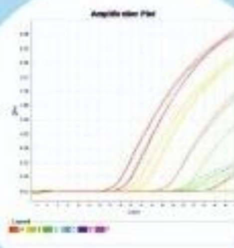
CIBA/TM Series No.59/2026/1

Training Manual on

MOLECULAR DIAGNOSIS OF IMPORTANT SHRIMP PATHOGENS

2nd Feb – 6th Feb, 2026



National Referral Laboratory for Brackishwater Aquatic Animal Diseases
NABL Accreditated Laboratory
Aquatic Animal Health and Environment Division
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Training Manual on
Molecular diagnosis of important shrimp pathogens

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1. ACUTE HEPATOPANCREATIC NECROSIS DISEASE (AHPND)

1.1 Introduction

Vibrio parahaemolyticus is found to be associated with number of diseases in fish and shellfish acts as opportunistic or secondary pathogen that can cause mortality from a few to 100% in affected populations under stress. Recently, it has been found that unique strain of *Vibrio parahaemolyticus* is found to cause disease in shrimp leading to severe economic loss in culture practices named acute hepatopancreatic necrosis disease (AHPND), most important bacterial disease characterized by mass mortality during the first 35 days of culture, where affected shrimp show massive sloughing of hepatopancreatic epithelial cells followed by death. It was first reported from China in 2009, followed by Vietnam in 2010, Malaysia in 2011, and in Thailand since 2012. The disease was identified as early mortality syndrome (EMS) before identification of the causative agent.

The causative agent was identified in 2013 as a bacterial agent, *V. parahaemolyticus*, carrying a specific toxin on a specific extra-chromosomal DNA. AHPND-causing *V. parahaemolyticus* strains carry a conjugative plasmid (~ 63–70 kb, pVA1 type) encoding the binary toxins PirA^{VP} and PirB^{VP} that damage shrimp hepatopancreas cells. Recent studies have shown the PirAB^{vp} binary toxin has been identified in other *Vibrio* species belonging to the *Harveyi* clade, such as *V. harveyi*, *V. campbellii*, and *V. owensii.* The presence of these genes in different bacterial species is a potential risk for the spread of emerging diseases. The clinical signs of shrimp affected with AHPND are a pale hepatopancreas, empty gut, anorexia, and lethargy accompanied by pathognomonic lesions: massive sloughing of tubule epithelial cells of the shrimp hepatopancreas. In addition, two variable regions have also been identified in the pVA1-type plasmids which have been linked to the geographical region of origin of the isolates. These regions correspond to a 4,243 bp *tn3*-like transposon and a 9 bp small sequence repetition (SSR). The presence of the *tn3*-like transposon has only been reported in *V. parahaemolyticus* isolates from Mexico, and a variation in the number of repeated units (RU) of SSR

has been reported in isolates from different geographical origins, although no evidence has been reported linking those regions with the virulence capacity of the strain.

The differences in the virulence have been reported with variations in the mortality rate caused by different isolates of AHPND causing *V. parahaemolyticus* (VP_{AHPND}) in shrimp populations have been observed during disease outbreaks in various regions of the world, as well as when strains are used under experimental conditions during challenge tests. It is reported low virulence in VP_{AHPND} strains due to a partial or total loss in the $PirAB^{vp}$ genes, but the disease could continue to occur. Therefore, there are other factors involved in the pathogenesis of AHPND. The differences in chromosomal and plasmid genes could be related to virulence factors associated with variations in pathogenicity. Secretion systems have major role in pathogenic pathways in *Vibrio* species, type 3 secretion systems (T3SS) and type 6 secretion systems (T6SS) are elements that have been studied and appear to be the most promising for explaining the virulence differences among *V. parahaemolyticus* strains. The involvement of genes related with transposases, DNA methyltransferases, anti-restriction proteins, post segregational killing systems, and secretion systems needs to be studied for their role in virulence mechanism of bacteria causing AHPND.

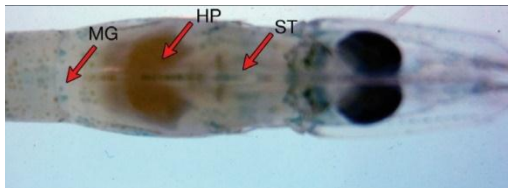
1.2 Transmission and Host Susceptibility

Horizontal transmission is the primary mode of spreading through contaminated water, feed and in-animate objects in the farm, the transmission also occurs due to cannibalism of infected dead shrimp, through infected carriers such as planktons, polychaetes, crabs and mollusks. Experimental studies have shown that *Vp* AHPND could not be transmitted via frozen infected shrimp as AHPND *V. parahaemolyticus* are known to be sensitive to freezing, refrigeration, heating and common disinfectants. *Penaeus vannamei* and *P. monodon* are highly susceptible when compared to *P. indicus* and *P. japonicus*. The post larvae life stages from PL 10 to PL 30 is highly susceptible than other life stages of shrimp, the susceptibility

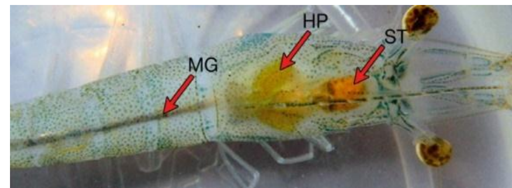
increase with high water temperature 28 to 32 ^{o}C, high salinity above 15 ppt, poor water quality, high stocking density and poor feed management.

1.3 Clinical Signs and Pathology

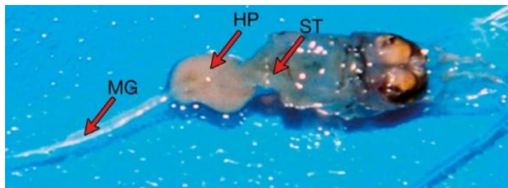
The infected shrimp were observed with lethargy, empty gut, soft shell, pale atrophied hepatopancreas, reduced feed intake and sudden mass mortality within 35 days of culture period. Black spots or streaks on the surface of hepatopancreas, does not squash easily between the thumb and forefinger (probably due to increased **fibrous connective tissue and haemocytes.**



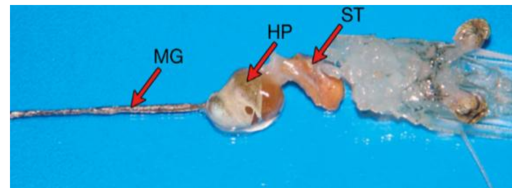
Pale and atrophied hepatopancreas



Normal size HP with dark orange color



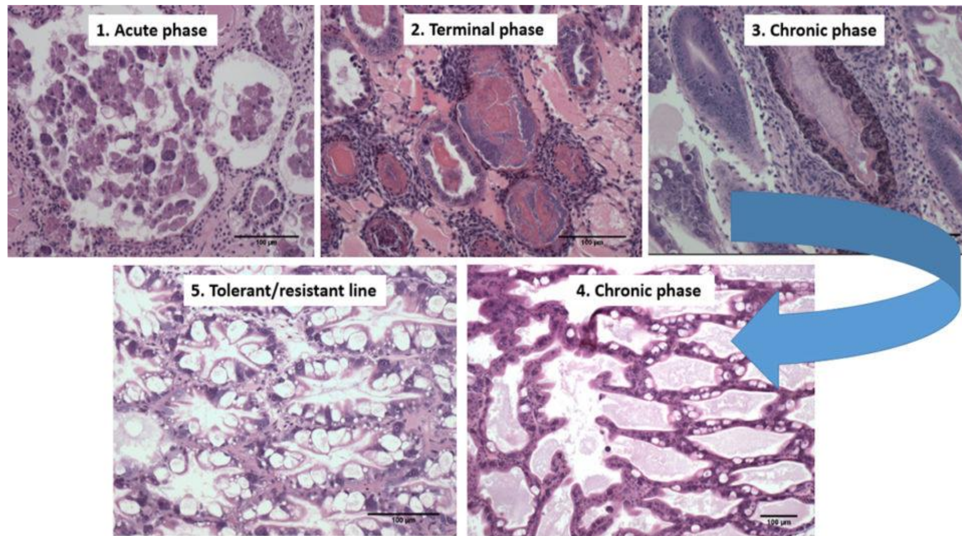
Empty stomach (ST) and midgut (MG)



Full stomach and midgut

Histopathological findings include acute sloughing of the hepatopancreatic epithelial cells, tubular necrosis, the acute phase is characterised by a massive and progressive degeneration of the HP tubules from proximal to distal, with significant rounding and sloughing of HP tubule epithelial cells into the HP tubules, HP collecting ducts and posterior stomach. No B-, F- and R-cells are seen in the hepatopancreatic tubule and some nuclei of tubule epithelial cells are enlarged (karyomegaly). No significant bacterial involvement appears during this phase.

The terminal phase is characterised by marked intra-tubular haemocytic inflammation and development of massive secondary bacterial infections that occur in association with the necrotic and sloughed HP tubule cells. In *Penaeus vannamei* AHPND tolerant lines, the chronic phase is characterised by only a few tubules with epithelial necrosis with bacteria and inflammation.



Evolution of the pathology of AHPND in Latin America, with microphotographs of the tubules of hepatopancreas of affected animals, from the acute phase (1), to the terminal phase, to the chronic phase, and eventually to the tolerant/resistant line. Courtesy: Luis Fernando Aranguren Caro, et.al. (2020)

1.4 PCR Methodology

PCR methods have been developed that target the *Vp* AHPND toxin genes. Two one-step PCR methods (AP1 and AP2) are described here for detection of the pVA1 plasmid in enrichment broth cultures (Flegel & Lo, 2014). The AP3 method is a single-step PCR that targets the 12.7 kDa PirA^{vp} gene (Sirikharin et al., 2015). Single-step PCRs such as the AP3 method and others, e.g. VpPirA-284, VpPirB-392 (Han et al., 2015a) and TUMSAT-Vp3 (Tinwongger et al., 2014), have relatively low sensitivity when used for detection of *Vp* AHPND at low levels (e.g. sub-clinical infections) or in environmental samples such as sediments and biofilms. Alternatively, a nested PCR method, AP4, has been developed with a 100% positive predictive value for *Vp* AHPND (Dangtip et al., 2015), and has greater sensitivity (1 fg of DNA extracted from *Vp* AHPND), allowing it to be used directly with tissue and environmental samples without an enrichment step. In addition, real-time PCR methods, for example the *Vp* AHPND-specific TaqMan real-time PCR developed by Han et al., 2015b, and an isothermal loop-mediated amplification protocol (LAMP) method developed by Koiwai et al., 2016 also have high sensitivity and can be used directly with tissue and environmental samples without an enrichment step.

1.5 PCR Primer Sequences

Method	Primers	Target gene	Expected Amplicon size	Reference
AP1	**AP1F:** 5'-CCT-TGG-GTG-TGC-TTA-GAG-GAT-G-3' **AP1R:** 5'-GCA-AAC-TAT-CGC-GCA-GAA-CAC-C-3'	*pVA1*	700 bp	Flegel & Lo, 2014
AP2	**AP2F:** 5'-TCA-CCC-GAA-TGC-TCG-CTT-GTG-G-3' **AP2R:** 5'-CGT-CGC-TAC-TGT-CTA-GCT-GAA-G-3'	*pVA1*	700 bp	Flegel & Lo, 2014
AP3	**AP3-F:** 5'-ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC-3' **AP3-R:** 5'-GTG-GTA-ATA-GAT-TGT-ACA-GAA-3'	*pir*Avp	333 bp	Sirikharin et al., 2014, 2015
TUMSAT-Vp3	**TUMSAT-Vp3 F**: 5'-GTG-TTG-CAT-AAT-TTT-GTG-CA-3' **TUMSAT-Vp3 R**: 5'-TTG-TAC-AGA-AAC-CAC-GAC-TA-3'	*pir*Avp	360 bp	Tinwongger et al., 2014
VpPirA-284	**VpPirA-284F:** 5'-TGA-CTA-TTC-TCA-CGA-TTG-GAC-TG-3' **VpPirA-284R:** 5'-CAC-GAC-TAG-CGC-CAT-TGT-TA-3'	*pir*Avp	284 bp	Han et al., 2015a
VpPirB-392	**VpPirB-392F:** 5'-TGA-TGA-AGT-GAT-GGG-TGC-TC-3' **VpPirB-392R:** 5'-TGT-AAG-CGC-CGT-TTA-ACT-CA-3'	*pir*Avp	392 bp	Han et al., 2015a
AP4 Step 1	**AP4-F1:** 5'-ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC-3' **AP4-R1:** 5'-ACG-ATT-TCG-ACG-TTC-CCC-AA-3'		1269 bp	Dangtip et al., 2015
AP4 Nested Step	**AP4-F2:** 5'-TTG-AGA-ATA-CGG-GAC-GTG-GG-3' **AP4-R2:** 5'- GTT-AGT-CAT-GTG-AGC-ACC-TTC-3'		230 bp	Dangtip et al., 2015

1.6 Primers and Probe for the Real-time PCR Method for Detection of VpAHPND

Primer/probe name	Sequence	Target gene	Reference
VpPirA-F	5'-TTG-GAC-TGT-CGA-ACC-AAA-CG-3'	pirA	Han et al., 2015b
VpPirA-R	5'-GCA-CCC-CAT-TGG-TAT-TGA-ATG-3'		
VpPirA Probe	5'-6FAM-AGA-CAG-CAA-ACA-TAC-ACC-TAT-CAT-CCC-GGA-TAMRA-3'		

1.7 Conclusion

PCR-based assays targeting the *pirA* and *pirB* toxin genes are the highly specific method for the detection of Acute Hepatopancreatic Necrosis Disease (AHPND) in shrimp. These methods enable identification of pathogenic strains, allowing timely implementation of management and biosecurity measures. Molecular diagnosis serves as a reliable tool for confirming AHPND outbreaks and monitoring the presence of virulent strains in hatcheries and grow-out systems.

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2. HEPATOPANCREATIC MICROSPORIDIOSIS (HPM)

2.1 Introduction

Hepatopancreatic microsporidiosis (HPM) is the disease caused by the emerging microsporidian pathogen EHP. The microsporidian parasite was reported to affect the black tiger shrimp Penaeus monodon in Thailand in the year 2009. Since then, EHP is widespread in most of the Southeast Asian countries, including India. It is referred to as hepatopancreatic microsporidiosis (HPM) since the parasite is confined to the shrimp hepatopancreas (HP). EHP infection did not cause mass mortality but was reported to be associated with stunted growth and WFS. EHP inflicts reduced feed consumption, stunted growth, and severe production losses.

2.2 Host Range, Distribution and Prevalence

The epizootics and spread of EHP were reported in different shrimp farming nations, including Taiwan, Malaysia, Brunei, Vietnam, Venezuela, Korea, Australia, and India. In India the spread of EHP has been recorded in both east and west coast. Co-infection of EHP has been recorded with other viral diseases such as WSSV, IMNV, TSV, HPV and with bacterial diseases such as AHPND and Vibrio spp. This microsporidian reported to infect *Penaeus monodon*, *P. vannamei*, *P.indicus* and *P. merguiensis* are known to get affected. Many aquatic invertebrates organisms such as polychaetes, artemia, wild crabs, small aquatic crustaceans, insects, molluscs etc. also act as carriers for cultured shrimp.

