "PL" followed by a number that is the number of day one since they passed from mysis to post larva stage. For example, "PL21" means shrimp that have been the post larvae stage for 21 days.
- Juvenile means shrimp that are 2-3 cm long and are the same as adults but have not yet reached reproductive maturity.
- Adult is a fully mature shrimp that can reproduce.
- Haemolymph means components of plasma and haemolytes in the circulatory system of shrimp.
- YHD is a disease occurring in shrimp caused by yellow head virus, characterized by yellowish discolouration of the cephalothorax caused by the underlying yellow hepatopancrease and pale body. Infected shrimps die rapidly at a high rate.

Diagnosis: test or inspection to analyze and determine the presence of the disease

- **Presumptive test**: A fast and convenient laboratory procedure to test for a disease, such as rapid staining, haemolymph smear or histopathological tests.
- **Confirmation test:** A laboratory procedure to confirm the results of a diagnosis, which is accepted to be highly specific and sensitive.
- **Specificity:** The ability of a diagnosis test method to produce negative results from uninfected samples.
- **Sensitivity**: The ability of a diagnosis test method to produce positive results from infected samples.
- **Positive control:** A test consisting of chemicals and the standard microbes to be studied for comparison with the unknown samples when the same diagnostic procedures are performed on both.
- **Negative control :** A test that does not contain the standard microbes to be studied for comparison with the unknown samples when the same diagnostic procedures are performed on both.

Clinical signs

Clinical signs of yellow head disease are usually noticeable in juvenile to sub adult shrimp. At first, they will have high feeding activity for several days and then they will abruptly stop eating 2-4 days after some of the shrimp have begun to show clinical signs or die (Spann *et al*., 1992). Large numbers of shrimp may be seen floating near the edges of the pond. The most noticeable external abnormalities are paleness, swelling of the cephalothorax and a yellowish discoloration due to the hepatopancreas turning yellow. The hepatopancrease will be softer than normal. Usually all the shrimp in the pond start to show clinical signs and die within only 2 or 3 days (Flegel *et al*., 1993).

Diagnosis

The main purpose of diagnosis of yellow head disease is to confirm the occurring of an outbreak throughout the hatcheries. It helps to ensure that brood stock shrimp and shrimp larvae are pathogen-free for disease surveillance (Flegel *et al.* 1993).

The different diagnostic methods have different levels of efficiencies. The histopathological method (Spann *et al.,* 1992), the staining of haemolymph smear method (Spann *et al.* 1992) are used as presumptive tests but it is necessary to use the RT-PCR method (Kasornchandra *et al*., 1993) to confirm the diagnosis, which is sensitive and specific enough to detect latent infection where YHV is present in minute amounts.

Figure 1. Infected samples Clinical signs of yellow head disease

2. MATERIAL AND METHODS

Sampling

Samples (n =150) were collected from several hatcheries in Ambakadawilla, Kalpitiya and several rural areas in Negombo lagoon annually. Samples were labelled and stored at -70 °C. Fifty one samples were collected in 2010, 65 in 2011 and 34 in 2012 respectively. Every sample was analyzed by using three different identification methods, namely haemolymph smear method, histological identification and RT-PCR method

Staining Of Haemolymph Smear method (Spann *et al.* 1992)

The most suitable sample for this method is haemolymph from shrimp that show clinical signs indicative of yellow head disease taken from the same place of moribund shrimp which were suspected to be infected with YHV. A haemolymph sample was taken from the haemocoel in the abdomen under the first pair of swimming legs (ventral sinus) or from the haemocoel under the third to last pair of walking legs (cardiac sinus), using a needle and syringe that is preloaded with 10-25% formalin of a volume equal to the volume of haemolymph to be taken. The syringe was shaken between the palms in order to mix the haemolymph completely with the formalin.

A smear was made from the sample on a microscope slide and this was allowed to air dry. The smear was then stained with H&E or other haemolymph stain such as Wright and Giemsa's stain. The slide was then examined under the light microscope at x40.

Figure 1. Shrimp tissue sample

Histological Method (Spann *et al.* 1992)

Principle

The principle of this method is to detect evidence of the disease in the shrimp tissue by staining fixed tissue with hematoxylin and eosin (H&E) and observing it under a light microscope (Spann et al., 1992).