2.3 Clinical Signs

Shrimp infected with EHP do not have any specific signs when they are small. The main clinical signs of EHP at the farm level are slow growth/growth retardation leading to size variation (Fig. 1). EHP infection in shrimp HP affect physiology, metabolism and in turn the shrimp growth. Also, EHP infected shrimp display reduced feed intake, increase food conversion ratio (FCR), empty gut, lethargy and display soft shells. EHP infection did not cause mass mortality but severe infection result in daily mortalities. It is also associated with occurrence of WFS as the infection progress. EHP-WFS affected shrimp exhibit gold brown/white intestine, loose shell, and excreting white fecal strings. Severe infections by EHP can increase the susceptibility to other bacterial infections due to Vibrio spp. in shrimp farms and can result in mortality.

2.4 EHP Transmission

EHP can infect shrimp at all stages of development and at various salinity levels of many penaeid species. Transmission of the disease mainly occurs by oral route. Shrimp may get infected by consuming feed contaminated with faeces and through cannibalism of infected shrimp or even by consuming spores present in the pond water and sediment. However, the vertical transmission of EHP from female brooders to the offspring is unlikely. Many invertebrates such as polychaetes, artemia, wild crabs, small aquatic crustaceans, insects, bivalves, etc. also act as carriers for cultured shrimp. In hatcheries, polychaete worms are considered as main carriers for shrimp broodstock.

2.5 Pathology

EHP is an intracellular spore-forming parasite. It replicates within the cytoplasm of the tubular epithelial cells of the hepatopancreas. EHP infection causes severe necrosis and dilation of HP epithelial tubules, increase in the hemal sinus, and sloughing of epithelial cells. Characteristic histopathological features of EHP include the presence of any life stages or free and mature EHP spores in HP cells. Early stages of EHP infection stained with haematoxylin and eosin are observed as eosinophilic to basophilic inclusions and mature spores are retractile.

2.6 EHP Diagnosis

The EHP can be diagnosed by demonstration of spores in fecal sample and in hepatopancreas by microscopic examination (Fig. 2). Spores can also be demonstrated in the affected animals by different stains such as Giemsa, phloxin, trichrome, calcofluor white, hematoxylin and eosin. Histology of infected tissues reveals several developmental stages, including plasmodium and spore stages. But microscopic demonstration remains successful only in severely affected cases and often undetectable in the early infection. However, many molecular methods such as PCR, qPCR, Loop mediated isothermal amplification (LAMP), are available for routine use for field applications. These techniques are faster, easier and far more accurate to detect the EHP in feces, post-larvae and hepatopancreatic tissue.

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3. INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS DISEASE (IHHNV)

3.1 Introduction

Infectious hypodermal and haematopoietic necrosis disease (IHHND) was first detected in 1981 at a shrimp farm in Hawaii, affecting post-larval and juvenile blue shrimp *Penaeus stylirostris* imported from Costa Rica and Ecuador. In this species, the disease caused mortalities of up to 90%. Subsequently, the infection was also reported in whiteleg shrimp *P. vannamei* and giant tiger prawn *P. monodon*. Infectious hypodermal and haematopoietic necrosis virus (IHHNV) is now recognised as one of the major viral pathogens of shrimp, responsible for significant economic losses to the global shrimp aquaculture industry, and has been listed as a reportable crustacean disease pathogen by the World Organisation for Animal Health (WOAH) since 1995.

3.2 Aetiology

IHHN disease caused by Decapod penstylhamaparvovirus 1, of the Genus Penstylhamaparvovirus belonging to the family *Parvoviridae*. It is a small, unenveloped, icosahedral DNA virus with a linear single-stranded genome of ≤4.1 kb, making it the smallest known penaeid shrimp virus. The genome contains three open reading frames (ORF1–ORF3), each regulated by upstream promoters P2, P11, and P61, respectively, which are transcriptionally active in shrimp as well as insect and fish cells. Five IHHNV genotypes have been described, of which Types I, II, and III are infectious, whereas Types A and B are non-infectious. Type I, II and III are infectious to Penaeid shrimp like *Penaeus vannamei* and *P. monodon*. In contrast, IHHNV-related sequences integrated into penaeid genomes occur as Type 3A (East Africa, India, Australia) and Type 3B (western Indo-Pacific, including Madagascar, Mauritius, and Tanzania). These endogenous viral elements in *P. monodon* are non-infectious to susceptible hosts.

3.3 Susceptible Host Species

IHHNV primarily infects penaeid shrimp; however, natural infections have also been reported in *Artemesia longinaris*, *Palaemon macrodactylus*, post-larval and subadult stages of *Macrobrachium rosenbergii*, and *Procambarus clarkii*. In contrast, bivalve molluscs and adult *M. rosenbergii* act as asymptomatic carriers of IHHNV. IHHNV exhibits species-dependent pathogenicity, being highly virulent in *P. stylirostris*, whereas infection in *P. vannamei* results in runt deformity syndrome (RDS), a chronic condition.

3.4 Transmission and Prevention

Transmission can occur through cannibalization, contaminated water, and infected eggs. Those purchasing live shrimp or breeding shrimp on a shrimp farm should review their biosecurity practices and take steps to address potential gaps as listed below. IHHNV is vertically transmitted via the trans ovarian route. Although egg and larval disinfection is a recommended management practice that may reduce surface contamination (Chen et al., 1992), it is ineffective in preventing trans ovarian transmission of IHHNV.

3.5 Distribution of the Pathogen in the Host

IHHNV exhibits broad tissue tropism, infecting the gills, haematopoietic nodules and haemocytes, ventral nerve cord and ganglia, antennal gland tubule epithelium, lymphoid organ, parenchymal and connective tissues, as well as the ovaries.

3.6 Clinical Signs

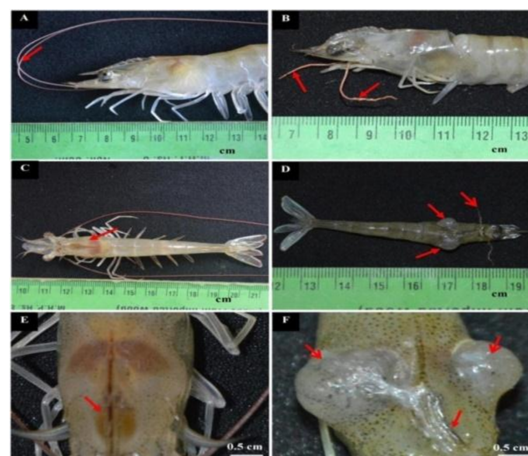
IHHNV infection may be subclinical, with the virus present in the absence of overt signs. When clinical signs occur, they are generally non-specific. In juvenile *P. stylirostris* with acute infection, a pronounced reduction in feed intake is followed by characteristic behavioural and postural changes, including slow ascent to the water surface, transient immobility, rollover, and gradual sinking in a ventral-up position. This cycle may repeat for several hours until affected shrimp become moribund or are cannibalised by conspecifics. In chronic infection, particularly runt deformity syndrome (RDS) in *P. vannamei* and *P. stylirostris*, cuticular deformities such as lateral bending of the rostrum may be observed, although this sign is inconsistently expressed in chronically infected populations. Behavioural alterations are prominent in acute disease of *P. stylirostris* but are not consistently associated with RDS. IHHNV infection also disrupts normal reproduction and early development, reducing egg hatchability and compromising larval and post-larval survival and culture performance when broodstock originate from enzootic populations.

3.7 Gross Pathology

Juvenile shrimp affected by runt deformity syndrome (RDS) commonly exhibit cuticular abnormalities, including lateral rostral bending (45°–90°), deformities of the sixth abdominal segment, wrinkling of the antennal flagella, cuticular roughness, and cephalic swelling (“bubble-head”). Affected populations show marked growth heterogeneity, with a wide size distribution and a high

proportion of undersized ("runted") individuals. The coefficient of variation (CV) in body size typically exceeds 30% and may reach up to 90% in RDS-affected stocks, compared with 10–30% in IHHNV-free populations of *P. vannamei* and *P. stylirostris*.

Comparative morphological observation of *P. vannamei* of normal and IHHNV infected. Normal straight rostrum (A), IHHNV infected with bent rostrum (B), Normal muscle (C), Deformed muscle (D), Normal round shaped eye (E), Infected shrunken eye (F). Image adapted from Zhoa et al., (2024).



Comparative morphological observation of *P. vannamei* of normal with infected. Normal long antennae (closed arrow) (A), Infected shrunken antennae (closed arrow) (B), Normal surface of carapace (closed arrow) (C), Hyperplasia on both sides of carapace (close arrows) and shrunken antennae (open arrow) (D), Normal straight gastrofrontal sulcus (closed arrow) (E), Close view of hyperplasia (open arrows) and bent gastrofrontal sulcus (closed arrow) (F). Image adapted from Zhoa et al., (2024).

3.8 Diagnosis

3.8.1 Ideal Samples for Pathogen Detection

Ectodermal and mesodermal origin tissues are infected by this pathogen and hence the primary tropism for the pathogen is connective tissues, gills, haematopoietic nodules and haemocytes, ventral nerve cord and ganglia, antennal gland tubule epithelium, and lymphoid organ parenchyma. Pleopods, gills, haemolymph, stomach or abdominal muscle are recommended for submission. Whole shrimp (e.g. larvae or post-larvae) or tissue samples containing these target tissues are suitable for molecular diagnostic assays. Haemolymph or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary. Enteric tissues, including the hepatopancreas, midgut, and midgut caeca, are unsuitable for IHHNV detection. In addition, shrimp eye tissues contain PCR inhibitors and should be avoided for molecular diagnostic assays.

3.8.2 Selection of Populations and Individual Specimens

Post-larvae (PL), juveniles, and adults are suitable for IHHNV testing. Although all life stages may be infected, viral loads in spawned eggs and early larval stages are often below detection limits; therefore, these stages are unsuitable for surveillance aimed at demonstrating freedom from IHHNV.

3.8.3 Real-Time PCR

Real-time PCR methods developed for the detection of IHHNV follows the method used in Tang & Lightner (2001).

3.8.4 Primer

Primer Name	Primer/probe (5'–3')
IHHNV1608F	TAC-TCC-GGA-CAC-CCA-ACC-A
IHHNV1688R	GGC-TCT-GGC-AGC-AAA-GGT-AA
IHHNV Probe	6FAM- CTG-CCC-ATC-TAA-CAC-CAT-CTC-CCG-CCC - TAMRA

3.8.5 PCR Reaction

Wear disposable powder free gloves and at all times when handling plates / strips/tubes. Avoid contaminating plates with tissue lint etc. Determine the number of reaction (N) to set up per assay in addition include positive, negative and method control in the test

Prepare one excess reaction cocktail for every 10 reactions to account for pipetting error, if no. of samples (n) including controls =1 to 10, then N = n + 1

Prepare the master mix in PCR work station in the reagent preparation room by following the table below:

Reagents	Stock concentration	Final concentration	Volume 1x Reaction (20µl)
Sterile water	-	-	4.8µl
2X Master mix with ROX	2X	1X	10.0 µl
Forward primer (IHHNV1608F)	10µM	0.4µM	0.4 µl
Reverse primer (IHHNV1688R)	10µM	0.4µM	0.4 µl
Probe (IHHNV Probe)	10µM	0.4µM	0.4 µl
DNA Template (50 to 100ng)			2.0 µl
Total			20.0 µl

A Master Mix is prepared in a tube by combining a 2X Master Mix Reagent and Primer & Probe Mix with water (protect from light). After mixing thoroughly 18µl volumes of the Master Mix are quickly dispensed into the plate / strips/ tubes. Before moving to template addition PCR work station, Add 2 µl of the sterile water in to NTC (negative control) tube. In the template addition UV cabinet add 2 µl of sterile water to Method control tube. Add 2 µl of each DNA sample to respective well as per plate/strips/ tubes set up. Finally add 2 µl of positive plasmid template into positive control tubes in positive control UV cabinet. Centrifuge the tubes for 10 sec to remove bubbles trapped in the reaction tubes. Amplification is performed in real time PCR machine.

The cycling profile is as follows

Cycling steps	Temperature	Time	No. of cycles
Step 1	95° C	30 sec	1
Step 2	94° C	5 sec	40
	60° C	31 sec	
	72° C	2 min	

3.8.6 Data Analysis

A threshold will be set to be above the baseline that begins to detect the increase in signal associated with an exponential increase of PCR product. After completion of the amplification reaction, amplification plots must be critically

assessed. The baseline and the threshold can be set either automatically or manually. The threshold should be placed above the background fluorescence noise, across the exponential phase of all the amplification curves. The PTC with a known target concentration can act as a calibrator to enable standardization of data analysis and an approximate estimation of the viral load.

3.8.7 Acceptance Criteria

The test reliability is assured by analyzing the results of the controls. If the controls did not yield the expected results the causative reason must be investigated and repeat the test from nucleic acid extraction.

Controls	Expected results	Corrective action to be taken
Positive control	Positive, there must be an increase in fluorescence from the FAM fluorophore yielding a sigmoidal amplification curve at the expected Ct value	Repeat the test from the nucleic acid extraction and check the positive control stock
Negative control	Negative, i.e. absence of fluorescence increases from the FAM fluorophore, with no sigmoidal amplification curve	Repeat the test from the nucleic acid extraction, check all the nucleic acid extraction reagents and disinfect the nucleic acid extraction, template addition chambers.
Method control	Negative, i.e. absence of fluorescence increases from the FAM fluorophore, with no sigmoidal amplification curve	Repeat the test from the nucleic acid extraction, Check /change all the PCR reagents.

3.8.8 Interpretation of Results

The following criteria must be followed for data interpretation of diagnostic samples.

Results	Interpretation
Increase in fluorescence from the FAM fluorophore yielding an amplification curve with Ct ≤ 37th cycle.	Positive
Absence of fluorescence increase from the FAM fluorophore, with no sigmoidal amplification curve	Negative
Weak increase in fluorescence from the FAM. Fluorophore yielding a sigmoidal amplification curve with Ct between 37th and 40th cycle.	Inconclusive, Repeat the test from the nucleic acids extraction

3.8.9 Conventional PCR

This is based on the method described by Tang et al., (2007) GenBank Accession No.: AF218266; amplicon size 309 bp

3.8.10 Primer Details

IHHNV ORF1	Fwd 309F: TCC-AAC-ACT-TAG-TCA-AAA-CCA-A
	Rev 309R: TGT-CTG-CTA-CGA-TGA-TTA-TCC-A

3.8.11 Procedure

Perform DNA extraction from the samples according to DNA extraction protocol. Perform PCR reaction with the primers for IHHNV gene. Determine the number of reactions (N) to set up per assay. In addition, include Negative control and Positive control in the test. Prepare excess reaction cocktail to account for pipetting error. If number of samples (n) including controls = 1 to 10, then N=n+1. In the clean reagent preparation room prepare the master mix: Calculate the amount of each reagent to be added for the given primer set reaction master mix with the following calculation

Component	Volume
Nuclease free water	8.5 µl
Taq DNA reaction mix	12.5 µl
Forward primer	1 µl
Reverse primer	1 µl
Template DNA	2 µl
Total	25 µl

Mix reaction mixtures by pipetting up and down. Do not vortex. Centrifuge for 5-6 sec to collect contents at bottom of the tube, and then place the tube in cold rack. Set up reaction tubes in PCR rack. Dispense 23 µl of each master mix into each PCR tubes. Before moving to nucleic acid handling area. Add 2 µl of the nuclease free water in to NTC (No template control) wells. Cap NTC wells. In the nucleic acid extraction room, add 2 µl of each sample to respective wells as per the set up. Cap the PCR tubes to which the samples have been added. Finally, pipette 2µl of positive plasmid template control into positive template control (PTC) wells in positive control addition area. Cap PTC wells. Centrifuge the tubes for 10 seconds. Make sure that bubbles are eliminated from the bottom of the reaction tubes. The reaction volume is 25µl. Run the programme as follows:

Cycling steps	Temperature	Time	No. of cycles
Step 1	94° C	4 min	1
Step 3	94° C	1 min	35
	55° C	1 min	
	72° C	2 min	
Step 3	72° C	5 min	1
Step 4	4° C	Infinite hold	

After completion PCR run, analyse the amplified product by agarose gel electrophoresis. According to agarose gel electrophoresis protocol in document.

3.8.12 Recommended Biosecurity Practices

Purchase live shrimp and germplasm from sources tested negative for the virus. Monitor all new shrimp stocks upon arrival to a farm, disinfect and properly dispose of water used to transport live shrimp, and note any unusual rise in mortality, followed by appropriate diagnostic testing. Implement measures to control virus spread such as pre-treating all water sources and minimizing or eliminating water exchange between populations. Routinely sanitize equipment with appropriate disinfectants. Report suspicious cases to state animal health official.

4. INFECTIOUS MYONECROSIS VIRUS (IMNV)

4.1 Aetiology

Infectious myonecrosis (IMN) is an emerging viral disease in shrimp aquaculture industry, caused by Infectious Myonecrosis Virus (IMNV). The disease was first recorded in Pacific white shrimp, *Penaeus vannamei* in Brazil in 2002 and then in 2006 in Indonesia including Java island. The disease causes significant economic losses to aquaculture due to associated mortalities in *P. vannamei* in grow out ponds. The estimated loss caused by IMNV infection exceeded $100 million from 2002 to 2006 in Brazil and $1 million by 2010 in Indonesia. In India, occurrence of IMN in *P. vannamei* has been recorded in 2016. Infectious myonecrosis (IMN) is a recently identified viral disease caused by dsRNA infectious myonecrosis virus (IMNV).

4.2 Causative Agent

Infectious myonecrosis virus (IMNV) is a non-enveloped, double-stranded RNA (dsRNA) virus tentatively classified under the family Totiviridae. The virus has a monopartite genome of approximately 7.5 kb, encoding a major capsid protein (MCP) and an RNA-dependent RNA polymerase (RdRp). IMNV virions are icosahedral, ~40 nm in diameter, and are highly stable in aquatic environments, contributing to efficient horizontal transmission in shrimp culture systems. It is not the same disease as white tail disease (WTD) of penaeid shrimp and *Macrobrachium rosenbergii*. These two diseases exhibit gross and histological signs that mimic IMN, but which is caused by two different types of virus such as a nodavirus named *P. vannamei* nodavirus (PvNV) and *Macrobrachium rosenbergii* nodavirus (MrNV), respectively. Molecular analyses indicate that IMNV is genetically distinct from nodaviruses causing white tail disease, despite similarities in clinical presentation. The virus primarily replicates in mesoderm-derived tissues, particularly skeletal muscle, leading to extensive myonecrosis and associated production losses in affected shrimp populations.

4.3 Susceptible Host Species

It causes mortalities in juvenile and sub adult pond-reared stocks of *L. vannamei* and the mortality range from 40 to 70 per cent. Outbreaks of the disease seems to be associated with certain types of environment and physical stresses (i.e. extremes in salinity and temperature, collection by cast net, etc.), and possibly with the use of low quality feeds. Experimental infection is observed in tiger shrimp, *P. monodon* and blue shrimp, *P. stylirostris*.

4.4 Geographical Distribution

This disease has been reported in north-eastern Brazil, Java Island, Sumatra, Bangkok, west Borneo, south Sulawesi, Bali, Lombok and Sumbawa in South-East Asia. There are unofficial and anecdotal reports of IMNV occurring in other South-East Asian countries.

4.5 Clinical Signs and Lesions

Mortalities from IMN can range from 40 to 70 per cent in cultivated *P. vannamei*, and feed conversion ratios (FCR) of affected populations can increase from a normal value of ~ 1.5 up to 4.0 or higher. IMN affected shrimp presents focal to extensive white necrotic areas in the striated (skeletal) muscle, especially of the distal abdominal segments and tail fan, which can become necrotic and reddened in some affected shrimp. By histopathology, shrimp with acute phase disease presents lesions with coagulative necrosis of skeletal muscle. In shrimp recovering from acute disease or those in the more chronic phase of the disease, the myonecrosis appears to progress from coagulative to liquefactive necrosis accompanied with haemocytic infiltration and fibrosis. Significant LO spheroid formation is typically present, and ectopic LO spheroids are often found in the hemocoel and loose connective tissues, especially in the heart lumen and adjacent to antennal gland tubules. In some histological preparations, perinuclear pale basophilic to dark basophilic inclusion bodies are evident in muscle cells, connective tissue cells, haemocytes, and in cells that comprise LO spheroids.

4.6 Target Organs

IMNV infects tissues of mesodermal origin (striated muscles - skeletal and cardiac muscle, connective tissues, haemocytes and LO tubule parenchymal cells). The enteric organs (endoderm-derived HP, midgut and midgut caeca) show no histological signs of infection by IMNV.

4.7 Disease Transmission

It is transmitted horizontally by cannibalism and via water. Vertical transmission from broodstock is suspected from anecdotal evidence but it is not known whether this occurs via transovarial mechanism or by surface contamination of newly spawned eggs.

4.8 Diagnosis

IMN is diagnosed using a nested RT-PCR protocol. IMN can be confirmed by histopathology. The histopathological characteristic lesions, including coagulative necrosis of striated skeletal muscle, marked inter-fibre oedema, and pronounced lymphoid organ hypertrophy associated with extensive formation of lymphoid organ spheroids (LOS).

5. TAURA SYNDROME DISEASE

5.1 Introduction

The disease Taura syndrome caused by the pathogenic agent Taura syndrome virus (TSV), is a highly contagious shrimp disease belonging to the genus Aparavirus, Family Dicistroviridae, Order Picornavirales often fatal, disease outbreaks in cultured penaeid shrimp, particularly *Penaeus vannamei* and *P. stylirostris*. Identified in the 1990s, it causes high mortality during the juvenile stage and, while it does not affect humans, it causes massive economic damage in the aquaculture industry.

5.2 Aetiology

Taura syndrome virus, the etiological agent of Taura syndrome disease, is classified into four distinct genotypes based on sequence polymorphisms in the VP1 gene, which encodes the largest and predominant capsid protein. These genotypes comprise the Americas, South-East Asia, Belize, and Venezuela lineages. TSV is a non-enveloped, icosahedral virus with a positive-sense, single-stranded RNA genome of approximately 10.2 kb, excluding the 3′ polyadenylated tail. The genome consists of two major open reading frames: ORF1 encodes non-structural proteins, including helicase, protease, and RNA-dependent RNA polymerase, whereas ORF2 encodes the viral structural proteins VP1, VP2, and VP3, with molecular masses of approximately 55, 40, and 24 kDa, respectively. Viral replication is confined to the cytoplasm of host cells.

5.3 Susceptible Host Species

Blue shrimp (*Penaeus stylirostris*), giant tiger prawn (*Penaeus monodon*), greasyback shrimp (*Metapenaeus ensis*), northern brown shrimp (*Penaeus aztecus*), northern white shrimp (*Penaeus setiferus*), and whiteleg shrimp (*Penaeus vannamei*) are found to be susceptible shrimp species. Infection with TSV has been documented in all life stages of *P. vannamei* shrimp except eggs, zygotes and larvae.

5.4 Distribution of the Pathogen in the Host

TSV could be detected in different body parts including gills, head, whole tail, tail muscle, pleopod and tail fan.

5.5 Transmission

Birds can remain as an important mechanical vector for the transmission of the virus within affected farms or farming regions. Aquatic insects, the water boatman (*Trichocorixa reticulata* [*Corixidae*]) found to harbour TSV within their intestinal contents, but are not directly infected by the virus.

5.6 Disease Pattern

Nursery- or grow-out-phase of *P. vannamei* were affected i.e within ~14–40 days of stocking PLs into grow-out ponds or tanks.

5.7 Mortality, Morbidity and Prevalence

At the farm level, outbreaks of TSV infection in *P. vannamei*, gave cumulative mortalities range from 40 to >90% in cultured populations of PL, juvenile, and subadult life stages.

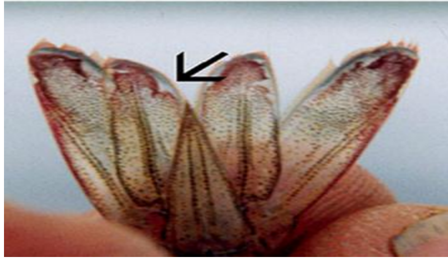
5.8 Clinical Signs

Only acute-phase clinical infection with TSV can be presumptively diagnosed from clinical signs.

5.9 Gross Pathology

Infection with TSV has three distinct phases, acute, transition, and chronic, which are grossly distinguishable. Gross signs presented by juvenile, subadult and adult shrimp in the transition phase of infection with TSV are unique and provide a suspicion of infection. During acute phase gross signs displayed by moribund *P. vannamei* are expansion of the red chromatophores giving the affected shrimp a general, overall pale reddish colouration and making the tail fan and pleopods distinctly red, hence named as 'red tail' disease. In such shrimp, close inspection of the cuticular epithelium in thin appendages such as the edges of the uropods or pleopods will have focal epithelial necrosis. During the transition phase shrimp show random, multifocal, irregularly shaped melanised cuticular lesions. Chronic phase: after successfully moulting, shrimp in the transition phase move into the chronic phase of infection with TSV in which persistently infected shrimp show no obvious signs of disease. However, *P. vannamei* that are chronically infected with TSV may be less resistant to normal environmental stressors (i.e. sudden salinity reductions) than uninfected shrimp.

Image adopted from Samocha, Sustainable biofloc system for marine shrimp, p. 23, Copyright (2019),



5.10 Diagnosis

5.10.1 Ideal Sample for Pathogen Detection

TSV infects tissues of ectodermal and mesodermal origin. The principal target tissue in the acute phase of infection with TSV is the cuticular epithelium. In chronic infections the lymphoid organ (LO) is the principal target tissue. Haemolymph or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary. Specimens for testing for infection with TSV are shrimp, artemia cyst, post larvae (PL), juveniles adults and Polychaetes. Samples of or from the pleopods, gills, haemolymph, stomach or abdominal muscle are recommended for submission. Haemolymph or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary

5.10.2 Samples or Tissues Not Suitable for Pathogen Detection

TSV is a systemic virus, and it does not replicate in enteric tissues (e.g. the hepatopancreas, the midgut, or its caeca). Hence, enteric tissues are inappropriate samples for detection of infection with TSV.

5.10.3 RNA Extraction

Perform RNA extraction of pathogenic samples according to RNA extraction protocol. Total RNA is first transcribed into complementary DNA (cDNA) by reverse transcriptase according to standard cDNA Synthesis Kit.

5.10.4 Real-time PCR

The real-time RT-PCR method described below for TSV follows the method used in Tang et al., 2004.

5.10.5 Primer Details

Primer Name	Primer/probe (5'–3')
TSV 1004F	5'-TTG-GGC-ACC-AAA-CGA-CAT-T-3'
TSV 1075R	5'-GGG-AGC-TTA-AAC-TGG-ACA-CAC-TGT-3'),
TSV Probe	6FAM- (5'-CAG-CAC-TGA-CGC-ACA-ATA-TTC-GAG-CAT-C-3' – BHQ 1

5.10.6 PCR Reaction

Wear disposable powder free gloves and at all times when handling plates / strips/tubes. Avoid contaminating plates with tissue lint etc. Determine the number of reaction (N) to set up per assay in addition include positive, negative and method control in the test

Prepare one excess reaction cocktail for every 10 reactions to account for pipetting error, if no. of samples (n) including controls =1 to 10, then N = n + 1

Prepare the master mix in PCR work station in the reagent preparation room by following the table below:

Reagents	Stock concentration	Final concentration	Volume 1x Reaction (20µl)
Sterile water	-	-	4.8µl
2X Master mix with ROX)	2X	1X	10.0 µl
Forward primer (TSV 1004F)	10µM	0.4µM	0.4 µl
Reverse primer (TSV 1075R)	10µM	0.4µM	0.4 µl
Probe (TSV Probe)	10µM	0.4µM	0.4 µl
DNA Template (50 to 100ng)			2.0 µl
Total			20.0 µl

A Master Mix is prepared in a tube by combining a 2X Master Mix Reagent and Primer & Probe Mix with water (protect from light). After mixing thoroughly 18µl volumes of the Master Mix are quickly dispensed into the plate / strips/ tubes. Before moving to template addition PCR work station, Add 2 µl of the sterile water in to NTC (negative control) tube. In the template addition UV cabinet add 2 µl of sterile water to Method control tube. Add 2 µl of each DNA sample to respective well as per plate/strips/ tubes set up. Finally add 2 µl of positive plasmid template into positive control tubes in positive control UV cabinet. Centrifuge the tubes for

10 sec to remove bubbles trapped in the reaction tubes. Amplification is performed with the Real time PCR machine. The cycling profile is as follows

Cycling steps	Temperature	Time	No. of cycles
Step 1	95° C	30 sec	1
Step 2	94° C	5 sec	40
	60° C	31 sec	
	72° C	2 min	

5.10.7 Data Analysis

A threshold will be set to be above the baseline that begins to detect the increase in signal associated with an exponential increase of PCR product. After completion of the amplification reaction, amplification plots must be critically assessed. The baseline and the threshold can be set either automatically or manually. The threshold should be placed above the background fluorescence noise, across the exponential phase of all the amplification curves. The PTC with a known target concentration can act as a calibrator to enable standardization of data analysis and an approximate estimation of the viral load.

5.10.8 Acceptance Criteria

The test reliability is assured by analyzing the results of the controls. If the controls did not yield the expected results the causative reason must be investigated and repeat the test from nucleic acid extraction.