Collection and storage of samples

Live shrimps were immersed in chilled Davidson's fixative reagent that is approximately ten times the volume of the shrimp which is as follows (Spann *et al*., 1992):

- When the shrimp are nauplius to PL20 stage, they were stained with Davidson's fixative overnight .
- When the shrimp are PL21 stage to ≤ 3 g - Stained Davidson's fixative but an inclusion was made lengthwise along the carapace to allow the fixative to reach the hepatopancreas.
- When the shrimps are 3-12 g - Davidson's fixative was injected into the shrimp's mouth, under the back of the carapace, into the hepatopancreas and the abdomen from the third to the last pair of walking legs (periopods) as well as all over the dorsal and ventral portions of the cephalothorax, using 1 ml to 10 ml of Davidson's fixative per shrimp, depending on shrimp's size. The shell was incised lengthwise from the sixth abdominal segment to the cephalothorax.
- When the shrimps are over 12 g - Davidson's fixative was injected thoroughly into the cephalothorax and the ventral side of the body from cephalothorax to tail, afterwards the shrimp was cut in half in cross section between the cephalothorax and abdomen.

The samples were immersed in Davidson's fixative for 24 h to 48 h, depending on the size and then it was transferred into 70% ethanol to extend the storage life.

Procedure (Spann *et al.* 1992)

Specimens stored as above were prepared for histopathological examination and the tissue samples on slides were dried as follows:

Paraffin was liquefied which was in the samples by heating the slides to 60 °C for 30 min and then it was immersed in xylene. Samples were rehydrated by immersing the slides in absolute ethanol and 95% ethanol in sequence for 5 min each. Slides were then rinse with running water for 5 min. Slides we immersed again in Mayer's hematoxylin for 5-7 min. Slides were again rinsed with running water for 15-30 s. then the slides were immersed in eosin for 30-60 s and those were then dehydratedby immersing in 95% ethanol and absolute ethanol in sequence. Slides were immersed in xylene for 5 min in order to remove ethanol from the samples. A drop of Immersion oil was added per mount and was covered with a cover slip. Slides were observed under a light microscope at 40x (Spann *et al.* 1992).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Method (Kasornchandra *et al.* 1993)

Principle

The principle of this method is to detect YHV RNA genome. First RNA was extracted, then RNA was transcribe to complementary DNA (cDNA) was synthesized. Then detect the particular segment by using specific primers and lastly to separate the DNA bands using electrophoresis.

Procedure

Extraction of RNA (the following steps were performed at a temperature under 4 ºC)

RNA was extracted using the IQ2000 kit (IQ2000™ YHV/GAV Detection and Typing System developed by CSIRO, Australia, BIOTEC, GeneReach) according to manufacturer's guidlines.

Sample preparation, depending on the type of specimen was as follows:

- For large (juvenile or adult) shrimp - Take 10 mg - 20 mg shrimp tissue and add 150 µl Trizol. Crush and mix, then add more Trizol for a final volume of 500 µl.
- For post larval shrimp (PL10-PL15) - Take 300 shrimp and add 1,000 µl Trizol.
- Haemolymph specimen, take 50 µl of sample and add 500 µl Trizol and mix for 20s.

The common procedure for all specimens

i. Next crush and mix sample seperately, then take just 150 µl of the mixture and add more Trizol for a final volume of 1,000 µl. Incubate the sample from at 25 °C for 5 min.
ii. Centrifuge at 12,000 **g** at 4 °C for 10 min; pipette up the supernatant and transfer it to a new microcentrifuge tube. Add 200 µl chloroform and mix for 20 s.

iii. Incubate at 25 °C for 10 min. Centrifuge at 12,000 g at 25 °C for 10 min; pipette up the supernatant and transfer it to a new microcentrifuge tube.
iv. Add 670 µl isopropanol and mix. Incubate at 25 °C for at least 10 min. Centrifuge at 12,000 g at 25 °C for 10 min; pipette off the supernatant and discard.
v. Rinse the pellet with 0.5 ml of 70% ethanol for at least 30 min at 25 °C. Centrifuge at 12,000 g at 25 °C for 10 min.
vi. Pipette off the supernatant and discard.
vii. Leave at room temperature for 20 min or until the pellet is dry. Add 25 µl diethylpyrocarbonate (DEPC)-treated water. Incubate at 56° C for 15 min. Mix gently and use for the next step to synthesize complementary DNA immediately or store at -70°C until ready for use.