Controls	Expected results	Corrective action to be taken
Positive control	Positive, there must be an increase in fluorescence from the FAM fluorophore yielding a sigmoidal amplification curve at the expected Ct value	Repeat the test from the nucleic acid extraction and check the positive control stock
Negative control	Negative, i.e. absence of fluorescence increases from the FAM fluorophore, with no sigmoidal amplification curve	Repeat the test from the nucleic acid extraction, check all the nucleic acid extraction reagents and disinfect the nucleic acid extraction, template addition chambers.
Method control	Negative, i.e. absence of fluorescence increases from the FAM fluorophore, with no sigmoidal amplification curve	Repeat the test from the nucleic acid extraction, Check /change all the PCR reagents.

5.10.9 Interpretation of Results

The following criteria must be followed for data interpretation of diagnostic samples.

Results	Interpretation
Increase in fluorescence from the FAM fluorophore yielding an amplification curve with Ct ≤ 37th cycle.	Positive
Absence of fluorescence increase from the FAM fluorophore, with no sigmoidal amplification curve	Negative
Weak increase in fluorescence from the FAM Fluorophore yielding a sigmoidal amplification curve with Ct between 37th and 40th cycle.	Inconclusive, Repeat the test from the nucleic acid extraction

5.10.10 Conventional RT-PCR

Tissue samples (haemolymph, pleopods, whole small shrimp etc) may be assayed for TSV using RT-PCR. The RT-PCR method outlined below for TSV follows the method used in Nunan et al. (1998) of amplicon size 231 bp

Primer (5'–3')	Cycling parameters
Fwd: 9992: AAG-TAG-ACA-GCC-GCG-CTT	Reverse transcription 60°C/30 min 40 cycles: 94°C/45 sec, 60°C/45 sec
Rev:9195R: TCA-ATG-AGA-GCT-TGG-TCC	

5.10.11 Procedure

Perform RNA extraction from the samples according to RNA extraction protocol. Perform PCR reaction with the primers for TSV gene. Determine the number of reactions (N) to set up per assay. In addition, include Negative control and Positive control in the test. Prepare excess reaction cocktail to account for pipetting error. If number of samples (n) including controls = 1 to 10, then N=n+1. In the clean reagent preparation room prepare the master mix: Calculate the amount of each reagent to be added for the given primer set reaction master mix with the following calculation

Component	Volume
Nuclease free water	8.5 µl
Taq DNA reaction mix	12.5 µl
Forward primer	1 µl
Reverse primer	1 µl
Template DNA	2 µl
Total	25 µl

Mix reaction mixtures by pipetting up and down. Do not vortex. Centrifuge for 5-6 sec to collect contents at bottom of the tube, and then place the tube in cold rack. Set up reaction tubes in PCR rack. Dispense 23 µl of each master mix into each PCR tubes. Before moving to nucleic acid handling area. Add 2 µl of the nuclease free water in to NTC (No template control) wells. Cap NTC wells. In the nucleic acid extraction room, add 2 µl of each sample to respective wells as per the set up. Cap the PCR tubes to which the samples have been added. Finally, pipette 2µl of positive plasmid template control into positive template control (PTC) wells in positive control addition area. Cap PTC wells. Centrifuge the tubes for 10 seconds. Make sure that bubbles are eliminated from the bottom of the reaction tubes. The reaction volume is 25µl. Run the programme as given in that table. After completion of PCR run, either it can be preserved in 4° C for further analysis or the amplified product should be resolved by agarose gel electrophoresis following agarose gel electrophoresis protocol. The sample is considered positive based on the amplification similar to that of positive control.

5.10.12 Recommended Biosecurity Practices

Purchase live shrimp from SPF brooders which are tested negative for the virus. Implement measures to control virus spread such as pre-treating all water sources and minimizing or eliminating water exchange between populations. Routinely sanitize equipment with appropriate disinfectants. Report suspicious cases to state animal health official. Routinely screen all the samples for the pathogen.

6. SHRIMP WHITE SPOT SYNDROME VIRUS (WSSV)

6.1 Background

Known for its high virulence nature, WSSV is a rapidly replicating shrimp virus that has wide spread presence throughout the world. The virus started its journey from Taiwan in 1992, and soon spread to Japan during 1993 and subsequently very quickly to all other Asian countries. By 1995 it had already spread to North America and further by 1999 to South America. The quick spread of this virus to different regions and simultaneous investigation by different scientists speculated the same agent to be different ones and thereby called them in different names such as Systemic Ectodermal and Mesodermal Baculovirus (SEMBV), rod shaped nuclear virus of *Penaeus Japonicus* (RV-Pj), Hypodermal and Haematopoeitic Necrosis baculovirus (HHNBV), third *Penaeus monodon* non-occluded virus (PmNOB III), penaeid rod shaped DNA virus or white spot baculovirus. On subsequent investigation and data analysis when it was known that all these names are for the same agent, it was unanimously called as white spot syndrome virus.

6.2 Causative Agent

The causative agent for WSD is the white spot syndrome virus. This is the largest known animal virus so far. The virus contains double stranded circular DNA with approximately 300 kb genome size. So far genome sequences of this virus from many of the countries have been reported with different degree of variation in genome size. Different genotypes of this virus with differential virulence properties have also been reported. The virus appears to maintain its genotype status even after several passages across different penaeid species. When compared with other viruses, genetic structure of WSSV is considered as unique in many aspects and therefore this is placed in an entirely new family, Nimiviridae and genus *Whispovirus*.

6.3 Clinical Sign

WSSV is known for its typical clinical sign, i.e. formation of circular white spots on the carapace and body part during the advanced stage of the disease. It is also observed that white spot are not always present. Similarly, the white spot can also be due to some bacterial infection. Reddish discoloration is also often observed in WSD affected shrimp. Shrimp suffer mortality within 3-10 days of onset of infection and farmers salvage their crops by emergency harvest. However, there are also few examples of shrimp affected by WSSV in farms without mortalities and achieving normal harvest. There can be many other reasons for the development of clinical signs and therefore the infection should be confirmed using diagnostic

protocols. WSSV affects all stages of penaied shrimp and has posed a serious challenge to aquaculturists all over the world.

6.4 Host Status

All penaeid shrimp species have been shown to be highly susceptible to WSSV infection resulting high mortality. Other important crustaceans such as crabs, crayfish, freshwater prawns, spiny lobsters and clawed lobsters are also reported to be susceptible to WSSV infection. Apart from the penaeid shrimps, a large number of other cultured and non-cultured crustaceans serve as carrier. Even some of the non-crustacean members such as polychaete worms have been reported to be career for this virus. Therefore, the virus resembles more like a bacterial pathogen and once in the system, it becomes virtually impossible to eradicate the virus from the culture system. However, morbidity and mortality consequence of infection is highly variable. Similarly, prevalence of WSSV is reported highly variable, from <1% in infected wild populations to up to 100% in captive populations.

6.5 Diagnosis

Suitability of stages: All stages of animals starting from post larvae to brooders are suitable for detection of WSSV by molecular methods. Healthy, apparently healthy, moribund and freshly died animals are suitable for disease diagnosis.

While considering post larvae, usually 20-30 animals, based on size, are pooled together and taken for DNA extraction. For other tissues, usually 100 – 200 mg will be sufficient. Fresh, frozen or preserved tissues can be taken for DNA extraction.

Sampling: Sampling can be done from hatchery, nursery or farms. Usually two types of sampling are done. In non-lethal sampling, tip of pleopod or haemolymph is collected where the animals are put back safe to their tanks. For high value brooders non-lethal sampling is recommended. In case of lethal sampling, the animal can be sacrificed and the desired tissues can be collected from any part.

Samples can be collected fresh and analysed immediately. Otherwise, these can be stored at -80 C for long time. Another way of collecting sample is preserving the entire shrimp (post-larvae or juvenile) or specific parts in 80-90% ethanol and store at room temperature. WSSV usually infects ectodermal and mesodermal

tissues. In general, pleopods, gills, haemolymph, stomach or abdominal muscle are recommended for WSSV testing.

6.6 DNA Extraction

There are several DNA extraction methods available including direct commercially available kits. Different column purification methods can also be used to obtain for pure DNA. As per WOAH method, the required tissue or whole larvae is taken in a 1.5 ml microfuge tube with 600 µl lysis solution (100 mM NaCl, 10 mM Tris/HCl, pH 8, 25 mM EDTA [ethylene diamine tetra-acetic acid], 0.5% SLS [sodium N-laurylsarcosinate] or 2% SDS [sodium dodecyl sulphate], and 0.5 mg ml–1 proteinase K added just before use). This is homogenised and incubated at 65 C for 1 hour. This is subjected to CTAB extraction method.

6.7 PCR (WOAH Method)

Primers for 1st step PCR: 146F1, 5'-ACT-ACT-AAC-TTC-AGC-CTA-TCTAG-3' and 146R1, 5'-TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A-3 and Primers for 2nd step PCR: 146F2 (5'-GTA-ACT-GCC-CCTTCC-ATC-TCC-A-3') and 146R2 (5'-TAC-GGC-AGC-TGC-TGC-ACC-TTG-T-3') with PCR reaction of; one cycle of 94°C for 4 minutes, 55°C for 1 minute, and 72°C for 2 minutes, followed by 39 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes and a final 5-minute extension at 72°C. Decapod-specific primers (143F 5'-TGC-CTT-ATC-AGCTNT-CGA-TTG-TAG-3' and 145R 5'-TTCAGN-TTT-GCA-ACC-ATA-CTT-CCC-3') can be used to check the quality of DNA extracted. Amplified products are visualized in 1% agarose gel.

Master mix is prepared with primers and other components to which DNA is added and loaded to a thermocycler. For the 2nd step PCR, the amplified product in the 1st step is used as DNA. All reactions should be accompanied by a positive control, negative control and a method control.

For the proper verification of amplified product, sequencing should be done.

For real time PCR, primes and probe are: WSS1011F: 5'-TGG-TCC-CGT-CCT-CAT-CTC-AG-3', WSS1079R: 5'-GCT-GCC-TTG-CCG-GAA-ATT-A-3', Taqman Probe: 5'-AGC-CAT-GAA-GAA-TGC-CGT-CTA-TCA-CAC-A-3' with reaction of; one cycle of 50°C for 2 minutes for AmpErase uracil-N-glycosylase (UNG) and 95°C for 10 minutes for activation of AmpliTaq, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

7. YELLOW HEAD VIRUS

7.1 Introduction

Yellow Head Disease (YHD) is caused by Yellow Head Virus genotype 1 (YHV-1). The disease is characterized by a distinct yellowing of the cephalothorax, resulting from enlargement and yellow discoloration of the hepatopancreas. YHV-1 was first recognized in the early 1990s following outbreaks that caused mass mortalities in *Penaeus monodon* farms in Thailand. Since then, the virus has been reported in several Asian countries, including Sri Lanka, Indonesia, the Philippines, China, Taiwan, and Malaysia. YHD is an extremely virulent disease, often resulting in up to 100% mortality within 3–5 days after the onset of clinical signs.

7.2 Aetiology

Yellowhead virus genotype 1 (YHV-1) is a bacilliform or rod-shaped, enveloped virus containing single-stranded RNA, belonging to the genus *Okavirus*, family *Roniviridae*, order *Nidovirales* with virions measuring approximately 44 ± 6 nm in diameter and 173 ± 13 nm in length. YHV-1 is classified as a Category C-1 pathogen, indicating its ability to cause rapid mass mortality and extensive spread within affected locations, making disease control extremely challenging. Yellowhead virus genotype 1 is the only confirmed causative agent of YHD. To date, ten genotypes have been identified within the yellowhead virus complex. Genotype 2 is known as Gill-Associated Virus (GAV) and is considered distinct from YHD. Genotypes 3, 4, 5, 6, 9, and 10 are commonly detected in *Penaeus monodon* populations across East Africa, Asia, and Australia, but are rarely or not associated with disease. In contrast, genotypes 7 and 8 have been reported to cause disease in *Penaeus monodon* and *Fenneropenaeus chinensis*, although they have not been detected in *Penaeus vannamei*.

7.3 Susceptible Host Species

Yellowhead virus (YHV) is highly infectious to most cultivated penaeid prawn species. Species shown to be susceptible include *Penaeus monodon*, *Penaeus merguiensis*, *Penaeus stylirostris*, and *Penaeus vannamei*. In *P. monodon*, early post-larval stages (PL15) have been reported to exhibit resistance to infection, whereas later post-larval stages (PL20–PL25), as well as growing juveniles and subadults, are highly susceptible to YHV infection.

7.3 Transmission and Prevention

Transmission of Yellowhead virus genotype 1 (YHV-1) occurs primarily through horizontal routes, including cannibalism and exposure to contaminated pond water, which may subsequently lead to infection of eggs. Vertical transmission has also been reported from infected male or female broodstock via surface contamination or infection of tissues surrounding fertilized eggs. The virus can remain infectious in water for up to 72 hours, facilitating its spread through contaminated water sources. YHV-1 may be introduced through uncertified broodstock, fry, or infected carrier hosts. Several farmed penaeid shrimp species, including *Penaeus monodon*, *P. japonicus*, *Litopenaeus vannamei*, and *P. stylirostris*, are known potential hosts, and some may act as asymptomatic carriers of the virus.

Preventive measures include minimizing stress in cultured shrimp by maintaining optimal water quality parameters, thoroughly removing accumulated sediments and organic matter after harvest, and stocking only certified virus-free shrimp fry.

7.5 Distribution of the Pathogen in the Host

Yellowhead virus genotype 1 (YHV-1) primarily targets tissues of ectodermal and mesodermal origin. Major tissues affected include the lymphoid organ, haemocytes, haematopoietic tissue, gill lamellae, and the spongy connective tissue of the subcutis. Additional sites of viral replication include the gut, antennal gland, gonads, as well as nerve tracts and ganglia.

7.6 Clinical Signs

Yellowhead disease is characterized by yellowing of the hepatopancreas and a swollen, yellowish cephalothorax. Affected shrimp show discoloration of the body and gills, stop feeding, and aggregate near pond edges or the water surface. Mass mortality of up to 100% can occur within 2–4 days after the onset of anorexia.

7.7 Gross Pathology

Early stages of the disease are marked by increased feeding, followed by reduced or complete cessation of feeding in later stages. Affected shrimp exhibit a pale or bleached body, yellow and swollen cephalothorax, yellow hepatopancreas, and white, yellow, or brown gills. The hepatopancreas is characteristically soft compared with the firm brown hepatopancreas of healthy shrimp.

7.8 Diagnosis

7.8.1 Ideal Samples for Pathogen Detection

In moribund shrimp suspected of YHV-1 infection, pleopods, gills, and the lymphoid organ are the most appropriate tissues for sampling. For routine screening or surveillance of apparently healthy juvenile or adult shrimp, pleopods or gills are the preferred sample tissues.

7.8.2 Selection of Populations and Individual Specimens

During disease outbreaks, moribund shrimp collected from pond edges are the preferred samples for diagnostic examination, and apparently healthy shrimp from the same ponds should also be included. For surveillance of apparently healthy populations, shrimp from post-larval stage 15 (PL15) onwards provide suitable tissue sources for testing.

7.9 Real-time PCR

7.9.1 RNA extraction

Perform RNA extraction of pathogenic samples according to RNA extraction protocol. Total RNA is first transcribed into complementary DNA (cDNA) by reverse transcriptase according to standard cDNA Synthesis Kit.

7.9.2 Primer

Primer Name	Primer/probe (5'–3')
YHV1-12-qF	AGT-CTA-CAG-TGC-TCT-GAT-CT
YHV1-12-qR	GAT-TCT-TGA-AGC-GCA-TGA-GT
YHV1-12-qPr	FAM-TCT-CAT-GTG/ZEN/TCA-TGA-TAT-TCT-CAA-GCG-AGT-IABkFQ

7.9.3 PCR reaction

Wear disposable powder free gloves and at all times when handling plates / strips/tubes. Avoid contaminating plates with tissue lint etc. Determine the number of reaction (N) to set up per assay in addition include positive, negative and method control in the test. Prepare one excess reaction cocktail for every 10 reactions to account for pipetting error, if no. of samples (n) including controls =1 to 10, then N = n + 1

Prepare the master mix in PCR work station in the reagent preparation room by following the table below:

Reagents	Stock concentration	Final concentration	Volume 1x Reaction (20µl)
Sterile water	-	-	4.8µl
2X Master mix with ROX	2X	1X	10.0 µl
Forward primer (YHVF)	10µM	0.4µM	0.4 µl
Reverse primer (YHVR)	10µM	0.4µM	0.4 µl
Probe (YHV Probe)	10µM	0.4µM	0.4 µl
cDNA Template (50 to 100ng)			2.0 µl
Total			20.0 µl

A Master Mix is prepared in a tube by combining a 2X Master Mix Reagent and Primer & Probe Mix with water (protect from light). After mixing thoroughly 18µl volumes of the Master Mix are quickly dispensed into the plate / strips/ tubes. Before moving to template addition PCR work station, Add 2 µl of the sterile water in to NTC (negative control) tube. In the template addition UV cabinet add 2 µl of sterile water to Method control tube. Add 2 µl of each cDNA sample to respective well as per plate/strips/ tubes set up. Finally add 2 µl of positive plasmid template into positive control tubes in positive control UV cabinet. Centrifuge the tubes for 10 sec to remove bubbles trapped in the reaction tubes. Amplification is performed in real time PCR machine.

The cycling profile is as follows

Cycling steps	Temperature	Time	No. of cycles
Step 1	95° C	10 sec	1
Step 2	95° C	15 sec	45
	60° C	60 sec	
	72° C	2 min	

7.9.4 Data Analysis

A threshold will be set to be above the baseline that begins to detect the increase in signal associated with an exponential increase of PCR product. After completion of the amplification reaction, amplification plots must be critically assessed. The baseline and the threshold can be set either automatically or manually. The threshold should be placed above the background fluorescence noise, across the exponential phase of all the amplification curves. The PTC with a known target concentration can act as a calibrator to enable standardization of data analysis and an approximate estimation of the viral load.

7.9.5 Acceptance Criteria

The test reliability is assured by analyzing the results of the controls. If the controls did not yield the expected results the causative reason must be investigated and repeat the test from nucleic acid extraction.

Controls	Expected results	Corrective action to be taken
Positive control	Positive, there must be an increase in fluorescence from the FAM fluorophore yielding a sigmoidal amplification curve at the expected Ct value	Repeat the test from the nucleic acid extraction and check the positive control stock
Negative control	Negative, i.e. absence of fluorescence increases from the FAM fluorophore, with no sigmoidal amplification curve	Repeat the test from the nucleic acid extraction, check all the nucleic acid extraction reagents and disinfect the nucleic acid extraction, template addition chambers.
Method control	Negative, i.e. absence of fluorescence increases from the FAM fluorophore, with no sigmoidal amplification curve	Repeat the test from the nucleic acid extraction, Check /change all the PCR reagents.

7.9.6 Interpretation of Results

The following criteria must be followed for data interpretation of diagnostic samples.

Results	Interpretation
Increase in fluorescence from the FAM fluorophore yielding an amplification curve with Ct ≤ 37th cycle.	Positive
Absence of fluorescence increase from the FAM fluorophore, with no sigmoidal amplification curve	Negative
Weak increase in fluorescence from the FAM. Fluorophore yielding a sigmoidal amplification curve with Ct between 37th and 40th cycle.	Inconclusive, Repeat the test from the nucleic acid extraction

7.10 Conventional PCR

The RT-PCR method outlined below for YHV follows the method used in Wongteerasupaya et al., (1997); of amplicon size 135 bp

7.10.1 Primer details

Primer (5'–3')	Cycling parameters
10F: CCG-CTA-ATT-TCA-AAA-ACT-ACG	Reverse transcription at 50°C/30 min and 94°C/2 min, 40 cycles of 94°C/30sec, 58°C/45 sec, 68°C/45 sec
144R: AAG-GTG-TTA-TGT-CGA-GGA-AGT	

7.10.2 Procedure

Perform RNA extraction from the samples according to RNA extraction protocol. Perform PCR reaction with the primers for YHV gene. Determine the number of reactions (N) to set up per assay. In addition, include Negative control and Positive control in the test. Prepare excess reaction cocktail to account for pipetting error. If number of samples (n) including controls = 1 to 10, then N=n+1. In the clean reagent preparation room prepare the master mix: Calculate the amount of each reagent to be added for the given primer set reaction master mix with the following calculation

Component	Volume
Nuclease free water	8.5 µl
Taq DNA reaction mix	12.5 µl
Forward primer	1 µl
Reverse primer	1 µl
Template DNA	2 µl
Total	25 µl

Mix reaction mixtures by pipetting up and down. Do not vortex. Centrifuge for 5-6 sec to collect contents at bottom of the tube, and then place the tube in cold rack. Set up reaction tubes in PCR rack. Dispense 23 µl of each master mix into each PCR tubes. Before moving to nucleic acid handling area. Add 2 µl of the nuclease free water in to NTC (No template control) wells. Cap NTC wells. In the nucleic acid extraction room, add 2 µl of each sample to respective wells as per the set up. Cap the PCR tubes to which the samples have been added. Finally, pipette 2µl of positive plasmid template control into positive template control (PTC) wells

in positive control addition area. Cap PTC wells. Centrifuge the tubes for 10 seconds. Make sure that bubbles are eliminated from the bottom of the reaction tubes. The reaction volume is 25µl. Run the programme as given in that table. After completion of PCR run, either it can be preserved in 4° C for further analysis or the amplified product should be resolved by agarose gel electrophoresis following agarose gel electrophoresis protocol. The sample is considered positive based on the amplification similar to that of positive control.

7.11 Recommended Biosecurity Practices

Procure live shrimp and germplasm exclusively from certified sources that have tested negative for the virus. All newly introduced shrimp stocks should be carefully monitored upon arrival at the farm. Water used for transporting live shrimp must be disinfected and disposed of safely. Any unusual increase in mortality should be promptly recorded and investigated through appropriate diagnostic testing. Measures to prevent viral spread should include pre-treatment of all water sources and minimization or complete avoidance of water exchange between different shrimp populations. Farm equipment should be routinely sanitized using suitable disinfectants. Suspected disease outbreaks must be reported to the state animal health authorities without delay. In the event of an outbreak, the affected pond should be disinfected with 30 ppm chlorine for a minimum of four days to eliminate infected shrimp and potential carriers. Dead shrimp should be disposed of by burial or incineration, followed by complete drying of the pond. Water used in the subsequent production cycle should also be disinfected with 20–30 ppm chlorine to eliminate any remaining carrier organisms.

8. COVERT MORTALITY NODA VIRUS

8.1 Introduction

Covert mortality noda virus (CMNV) is a newly found virus isolated from *Penaeus vannamei* was known to be the pathogenic agent of shrimp viral covert mortality disease (VCMD), which has caused serious production losses in the shrimp farming. CMNV is prevalent in China and countries in Southeast Asia, and Latin America. CMNV mainly infects shrimp nervous tissues, hepatopancreas, muscle tissues and ovary. The infected and moribund shrimp will not come to the surface of pond water as seen in other shrimp viral diseases, rather it will move to the pond bottom and eventually die in the deep water.

In shrimp ponds, in addition to cultured shrimp, CMNV was also detected positive in the coexisting annelid worm *Perinereis aibuhitensis* and a related brine shrimp *Artemia sinica*, which are the important fresh food for shrimp culture, infection with CMNV is not a WOAH listed disease of shrimp. In outdoor ponds, VCMD causes low mortality in the affected shrimp every day. This daily continuous mortality occurs throughout the culture period; so VCMD was initially called "running mortality syndrome (RMS)" (Zhang et al., 2017; Zhang, 2019). Higher mortalities occur at water temperatures above 28°C and with sudden changes in weather (Zhang et al., 2014,2017). In indoor tank farming, VCMD usually does not cause obvious mortality in the affected shrimp unless there are sudden changes in environmental conditions.

8.2 Etiology, Transmission and Host Distribution

CMNV was identified as the viral pathogen belongs to the genus Alphanodavirus (Nodaviridae), spherical and non-enveloped RNA virus. Similar to other viruses in Nodaviridae, it contained segmented, bipartite linear, positive-stranded ribonucleic acid (RNA) genome. The deduced amino acid sequence of an 1185 bp fragment of CMNV RNA dependent RNA polymerase (RdRp) gene shared only 39% identity with that of Macrobrachium rosenbergii nodavirus (MrNV).

The virus has a wide host range among invertebrates, and it is known to infect the major cultured shrimp species, Chinese white shrimp (*Penaeus chinensis*), Kuruma prawn (*Penaeus japonicus*), giant tiger prawn (*Penaeus monodon*), giant river prawn (*Macrobrachium rosenbergii*), ridgetail prawn (*Palaemon carinicauda*), white leg shrimp (*Penaeus vannamei*) (Liu et al., 2021), as well as the co-inhabiting

organisms in shrimp ponds. Recently, natural infections of CMNV were reported in several species of fish such as Japanese flounder (*Paralichthys olivaceus*) (Wang et al., 2019), large yellow croaker (*Larimichthys crocea*) (Xu et al., 2022), zebrafish (*Danio rerio*) (Wang et al., 2021a), *Mugilogobius abei*, a common marine fish in shrimp ponds and coastal water in China, and marine fish *Chaeturichthys hexanema* and *Larimichthys polyactis* from the Yellow Sea, and farmed Japanese flounder *Paralichthys olivaceus*.

Thus, this virus is capable of naturally crossing the species barrier and infecting both vertebrates and invertebrates. The vertical transmission of CMNV through sperm and oocytes in *Exopalaemon carinicauda* has been demonstrated (Liu et al., 2017), while the horizontal transmission has been reported among common species of invertebrates inhabiting shrimp ponds (Liu et al., 2018). However, there is currently no reported evidence of CMNV's horizontal and vertical transmission in fish.

8.3 Clinical Signs and Pathology

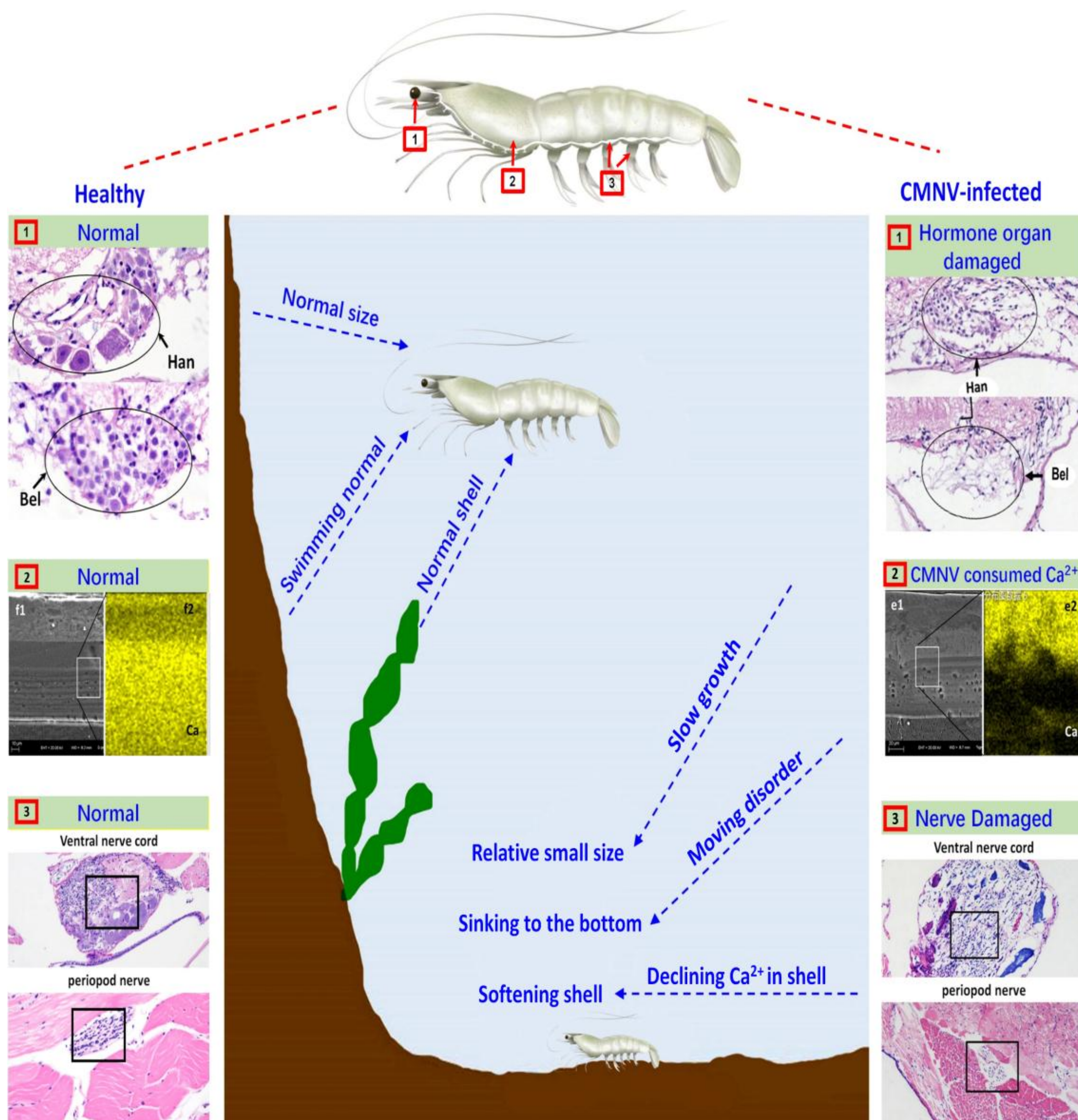
The infected shrimp observed with empty stomach and gut, soft shell, slow growth. In many cases, it also leaded to abdominal muscle whitening and necrosis. In the ponds with CMNV-infection, slowly developing mortality could be observed during daily management, and cumulative mortality of *Litopenaeus vannamei* was variable and might be up to 80–90%. CMNV could experimentally infect *L. vannamei* under laboratory condition and cumulative mortality of individuals in *per os* infection could reach 84.9% by day 10 post-injection. In the cases of VCMD prior to 2013, most mortality usually occurred 60–80 days post-stocking in the summer. However, in recent years, occur as early as one- or two-weeks post-stocking, and cause massive mortality of cultured shrimps.