cDNA synthesis

Use RNA extracted from shrimp haemolymph or shrimp tissue with a concentration of approximately 1 µg RNA as the template RNA. Synthesize the first strand of cDNA using the specific primer 144R (see Table 1). For best results, approximately 20 µl of substrate is needed with the following composition:

- antisense primer 144R 0.75 µM
- dNTPs 1 mM (each)
- Moloney murine leukemia virus reverse transcriptase (M-MLV) 2.5 units
- $MgCl_2$ 5 mM
- PCR buffer (10 mMTris/HCl. pH 8.3, 50 mMKCl)

Insert the sample into the thermocycler for the heating stage. If the thermocycler does not have a temperature controlled cover, add 50 µl mineral oil on top of the samples in the microcentrifuge tubes to prevent evaporation or use hot water bath according to the particular temperature. Set the temperature at 45 °C for 15 min for the reverse transcription of the template RNA to produce cDNA. Then increase the temperature to 100 °C for 5 min to stop the reverse transcriptase reaction and adjust the temperature of the solution down to 5 °C before the next step.

Table 1. Nucleotide sequence of primers 10F and 144R (Phromjai *et al.* 2000)

Primer	Sequence
10F	5'-CCG-CTA-ATT-TCA-AAA-ACT-ACG-3'
144R	5'-AAG-GTG-TTA-TGT-CGA-GGA-AGT-3'

Detection of PCR

One set of primers were used for the detection of YHV. The PCR protocol and primer set described by Phromjai *et* al. 2002 was expected to yield a product of 135 bp. The cycling

conditions for the reaction consisted of an initial C for °C for 30 sec and 72 °C for 30 sec, 55 °C for 5 min, followed by 30 cycles of 94 °C for 5 min. The protocol and primer set described by °30 sec, with a final extension at 72 (Phromjai *et al.* 2000).

Precautions

To prevent contamination of DNA and RNase, the materials, equipment and work space shall be kept separately when extracting DNA and mixing the PCR cocktail. Filtered tips should be used for mixing the PCR cocktail and preparing the template RNA.

3. RESULTS

Each and every sample has proceeded with these three diagnostic methods. The identification tests results have described in the below.

Interpretation - Staining of Haemolymph Smear method

In shrimp that are infected with YHV, the nucleus of dead cells can be seen as dark spot (pycnotic nucleus) or may be deteriorated to appear as tiny fragments (karyorrhectic nucleus). However, there shall be no evidence of bacterial infection in the same specimen because it may cause similar changes in haemocyt. This may cause misinterpretation (Nash *et al*. 1992).

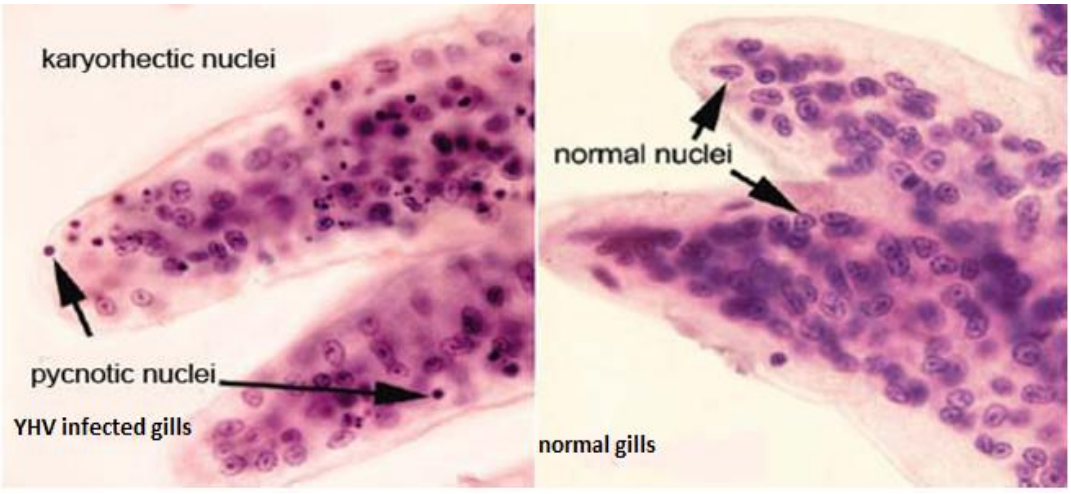

Figure 2. Smear appearance (Infected gill smear and normal gill smear)