Vacuolation in the cytoplasm of hepatopancreocytes and multifocal myonecrosis in the striated muscle could be found in the infected individuals. Presence of eosinophilic inclusions within the tubular epithelium of hepatopancreas and lymphoid spheroids was another typical pathological feature (Zhang et al., 2014). The nerve tissues distributing both in the eyestalks, abdominal segment and pleopods were also the important target tissue of CMNV infection. Previous preliminary studies demonstrated that CMNV infection could result in cytoplasm vacuolation of the hepatopancreocytes, multifocal myonecrosis of the striated muscle, hemocytic infiltration, and karyopyknosis of hemocytes in the target tissues, as well as the presence of the eosinophilic inclusions in the tubular epithelium of hepatopancreas and lymphoid spheroids.

The TEM histological examinations upon experimental trials revealed that severe vacuolation, karyopyknosis, and structural failure not only occurred in the nerve enrichment zone like the medulla externa, the sinus gland, the medulla interna, and the medulla terminalis, but also occurred in the hormone secretion zone including the globuli cells, the Hanstrm organ, and the organ of Bellonci. ISH demonstrated that the intense purple positive hybridization signals of CMNV probe could be observed in the sinus gland, in the Hanstrm organ, and in the organ of Bellonci, as well as in the globuli cells.

The tissues of the medulla externa, the medulla interna, and the medulla terminalis presented mild hybridization signals of the CMNV probe. The results of TEM analysis indicated that large quantities of CMNV-like particles assembled in the cytoplasm of the globuli cell and the cells of the sinus gland. TEM examination results revealed the similar ultrastructural pathological changes of the nerve tissue in swimming legs to that occurred in the nerve cord of the abdominal segment, that is, a vast amount of CMNV like particles distributed only in the starting or middle-stage vacuolar cytoplasm of nerve cells, no viral particles present in the cytoplasm of entirely vacuolar nerve cells.

The feature of the vast amount of calcium ions consumed in viral capsid assembly of the family Nodaviridae clued that the mass CMNV assembly in the diseased shrimp would consume a huge amount of calcium ions in the stage of acute infection of the virus as well. An experimental research result was that the carapace synthesis of diseased shrimp might not be able to obtain enough calcium ions, which led to softening of the diseased shrimp carapace, as well as molting difficulty and ultimately affecting the growth rate of the CMNV infected shrimp individuals. The microsporidian *Enterocytozoon hepatopenaei* (EHP) was first described in Thailand in 2009 and it caused hepatopancreatic microsporidiosis (HPM) in farmed black tiger shrimp *Penaeus monodon*. It was suspected that EHP infection was related to growth retardation in farming shrimp in previous reports. Recently the information from shrimp farmers indicated that it was associated with significant growth retardation that was not clearly noticeable until 2– 3 months of cultivation, whereas HPM was not normally associated with shrimp mortality. During the past 5-years epidemiological investigation of farming shrimp, we found that the diseased shrimp suffering merely EHP infection did not show syndromes of carapace softening. So, both syndromes of slowing growth and carapace softening were the typical characteristic of CMNV acute infection, which was different from the EHP infection.



Schematic diagram of the pathogenic mechanism of CMNV infection in P. vannamei. Figures in the left showed the hanstrm organ (Han) and Bellonci (Bel) organ (1), ventral nerve cord and periopod nerve (2), TEM, and FESEM of the longitudinal section of cephalothorax carapace (3), of the shrimp individuals in the control group. Figures in the middle part described the clinical symptoms and behavioral characteristics of healthy shrimp and CMNV-infected shrimp. Figures in the right showed the hanstrm organ (Han) and Bellonci (Bel) organ (1), ventral nerve cord and periopod nerve (2), TEM and FESEM of the longitudinal section of cephalothorax carapace (3), of the shrimp individuals in the CMNV infection group. The severe cell damage of the Han and Bel (1). The severe pathological damages in the nerve cells of both the ventral nerve cord and segmental nerve of the pleopods (2). The obvious lower calcium content (the more yellow means more calcium) in

the FESEM figure of (f2) contrasting with the health one in the left figure of (e2) (3). Courtesy Liu et al. 2022.

8.4 Molecular Diagnosis

Several molecular techniques such as one-step RT-PCR, nested RT-PCR, LAMP, ISH are available (Wang et al., 2021a; Li et al., 2018; Liu et al., 2018; Zhang et al., 2014). A pair of RT-PCR primers and a TaqMan probe (Table. 1) were designed from the RNA-dependent RNA polymerase (RdRp) gene of CMNV from which 130-bp amplicon (981-1110 in GenBank KM112247.1) was obtained. The CMNV probe was synthesized and labeled with 5-carboxyfluorescein (FAM) dye at the 5′ end and carboxytetramethylrhodamine (TAMRA) at the 3′ end (5′ FAM/3′ TAMRA) developed by Pooljun et al., 2016.

Primer probe	Nucleotide Sequences
CMNV-F	5′-ACC TCC GCA ATC TGA TTG-3′
CMNV-R	5′-GGG TCT ACT TTC GTT GGA-3′
CMNV-TaqMan	5′-CGC TAC CAC TGTC GGC TTG T-3′

The cycling consisted of 15 min at 95 °C followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min.

Wang et al. (2022) developed real time PCR primers of coat protein (CP) gene in the RNA2 genome of CMNV (GenBank accession number MZ643944) was chosen as the targeted gene for designing primers.

Primer probe	Nucleotide Sequences
CMN-CP-TaqIDT-F2	5′ -AACTACATCTGCACCCCATG-3′
CMNCP-TaqIDT-R2	5′ -TTGATGGTGTCGCTAGTCTTC-3′
TaqMan probe	5′-ATCCCTGCCGCTTAATGTGAGATCG-3′

The TaqMan probe was synthesized by Sangon (Shanghai, China), labeled with a 6-carboxyfluoresin (FAM) at the -5′ end and with a TAMRA^{TM} quencher (TAMRA) at the -3′ end, and purified by high-performance liquid chromatography (HPLC), the optimized reaction procedure is shown as the following: 54.9 ◦C for 15 min, 95 ◦C for 1 min; 40 thermal cycling amplifications (95 ◦C for 10 s, 54.9 ◦C for 25 s), and the optimized reaction mixture contained 0.3 µM of each primer, 0.3 µM probe, 0.8 µL Luna WarmStart® RT Enzyme Mix, 10 µL reagent 1 Luna® Universal Probe One-step Reaction Mix (2×), 1 µL template, 6.4 µL RNA-free H2O.

8.5 Conclusion

The Covert mortality noda virus is an important pathogen to be monitored in the aquafarms having the symptoms of running mortality syndromes in shrimp. The vertical transmission studies in broodstock will provide more knowledge on disease spread and biosecurity methods to prevent the outbreak of CMNV.

8.6 References

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9. NECROTIZING HEPATOPANCREATITIS BACTERIUM (NHPB)

9.1 Introduction

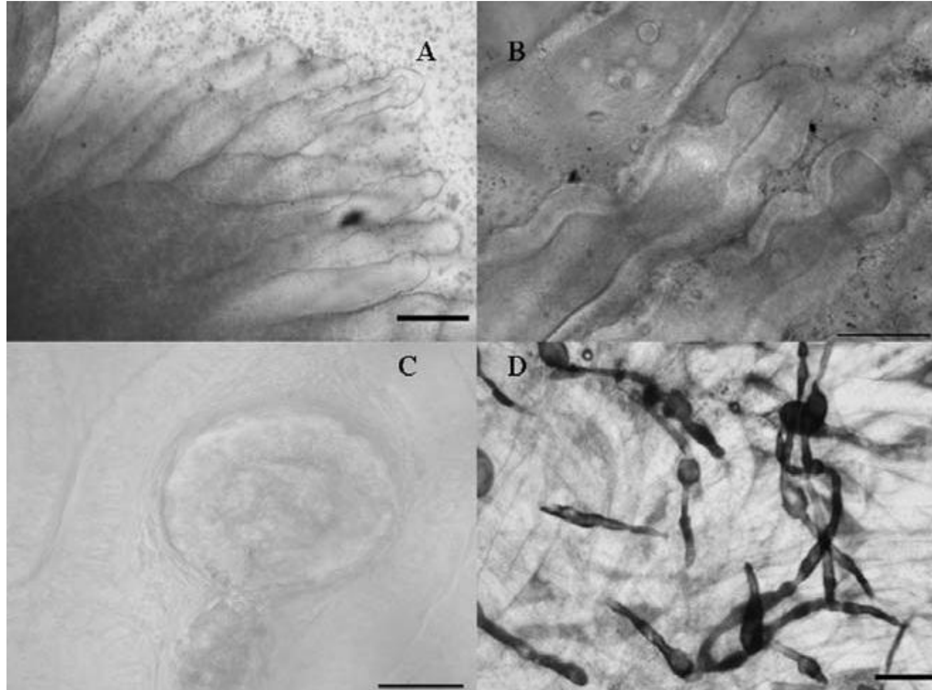
Necrotizing Hepatopancreatitis Bacterium (NHPB) is an intracellular bacterium capable of causing severe disease and mortality in shrimp aquaculture. The bacterial pathogen that produces necrotizing hepatopancreatitis (NHP) is a major problem to the shrimp farming industry. Although distribution of NHP has been virtually limited to the Americas, NHP is under consideration for listing by the World Organization for Animal Health (WOAH) because of the potential to produce disease worldwide. Mortalities from NHP may reach up to 95% in affected shrimp ponds, resulting in loss of nearly entire shrimp crops during a grow-out season (Johnson,1990; Lightner, 1996). NHP in shrimp is often associated with high salinity ranging from 30 to 38 ppt and higher temperature from 30 to 35°C in America and Mexico. NHPB was first reported in 1985 in a Texas shrimp farm, NHP has since affected shrimp farming in several North and South American countries, causing mortalities up to 95% and devastating economic losses to aquaculture crops.

9.2 Etiology, Host and Transmission

The NHPB is gram-negative intracellular, pleomorphic aften rod shaped bacteria, belonging to the subclass α- Proteobacteria highly pathogenic to penaeid shrimp, are difficult to propagate *in vitro* hence it is necessary to propagate in live shrimp through *per os*. After the first report since been observed in cultured penaeids in Peru, Ecuador, Venezuela, Brazil, Panama, Costa Rica, and Mexico, where it produces significant pathology and mortalities in affected ponds. The reported hosts include *Litopenaeus vannamei, L. setiferus,L. stylirostris, Farfantepenaeus aztecus, and F. californiensis*. An experimental infection study using American lobster (*Homarus americanus*) by force feeding of bacterial inoculum in the oral cavity with insulin syringe, the infected lobsters were found to be PCR positive at 15 days post infection. Necrotized spots were observed in hepatopancreas, showing the wide diversity of crustaceans can be host for the bacterium.

The cannibalism from infected environment resources and transportation of frozen NHPB infected shrimp can be the source of spreading of the bacterium. NHPB has been detected in faecal tissue from infected juveniles and broodstock. Faeces may be a possible source of infectious NHPB in the shrimp pond. In fact, waterborne transmission of NHPB was observed through continuous exposure of

susceptible shrimp to water containing NHPB-infected faeces (Pantoja et al., 2006). It is thought that the flagellated form of the bacterium is able to swim and survive in the water column.



Litopenaeus vannamei. Photomicrographs showing wet-mount detection of NHP disease. In hepatopancreas. (a) atrophied hepatopancreas tubules, (b) hepatopancreas tubules with desquamation cells, (c), lesions involving one or more of the tubules (encapsulation) and (d) Heavy melanized hepatopancreas tubules. Scale bar = 20μm.(Courtesy: Morales-Covarrubias et.al. 2012)

Faecal transmission of NHPB adds another level of transmission to the epidemic model, where transmission may be occurring from an acute host. However, the rate of NHPB transmission from an acute host is likely much lower than from a dead infected host. The bacteria are highly concentrated in tubular epithelial cells of hepatopancreatic cells of penaeid shrimp. Collecting the infected hepatopancreas and freezing at -80 °C can preserve the bacterium for longer period. The sucrose gradient purified preparations of NHPB can also be preserved (Frelier et al. 1993).

Vincent et al. (2004) transmitted NHPB to susceptible individuals of Kona *L. vannamei* through oral exposure in laboratory challenges. Survival of shrimp infected with NHPB orally is significantly lower than that of controls, and mortalities from NHPB are observed 16–51 d post-exposure. No experimentally infected animal has been observed to recover from NHPB infection, all infected animals eventually died from disease. The daily mortality rate or virulence of NHPB

is considerably lower (0.08) than that observed for TSV (0.3) and WSSV (0.4) in laboratory challenges.

9.3 Clinical Symptoms, Gross Changes and Histopathology

Reduced feed intake, empty guts, soft shells, flaccid bodies, heavy surface fouling by epicommensal organisms, black or darkened gills, chromatophore expansion giving the appearance of darkened edges on pleopods, lethargy, and marked atrophy of the hepatopancreas (Lightner, 1996). The shrimp hepatopancreas is the primary site of infection for NHPB and undergoes distinct changes over the course of infection. There is a marked reduction in lipids, and the hepatopancreas may appear pale with a whitish center rather than the normal tan to orange in NHPB-infected shrimp. Severe cases have pale hepatopancreas with black streaks, indicating the melanization of hepatopancreatic tubules, and the hepatopancreas may appear soft and watery with a fluid-filled centre (Lightner, 1996).

Courtesy: Vincent et al. 2007

Histopathological signs of NHPB infection include hepatopancreatic atrophy and multifocal granulomatous lesions. Lipids in resorptive (R) cells are reduced, and the number of blister-like (B) cells is drastically reduced or absent in the hepatopancreatic tubules. Cells present in the granulomatous lesion may be hypertrophied and contain masses of pale basophilic, non-membrane bound NHP-bacteria free in the cytoplasm. The nuclei of hypertrophied cells may appear normal or pycnotic.

9.4 Diagnostic Methods

Intracellular masses of bacteria free in the cytoplasm of the hepatopancreatic epithelial cells are visible through light microscopy using Hematoxylin and Eosin (H&E) stain, a modified Steiner's silver stain, or Giemsa stain. The pleomorphic forms of NHPB are visible using transmission electron microscopy. A DIG (digoxigenin-11-dUTP)-labeled DNA probe specific for the NHP-bacterium is used

for *in situ* and dot blot hybridization (Loy and Frelier, 1996). Polymerase chain reaction (PCR) and monoclonal antibodies are also available for NHPB detection, and the bacterium can be quantified through real-time PCR. The NHP-bacterium is also detectable in shrimp fecal tissue through PCR and real-time PCR. Hepatopancreas and faeces may be assayed for *H. penaei* using RT-PCR. Primers designated as NHPF2: 5'-CGT-TGG-AGG-TTC-GTC-CTT-CAGT-3' and NHPR2: 5'-GCC-ATG-AGG-ACC-TGA-CAT-CAT-C-3', amplify a 379 base pair (bp) fragment corresponding to the 16S rRNA of *H. penaei*

Pathogen/ target gene	**Primer Name**	**Real Time Primer/probe (5'–3')**
H. penaei/16S rRNA gene	NHP1300	CGT-TCA-CGG-GCC-TTG-TAC-AC
	NHP1366	GCT-CAT-CGC-CTT-AAA-GAA-AAG-ATA-A
	NHP Probe	6FAM-CCG-CCC-GTC-AAG-CCA-TGG-AA-TAMRA

9.5 Conclusion

There were only limited experimental studies due to difficulty in preserving NHPB, and *in-vitro* propagation was unsuccessful. The isolation and identification of NHPB is geographically limited. Continuous monitoring of shrimp culture, and surveillance of NHPB in faecal and suspected tissue samples of the imported stocks and allied frozen shrimp will prevent the risk of introduction in our country.