Interpretation - Histological Method

In shrimp that are infected with YHV, the following abnormalities can be observed: In the moribund shrimp, many dead cells can be observed in tissues originate from the ectoderm and mesoderm with pycnotic nuclei or karyorrhectic nuclei. Simultaneously basophilic cytoplasmic inclusion bodies may be observed, therefore it may cause for an misinterpretation (Nash *et al*. 1992). Especially in the haemocytes, lymphoid organ, gills, related subcuticular

tissues, muscles, digestive tract, green gland or antennal gland, reproductive organs, nerve tracts and nerve ganglia. The most suitable tissues to examine are the hepatopancreas, lymphoid organ and subcuticular tissues of the stomach and gills (Phromijai *et al.,* 2000).

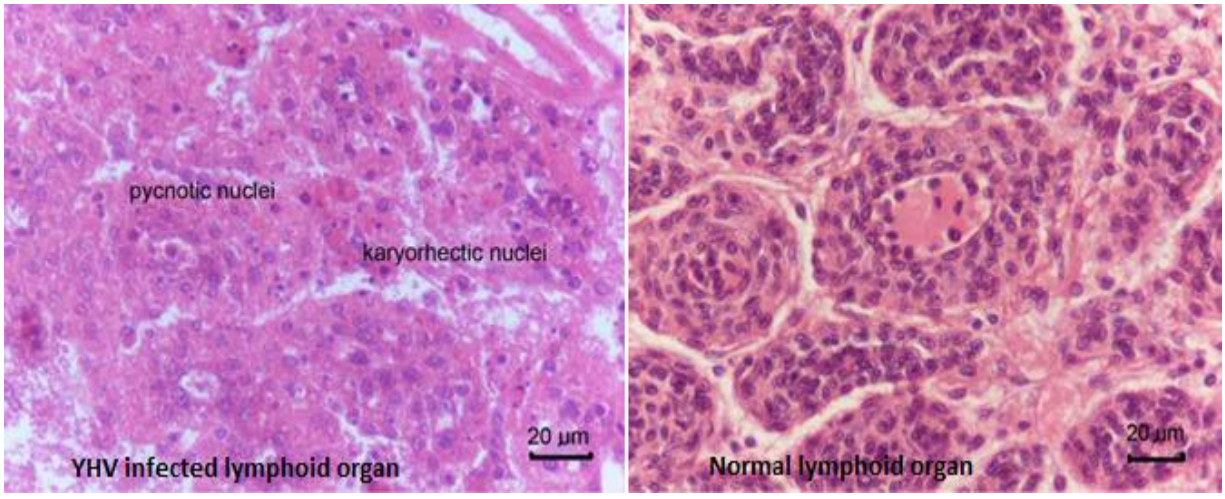

Figure 3. YHV infected and Normal lymphoid tissue identification

Interpretation – RT - PCR analysis

The results should analyze with the positive control and the negative control. Because if there is any occurrence of a contamination or inhibition of the PCR.

- A 135 bp DNA band means a positive reading compared to the positive control.
- No 135 bp DNA band means a negative reading compared to the negative control

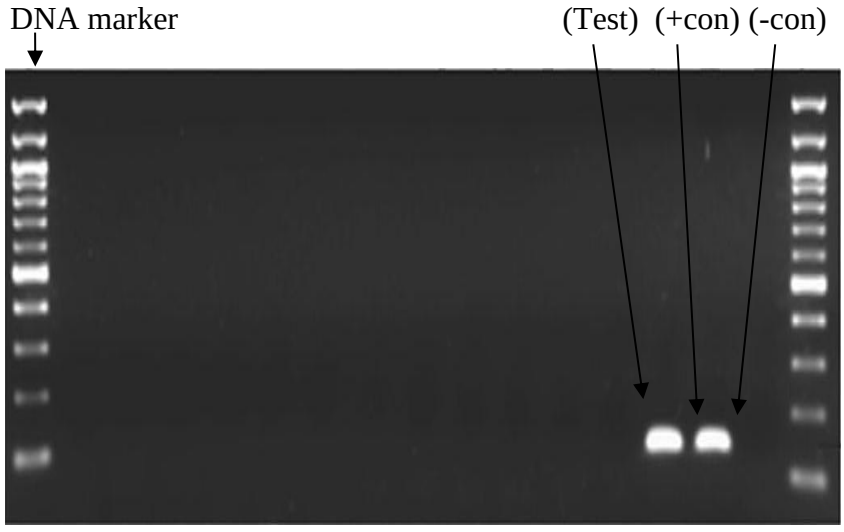

Figure 4. 135bp amplification – DNA marker, Test sample, Positive control and Negative control

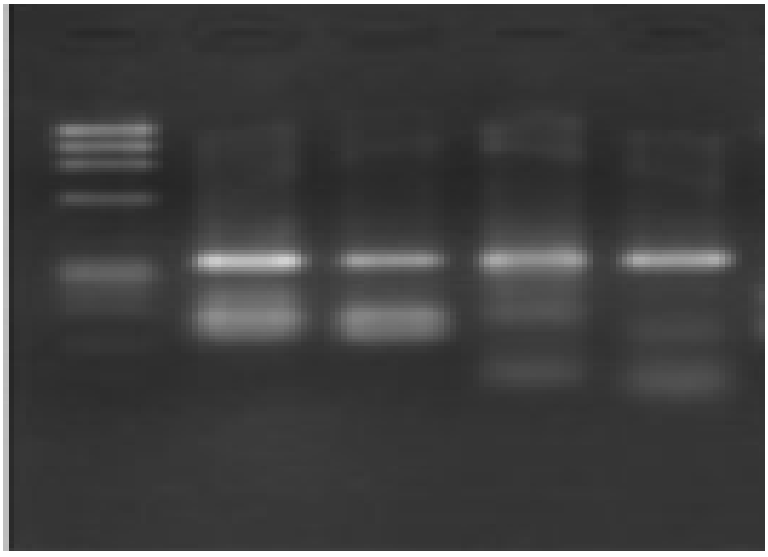

Figure 5. 135bp amplification – positive samples

4. DISCUSSION

Shrimp can get infected with YHV from the late post-larval stage onwards. The clinical signs are most commonly observed and the mortality rate is the highest during the early to late juvenile stages (Spann *et al*., 1992). In hatcheries, shrimp can get infected from other shrimp when the virus is transmitted through the food materials ,water or when they ingest tissues that are contaminated by dead shrimp (Wang *e*t *al*., 2005). Sometimes crustaceans and other animals may also act as disease vectors.

In haemolymph Smear method shrimp that are infected with YHV, the nucleus of dead cells can be seen as pycnotic nucleus or may be deteriorated to appear as karyorrhectic nucleus. However, there shall be no evidence of bacterial infection in the same specimen because it may cause similar changes in haemocyt. This may cause misinterpretation

Histopathological studies have revealed that YHV causes septicemia and destroys tissues that originate from the ectoderm and mesoderm. Once infected with the virus, it spreads rapidly to other organs such as the lymphoid, gills, cuticular epithelium, digestive tract, heart, antennal gland, haemolymph generating tissue, midgut cecum, myoepithelial cells of the hepatopancreas and endocardium (Spann *et al*., 1992). Inclusion bodies can be observed in the lymphoid organ, gills and haemocytes, Simultaneously basophilic cytoplasmic inclusion bodies may be observed. but sometimes it can be misidentified. YHV can also be detected in the haemolymph of shrimp with early stage infections that have not yet begun to show advanced clinical signs, but the suspected samples (without symptoms) can be used for the detection.

RT – PCR detection always analyze with the positive control, the negative control and the test samples. The proper identification is occurring of a positive band at the positive control lane and occurring of non band at the negative control lane in the gel comparing with DNA marker. Then test sample can observe by comparing with those lanes. Therefore it helps to distinguish the infected samples and the uninfected samples. Therefore RT-PCR analysis is

highly sensitive, accurate and an appropriate method to detect the infection though it is cost effective method.