9.6 References

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PCR – TRAINING PRACTICAL

1. ANIMAL OR TISSUE PRESERVATION FOR MOLECULAR ANALYSIS

1.1 Introduction

Collection, storage and archiving of specimens and tissue samples are prerequisites for the successful acquisition of molecular data for any systematic study. This chapter reviews the important practical aspects of the sampling and storage: 1) selection of appropriate tissues for nucleic acid extraction 2) storage of freshly collected tissues in the field 3) transportation, long-term storage and archiving of tissue samples.

1.2 Tissue Samples

Sampling must ensure an accurate representation of the health status of the population or individual. The healthy animals should also be tested along with the diseased animals during sampling.

1.3 Tissue Tropism

Tissue Tropism is the cells and tissues of a host which support growth of a particular virus. Some viruses have a broad tissue tropism and can infect many types of cells and tissues. Other viruses may infect primarily a single tissue. For example White Spot Syndrome Virus (WSSV) infects ectodermal and mesodermal origin tissues such as epidermis, gills, pleopod and hemolymph . But Monodon Baculovirus(MBV) infects only endodermal origin tissue hepatpancreas. So the selection of particular tissue type is mandatory in the accurate diagnosis of viral infection. The selected tissue of the organism should be relatively free of compounds potentially damaging to the nucleic acid or interfere with PCR. For example, Eye balls are known to contain PCR inhibitors.

1.4 Preservation of Tissue

Fresh material from live animals consistently provides the highest yield and quality of DNA for amplification. The live animals or moribund animals can be frozen in dry ice and rapidly placed in the cold and away from light. The tissues should be packed in plastic cryotubes or Ziploc bags excluding as much air as possible to avoid cross contamination.

The tissue samples can routinely stored and transported in 95– 100% ethanol at ambient temperature for molecular studies. The larger size or exoskeleton of the animal does not allow the penetration of ethanol of the tissue and causes degradation of the tissues. These samples should be injected with ethanol, dissected into smaller pieces to allow the ethanol to diffuse directly into the internal tissues. There should be about 10 volumes of ethanol to 1volume of sample for the proper preservation of the sample. Ethanol should be replaced after the initial fixation and periodically at a regular interval.

Long-term storage conditions should minimise variation in temperature. The animal tissues will remain indefinitely stable for extraction of nucleic acids at –70–80 °C.

This will allows the archiving of samples for reanalysis. There are also several commercial preservative available specifically to preserve nucleic acid in tissue.

1.5 Steps to Avoid Contamination

The investigator should be aware of the importance of keeping their instruments, containers and reagents clean in order to prevent cross-contamination. The individual tissue samples should be stored in separate containers. The investigator should Label and document all materials they collect with the details such as date of collection, collector, voucher number, etc with the permanent ink markers.

1.6 Target organ of DNA, RNA viruses, bacteria and fungus infecting shrimp

DNA Viruses	Abbreviation	Target organ	Genome
White spot syndrome virus	WSSV	Post larvae, Pleopod, gill, hypodermis , hemocytes	dsDNA
Infectious hypodermal haeamtopoietic necrosis virus	IHHNV	Post larvae, Pleopod, gill, hypodermis,haematopoietic tissues, lymphoid organ	ssDNA
Decapod iridescent virus 1	DIV 1	Post larvae, Hepatopancreas, stomach, gut	dsDNA
RNA Viruses	**Abbreviation**	**Target organ**	**Genome**
Yellow head virus	YHV	Post larvae, gill, gut, gonads, pleopod,hemocytes, lympoid organ	(+)ssRNA
Taura syndrome virus	TSV	Post larvae, gill, gut, striated muscle, pleopod, hypodermis, lympoid organ	(+)ssRNA
Infectious myonecrosis virus	IMNV	Post larvae, Skletal muscles, lympoid organ, hemocytes	(+)ssRNA
Bacteria	**Abbreviation**	**Target organ**	**Genome**
Acute Hepatopancreatic Necrosis Disease	AHPND	Post larvae, Hepatopancreas, gut	Plasmid
Necrotising Hepatopancreatitis bacterium	NHPB	Post larvae, Hepatopancreas, gut	dsDNA
Fungi	**Abbreviation**	**Targer organ**	**Genome**
Enterocytozoon hepatopenaei	EHP	Post larvae, Hepatopancreas	ds DNA

2 EXTRACTION OF NUCLEIC ACID FROM SHRIMP TISSUE

2.1 Introduction

The extraction of DNA, RNA, and protein, is the most crucial method used in molecular biology. These biomolecules can be isolated from any biological material for subsequent downstream processes, analytical, or preparative purposes. In the past, the process of extraction and purification of nucleic acids used to be complicated, time-consuming, labour-intensive. Currently, there are many specialized methods that can be used to extract nucleic acids, such as solution-based and column-based protocols. Manual method has certainly come a long way over time with various commercial offerings. Automated systems designed for medium-to-large laboratories have grown in demand over recent years. It is an alternative to labor-intensive manual methods. The technology should allow a high throughput of samples; the yield, purity, reproducibility, and scalability of the biomolecules as well as the speed, accuracy, and reliability of the assay should be maximal, while minimizing the risk of cross-contamination.

2.2 Components

The role various components of nucleic acid extraction protocol is as follows:

A. The extraction buffer: It includes a detergent such as SDS which disrupts the cell membranes, a chelating agent such as EDTA which chelates the magnesium ions required for DNase activity, a buffer which is almost always Tris at pH 8 and a salt such as sodium chloride which aids in precipitation by neutralizing the negative charges on the DNA so that the molecules can come together.

B. Precipitation of nucleic acids: Alcohol precipitation is the most commonly used method for nucleic acid precipitation. This requires diluting the nucleic acid with a monovalent salt, adding alcohol to it and mixing gently. The nucleic acid precipitated spontaneously. Ethanol (twice the volume) or isopropanol (two thirds volume) are the standard alcohols used for nucleic acid precipitation.

C. Elution of Nucleic acid: The nucleic acid pellet can be resuspended in either sterile distilled water or TE (10 mM Tris:1mM EDTA)

2.3 Protocol for Isolation and Purification of DNA using Silica Membrane Spin Column

Lysis: Transfer 0.2ml the lysis buffer in to a fresh micro centrifuge tube and take 20 to 100mg tissue sample and homogenize. Make up to 0.5ml with lysis buffer.

Incubate the sample at 95°C for 10 minutes.

Centrifuge the sample at 8,000 rpm for 5 min.

Take out 250µl of clear supernatant without disturbing the pellet in to a fresh tube and add 350µl neutralisation or binding buffer.

Centrifuge the sample at 8,000 rpm for 5 min.

Take out 350µl clear supernatant and add 350µl 100% isopropanol and mix thoroughly.

Apply the supernatant to the column and centrifuge at 8000 rpm for 1 min.

Wash the spin column with 700µl wash buffer 1.

Wash the spin column with 700µl wash buffer 2.

Discard the flow through and spin the column at 8000 rpm for 2 mins to dry the membrane completely.

Elute the DNA with 50µl water or TE buffer and quantity the DNA using Nano spectrophotometer.

2.4 Buffer Recipe

2.4.1 Tissue Lysis Buffer

Tris base (pH 8 – 8.5)	-	100mM
Sodium chloride	-	200mM
Sodium dodecyl sulfate	-	0.2%
EDTA	-	5mM

It can be stored at 4° C for up to 12 months.

2.4.2 Neutralisation buffer or Binding buffer

5M guanidine hydrochloride	(477.65 gms)
0.5M Potassium acetate	(49.09 gms)

Dissolved the content in 500 ml distilled water. Adjust the pH of the solution to more or less 4.2 with acetic acid and make up the solution to 1000ml with distilled water and filter sterilise and store indefinitely at 4°C.

2.4.3 Wash Buffer 1

Tris base (pH7.5)	-	2mM
Sodium chloride	-	20mM
EDTA	-	0.1mM

In 90 % ethanol

2.4.4 Wash Buffer 2

Tris base (pH7.5)	-	2mM
Sodium chloride	-	20mM

In 70 % ethanol

2.4.5 Elution Buffer

Tris base (pH 8)	-	10mM
EDTA	-	1mM

2.5 Protocol for Isolation and purification of RNA using Silica Membrane Spin Column

Lysis: Transfer 0.2ml the TRIZOL reagent in to a fresh micro centrifuge tube and take 20 to 100mg tissue sample and homogenize. Make up to 0.5ml with TRIZOL reagent.

Incubate the homogenized sample for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes.

Add 0.2 ml of chloroform to the supernatant. Vortex samples vigorously for 15 seconds and incubate them at room temperature for 2 to 3 minutes.

Centrifuge the samples at 12,000 x g for 15 minutes at 2 to 4°C.

Take out 250µl of clear supernatant without disturbing the interphase in to a fresh tube and add 350µl neutralisation or binding buffer.

Centrifuge the sample at 8,000 rpm for 5 min.

Take out 350µl clear supernatant and add 350µl 100% isopropanol and mix thoroughly.

Apply the supernatant to the column and centrifuge at 8000 rpm for 1 min.

Wash the spin column with 700µl wash buffer 1.

Discard the flow through and spin the column at 8000 rpm for 2 mins to dry the membrane completely.

Discard the flow through and spin the column at 8000 rpm for 2 mins to dry the membrane completely.

Elute the RNA with 50µl water or TE buffer and quantity the RNA using Nano spectrophotometer.

2.6 Nucleic Acid Quantification

S. No.	Sample Code	DNA/RNA Code	Quantity in ng / µl	A260/280
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				

3 cDNA SYNTHESIS

3.1 Introduction

This procedure summarizes the reagents, materials and method for the conversion of RNA in to complementary DNA for real time PCR.

3.2 Contents of the Kit

- 5x iScript reaction mix 100 µl
- Nuclease-free water 1.5 ml
- iScript reverse transcriptase 25 µl

3.3 Storage of Kit Components

- 5x iScript reaction mix – store at -20°C
- iScript reverse transcriptase-– store at -20°C.
- Nuclease-free water - can be stored at room temperature.

3.4 Reaction Setup

Reagents	Vol. x1 rxn in µl	___X in µl
5x iScript reaction mix	2.0	
iScript reverse transcriptase	0.5	
Nuclease-free water	5.5	
RNA Template (100 fg to 1 µg total RNA)	2.0	
Total Volume	10.0	

3.5 Protocol

- A Master Mix is prepared in a tube by combining a 5x iScript reaction mix, iScript reverse transcriptase with water.
- After mixing thoroughly 8µl volumes of the Master Mix are quickly dispensed into the PCR tubes.
- Add 2µl of RNA extracted from test samples.
- Mix the contents of tube gently and pulse spin tubes at 3000rpm.
- Keep the tubes in thermal cycler.
- PCR program is 25°C for 5 mins, 42°C for 30 mins, 85°C for 5 mins and hold at 4°C.
- cDNA can be stored at . -20°C for further use.

4. WHITE SPOT SYNDROME VIRUS (WSSV) TAQMAN ASSAY

4.1 Objective

The aim of this procedure is to provide guidance to detect the pathogenic agent White spot syndrome virus (WSSV) in aquatic animals (Crustacean, Polychaetes, Faecal, Mollusc, Algae, Copepod, Artemia and Artemia Products, Post Larvae, Live/Frozen Shrimp and Crab, Feed and Feed additives for Aquaculture) using Real**-Time PCR (RT-PCR)**

4.2 Definitions

A. Cycle threshold (Ct) – The cycle number at which the fluorescence passes a determined threshold

B. Positive control (PC) – The PC typically consists of the plasmid construct with respective target sequence. The successful performance of this control (Ct value falls within pre-defined ranges) set indicates that the PCR reaction was properly performed and all components of master mix are working properly

C. No template control (NTC) – The successful performance of this control (Ct value undetermined or zero) indicates that contamination did not occur during the PCR master mix setup protocol. Nuclease-free water serves as the NTC, as the NTC does not contain template

D. Method control (MC)- Successful performance of this control (Ct value undetermined or zero) indicates that contamination did not occur during the PCR template and positive control addition protocol. Nuclease-free water serves as the NTC, as it does not contain template

4.3 Materials Required

- PCR stripes or plates
- TaqMan probe master mix
- Primers and probe
- Sterile water

4.4 Equipment Required

- Micro Pipette (2.5µl, 10µl, 20µl, 100µl, 200µl)
- Real-Time –PCR thermal cycler

4.5 Storage of Kit Components

- Primer Probe mix – store -20°C and protect from light
- Mastermix – store at -20°C for long term storage. Avoid repeated freeze thawing may reduce the sensitivity of the assay.
- Lysis buffer can be stored at room temperature

4.6 Primer

Pathogen/ Target gene	Primer Name	Primer/probe (5'–3')
WSSV	WSS1011F	5'-TGG-TCC-CGT-CCT-CAT-CTC-AG-3'
	WSS1079R	5'-GCT-GCC-TTG-CCG-GAA-ATT-A-3'
	WSSV Probe	6FAM- 5'-AGC-CAT-GAA-GAA-TGC-CGT-CTA-TCA-CAC-A-3'- TAMRA
House keeping gene	EF94 F	5'-CAAGATCTGTAAGCTCTCGGT-3'
	EF94 R	5'-CTTGCCAGAGTCTACGTG-3'
	EF94 Probe	6 HEX 5'- TGGATCTTCTCCTTGCCCATGGTTG -BHQ- 3'

4.7 Samples

- Specimens for testing for infection with WSSV are aquatic animals (Crustacean, Polychaetes, Faecal, Mollusc, Algae, Copepod, Artemia and Artemia Products, Post Larvae, Live/Frozen Shrimp and Crab, Feed and Feed additives for Aquaculture)
- Samples such as pleopods, gills, haemolymph, stomach and abdominal muscle of crustacean are recommended for submission
- Haemolymph, faeces or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary

4.8 Procedure

4.8.1 PCR reaction

- Wear disposable powder free gloves and at all times when handling plates / strips/tubes. Avoid contaminating plates with tissue lint etc. Determine the number of reaction (N) to set up per assay in addition include positive, negative and method control in the test

- Prepare one excess reaction cocktail for every 10 reactions to account for pipetting error, if no. of samples (n)including controls =1 to 10, then N = n + 1

- Prepare the master mix in PCR work station in the reagent preparation room by following the table below:

Reagents	Volume 1x Reaction (10µl)
Sterile water	1.4µl
2X Taq Master mix	5.0 µl
WSSV Primer probe mix	0.8 µl
EF 94 primer probe mix	0.8 µl
DNA Template (100 to 200ng)	1.0 µl
Total	10.0 µl

- A master mix is prepared in a tube by combining a 2X master mix reagent and primer and probe mix with water (protect from light)

- After mixing thoroughly 9 µl volumes of the master mix are quickly dispensed into the plate / strips/ tubes

- Before moving to template addition PCR work station, Add 2 µl of the sterile water into NTC (negative control) tube

- In the template addition UV cabinet add 1 µl of sterile water to method control tube. Add

1 µl of each DNA sample to respective well as per plate/strips/tubes set up

- Finally add 1 µl of positive plasmid template into positive control tubes in positive control UV cabinet

- Centrifuge the tubes for 10 sec to remove bubbles trapped in the reaction tubes

- Amplification is performed with the Real time PCR thermal cycler. The cycling profile is as follows

Cycling steps	Temperature	Time	No. of cycles
Step 1	95° C	30 sec	1
Step 2	95° C	5 sec	40
	60° C	31 sec	

4.9 Data Analysis

After completion of the amplification reaction, amplification plots must be critically assessed. The baseline and the threshold can be set either automatically or manually. The threshold should be placed above the background fluorescence noise, across the exponential phase of all the amplification curves. The PTC with a known target concentration can act as a calibrator to enable standardization of data analysis and an approximate estimation of the viral load.