5. CONCLUSION

The smear preparations (histological and haemolymh detection) and the RT - PCR analysis are the best diagnostic methods to detect the infection. All samples were subjected to all three diagnostic methods, in order to determine the most accurate method out of all three diagnostic methods. In histopathological method, the positive samples showed as the necrotic cells in the smear, but sometimes it can be a misidentification.

In haemolymph smear methodpycnotic nucleus or karyorrhectic nucleus can be observed, but the same results appear with the bacterial infection. Therefore, it also can be misidentified. When the same samples were subjected to RT - PCR, results showed variations among the samples which were not positive for the haemolymph smear method or histological method. In RT - PCR it always analyze with the positive control and the negative control.

Therefore RT-PCR method can be used as the confirmation method as well as the most accurate, appropriate and highly sensitive detection method by comparing to other two methods of detection.

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References

[1] Boonyaratpalin, Direkbusarakom, S. S., Kasornchandra, U. Ekpanithanpong, C. Chantanachookin K. Supamattaya. Baculovirus, causative agent of yellow-head disease in penaeid shrimp *Penaeus monodon*. Proceedings of the Seminar on Fisheries (1992), 200-205.

[2] World Organization for Animal Health (OIE) 2009/Yellow Head Diseases/Manual of Diagnostic Tests for Aquatic Animals, Paris, France.

[3] IQ2000™ YHV/GAV Detection and Typing System developed by CSIRO, Australia, BIOTEC, GeneReach.

[4] Boonyaratpalin, S., K. Supamattaya, J. Kasornchandra, S. Direkbusarakom, U. Ekpanithanpong and C. Chantanachookin..Non-occluded baculo-like virus, the causative agent of yellow-head disease in the black tiger shrimp (*Penaeus monodon*) Fish Pathology (1993), 28, 103-109.

[5] Flegel, T. W. Fegan, D. F., Kongsom, S. Vuthikomudomkit, S. Sriurairatana, S. Boonyaratpalin, C. Chantanachookhin, MacDonald O. D., Occurrence, diagnosis and

treatment of shrimp diseases in Thailand. Diseases of Cultured Penaeid Shrimp in Asia (1992), 57-112.

[6] Flegel, TW, Sriurairatana,S**.** Black tiger prawn diseases in Thailand. Technical Bulletin of the American Soybean Association.American Soybean Association, Singapore (1993), 1-31.

[7] Kasornchandra, J. Supamattaya, K. Booynaratpalin, S**..** Electron microscopic observations on the replication of yellow-head baculovirus in then lymphoid organ of *Penaeus monodon*, (1993), 2-3.

[8] Nash, G. Arkarajamon, A .Withyachumnarnakul, B. Routine and rapid diagnosis of Yellow-head disease in *Penaeus monodon*. *Asian Shrimp News* 12(4) (1992) 2-3.

[9] Nash, G. Arkarajamon, A. Withyachumnarnakul, B. Rapid diagnosis of yellowhead disease in black tiger shrimp. Aquatic Animal Health Research Institute (1992), 3-4.

[10] Spann, K. M. Vickers, J. E. Lester, R. J. G.. Lymphoid organ virus of *Penaeus monodon* from Australia. *Diseases of Aquatic Organisms* 23 (1995) 127-134.

[11] Infante C., Gaetano C., Ponce M. and Manchado M. (2005). Novel Method for the Authentication of Frigate Tunas (*Auxisthazard* and *Auxisrochei*) in Commercial Canned Products. *Journal of Agricultural and Food Chemistry* 52(25), 7435-43.

[12] Raghavendra S. Kulkarni. Sex differences in the blood biochemical parameters of the fresh water fish, *Notopterus notopterus* (Pallas, 1789). *World News of Natural Sciences* 6 (2017) 44-51.

[13] Sangita Devi Sharma, Kaushilya Sahu and Pankaj Kumar Jain. Cyanobacterial species Biodiversity in Mahasamund district of Chhattisgarh region, India. *World News of Natural Sciences* 7 (2017) 1-15.

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