The test reliability is assured by analyzing the results of the controls. If the controls did not yield the expected results the causative reason must be investigated and corrective must be taken,

Controls	Expected results	Corrective action to be taken
Positive control	Positive, There must be an increase in fluorescence from the FAM fluorophore yielding a sigmoidal amplification curve at the expected Ct value	Repeat the test from the nucleic acids extraction and check the positive control stock
Negative control	Negative, i.e. absence of fluorescence increase from the FAM fluorophore, with no sigmoidal amplification curve	Repeat the test from the nucleic acids extraction and check all the nucleic acid extraction reagents and disinfect the nucleic acid extraction, template addition chambers.
Method control	Negative, i.e. absence of fluorescence increase from the FAM fluorophore, with no sigmoidal amplification curve	Repeat the test from the nucleic acids extraction, check or change all the PCR reagents.

4.10 Interpretation of Results

The following criteria must be followed for data interpretation of diagnostic samples.

Results	Interpretation
Increase in fluorescence from the FAM fluorophore yielding an amplification curve with Ct ≤ 37th cycle.	Positive
Absence of fluorescence increase from the FAM fluorophore, with no sigmoidal amplification curve	Negative
Weak increase in fluorescence from the FAM Fluorophore yielding a sigmoidal amplification curve with Ct between 38th and 40th cycle.	Inconclusive, repeat the test from the nucleic acids extraction

4.11 Plate Display

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

4.12 Mastermix Preparation

Reagents	Volume 1x Reaction (10µl)	Volume __x Reaction (10µl)
Sterile water	1.4µl	
2X Taq Master mix	5.0 µl	
WSSV Primer probe mix	0.8 µl	
EF 94 primer probe mix	0.8 µl	
DNA Template (100 to 200ng)	1.0 µl	
Total	10.0 µl	

5. INFECTIOUS MYONECROSIS VIRUS (IMNV) TAQMAN ASSAY

5.1 Objective

The objective of this document is to detect **infectious myonecrosis caused by Infectious Myonecrosis Virus (IMNV)** in aquatic animals (Crustacean, Polychaetes, Faecal, Mollusc, Algae, Copepod, Artemia and Artemia Products, Post Larvae, Live/Frozen Shrimp and Crab, Feed and Feed additives for Aquaculture) using **Real-Time PCR (RT-PCR).**

5.2 Definitions

A. Cycle threshold (Ct) – The cycle number at which the fluorescence passes a determined threshold

B. Positive control (PC) – The PC typically consists of the plasmid construct with respective target sequence. The successful performance of this control (Ct value falls within pre-defined ranges) set indicates that the PCR reaction was properly performed and all components of master mix are working properly

C. No template control (NTC) – The successful performance of this control (Ct value undetermined or zero) indicates that contamination did not occur during the PCR master mix setup protocol. Nuclease-free water serves as the NTC, as the NTC does not contain template

D. Method control (MC)- Successful performance of this control (Ct value undetermined or zero) indicates that contamination did not occur during the PCR template and positive control addition protocol. Nuclease-free water serves as the NTC, as it does not contain template

5.3 Materials Required

- PCR stripes or plates
- TaqMan probe master mix
- Primers and probe
- Sterile water

5.4 Storage of Kit Components

- Primer Probe mix – store -20°C and protect from light
- Mastermix – store at -20°C for long term storage. Avoid repeated freeze thawing may reduce the sensitivity of the assay.
- Trizol reagent – store at 4°C.

5.5 Equipment Required

- Micro Pipette (2.5µl, 10µl, 20µl, 100µl, 200µl)
- Real-Time –PCR thermal cycler

5.6 Primer

Pathogen/ Target gene	Primer Name	Primer/probe (5'–3')
IMNV Capsid protein gene	IMNV 412F	GGA-CCT-ATC-ATA-CAT-AGC-GTT-GCA
	IMNV 545R	AAC-CCA-TAT-CTA-TTG-TCG-CTG-GAT
	IMNV Probe	6FAM- CCA-CCT-TTA-CTT-TCA-ATA-CTA-CAT-CAT-CCC-CGG – BHQ 1
Housekeeping gene	EF94 F	5'-CAAGATCTGTAAGCTCTCGGT-3'
	EF94 R	5'-CTTGCCAGAGTCTACGTG-3'
	EF94 Probe	6 HEX 5'- TGGATCTTCTCCTTGCCCATGGTTG -BHQ- 3'

5.7 Samples

- Specimens for testing for infection with IMNV are aquatic animals (Crustacean, Polychaetes, Faecal, Mollusc, Algae, Copepod, Artemia and Artemia Products, Post Larvae, Live/Frozen Shrimp and Crab, Feed and Feed additives for Aquaculture)
- Samples such as pleopods, gills, haemolymph, stomach and abdominal muscle of crustacean are recommended for submission
- Haemolymph, faeces or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary

5.8 Procedure

5.8.1 RNA Extraction

- Perform RNA extraction of pathogenic samples according to RNA extraction protocol in nucleic acid extraction room.
- Total RNA is first transcribed into complementary DNA (cDNA) by reverse transcription using Bio-Rad iScript ™ cDNA synthesis kit according to manufacturer's protocol

5.8.2 PCR Reaction

- Wear disposable powder free gloves and at all times when handling plates / strips/tubes. Avoid contaminating plates with tissue lint etc. Determine the number of reaction (N) to set up per assay in addition include positive, negative and method control in the test
- Prepare one excess reaction cocktail for every 10 reactions to account for pipetting error, if no. of samples (n)including controls =1 to 10, then N = n + 1
- Prepare the master mix in PCR work station in the reagent preparation room by following the table below:

Reagents	Volume 1x Reaction (20µl)
Sterile water	1.4µl
2X Taq Master mix	5.0 µl
IMNV Primer Probe mix	0.8 µl
EF 94 Primer Probe mix	0.8 µl
cDNA Template	1.0 µl
Total	10.0 µl

- A master mix is prepared in a tube by combining a 2X master mix reagent and primer and probe mix with water (protect from light)
- After mixing thoroughly 9 µl volumes of the master mix are quickly dispensed into the plate / strips/ tubes
- Before moving to template addition PCR work station, Add 2 µl of the sterile water into NTC (negative control) tube
- In the template addition UV cabinet add 1 µl of sterile water to method control tube. Add
- 1 µl of each cDNA sample to respective well as per plate/strips/tubes set up
- Finally add 1 µl of positive plasmid template into positive control tubes in positive control UV cabinet
- Centrifuge the tubes for 10 sec to remove bubbles trapped in the reaction tubes
- Amplification is performed with the Real time PCR thermal cycler. The cycling profile is as follows

Cycling steps	Temperature	Time	No. of cycles
Step 1	95° C	30 sec	1
Step 2	95° C	5 sec	40
	60° C	31 sec	

5.9 Data Analysis

After completion of the amplification reaction, amplification plots must be critically assessed. The baseline and the threshold can be set either automatically or manually. The threshold should be placed above the background fluorescence noise, across the exponential phase of all the amplification curves. The PTC with a known target concentration can act as a calibrator to enable standardization of data analysis and an approximate estimation of the viral load.

5.9.1 Acceptance Criteria

The test reliability is assured by analyzing the results of the controls. If the controls did not yield the expected results the causative reason must be investigated and corrective must be taken,

Controls	Expected results	Corrective action to be taken
Positive control	Positive, There must be an increase in fluorescence from the FAM fluorophore yielding a sigmoidal amplification curve at the expected Ct value	Repeat the test from the nucleic acids extraction and check the positive control stock
Negative control	Negative, i.e. absence of fluorescence increase from the FAM fluorophore, with no sigmoidal amplification curve	Repeat the test from the nucleic acids extraction and check all the nucleic acid extraction reagents and disinfect the nucleic acid extraction, template addition chambers.
Method control	Negative, i.e. absence of fluorescence increase from the FAM fluorophore, with no sigmoidal amplification curve	Repeat the test from the nucleic acids extraction, check or change all the PCR reagents.

5.9.2 Interpretation of Results

The following criteria must be followed for data interpretation of diagnostic samples.

Results	Interpretation
Increase in fluorescence from the FAM fluorophore yielding an amplification curve with Ct ≤ 37th cycle.	Positive
Absence of fluorescence increase from the FAM fluorophore, with no sigmoidal amplification curve	Negative
Weak increase in fluorescence from the FAM Fluorophore yielding a sigmoidal amplification curve with Ct between 38th and 40th cycle.	Inconclusive, repeat the test from the nucleic acids extraction

6. WHITE SPOT SYNDROME VIRUS (WSSV) PCR ASSAY

6.1 Objective

The aim of this procedure is to provide guidance to detect the pathogenic agent White spot syndrome virus (WSSV) in aquatic animals (Crustacean, Polychaetes, Faecal, Mollusc, Algae, Copepod, Artemia and Artemia Products, Post Larvae, Live/Frozen Shrimp and Crab, Feed and Feed additives for Aquaculture) using PCR. https://www.woah.org/fileadmin/Home/eng/Health_standards/ aahm/current/chapitre_wsd.pdf

6.2 Definitions

A. Positive control (PC) – The PC typically consists of the plasmid construct with respective target sequence. The successful performance of this control (Ct value falls within pre-defined ranges) set indicates that the PCR reaction was properly performed and all components of master mix are working properly

B. No template control (NTC) – The successful performance of this control (Ct value undetermined or zero) indicates that contamination did not occur during the PCR master mix setup protocol. Nuclease-free water serves as the NTC, as the NTC does not contain template

C. Method control (MC)- Successful performance of this control (Ct value undetermined or zero) indicates that contamination did not occur during the PCR template and positive control addition protocol. Nuclease-free water serves as the NTC, as it does not contain template

6.3 Equipment / Instruments

- Autoclave
- Micropipettes (2.5, 10, 100, 200, 1000 µL)
- Spectrophotometer
- Refrigerated centrifuge
- Thermal cycler
- Gel electrophoresis system
- Gel doc or UV transilluminator with desk top PC

6.4 Reagents

- 2x PCR master Mix (Taq DNA Reaction Mix)
- primers
- WSSV positive control
- Ladder - 100 bp
- Agarose
- 6x gel loading dye
- Green R DNA Gel stain
- 1x TAE buffer (0.4M Tris-acetate, 1 mM EDTA)

6.5 Storage of Kit Components

- 2x PCR master Mix, Primers & DNA ladder – store -20°C
- Positive control – store at -20°C in a separate refrigerator.

6.7 Primers

Oligonulceotide	Sequence (5'- 3')	Amplicon size	Reference
WSSV 1F	ACT ACT AAC TTC AGC CTA TCT AG	1447 bp	WOAH, 2023
WSSV 1R	TAA TGC GGG TGT AAT GTT CTT ACG A		
WSSV 2F	GTA ACT GCC CCT TCC ATC TCC A	941 bp	
WSSV 2R	TAC GGC AGC TGC TGC ACC TTG T		

6.8 Samples

- Specimens for testing for infection with WSSV are aquatic animals (Crustacean, Faecal, Post Larvae, Live/Frozen Shrimp and Crab, Feed and Feed additives for Aquaculture)
- Samples such as pleopods, gills, haemolymph, stomach and abdominal muscle of crustacean are recommended for submission
- Haemolymph, faeces or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary

6.9 Procedure

6.9.1 Isolation of DNA

- Nucleic acid extraction performed according to the DNA Extraction in the nucleic acid extraction room.

6.9.2 PCR Reaction

- Perform PCR reaction with the given primers for WSSV gene Determine the number of reactions (N) to set up per assay In addition include Negative control and Positive control in the test
- Prepare excess reaction cocktail to account for pipetting error If number of samples (n) including controls = 1 to 10, then N=n+1
- In the clean reagent preparation room prepare the master mix: Prepare the reaction mix with the following amount of each reagent and the primer set

Component	Volume (µl)
Nuclease free water	8.5
Taq DNA reaction mix	12.5
Forward primer	1.0
Reverse primer	1.0
Template DNA	2.0
Total	25.0

- Mix reaction mixtures by pipetting up and down Do not vortex
- Centrifuge for 5-6 seconds to collect contents at bottom of the tube, and then place the tube in cold rack
- Set up reaction tubes in PCR rack
- Dispense 23 µl of each master mix into each PCR tubes
- Before moving to nucleic acid handling area Add 2 µl of the nuclease free water in to NTC (No template control) tubes Cap NTC tubes

- In the Pre-PCR room, add 2 µl of each sample to respective tubes as per the set up
- Cap the PCR tubes to which the samples have been added
- Finally, pipette 2 µl of positive plasmid template control into positive template control (PTC) tubes in positive control addition area Cap PTC tubes Centrifuge the tubes for 10 seconds Make sure that bubbles are eliminated from the bottom of the reaction tubes
- The reaction volume is 25 µl Program schedule as follows:

Cycling steps	Temperature	Time	No. of cycles
Step 1	94° C	4 min	1
Step 3	94° C	1 min	35
	55° C	1 min	
	72° C	2 min	
Step 3	72° C	5 min	1
Step 4	4° C	Infinite hold	

- Prepare nested reaction mix as per I step with II step primer(WSSV 2F and WSSV 2R)
- Add 2 µl of the product from I step reaction as template for the nested reaction. Add each sample to respective wells as per the set up in I step Centrifuge the tubes for 10 seconds. Make sure that bubbles are eliminated from the bottom of the reaction tubes
- After completion of the run, the PCR tubes and strips can be preserved in 4°C for further analysis
- Use the same PCR amplification protocol as above for the II step also
- After completion PCR run, analyse the amplified product by agarose gel electrophoresis in Post - PCR room
- After completion of PCR, either it can be preserved in 4°C for further analysis

6.10 Data Analysis

The amplified product should be resolved through an agarose gel electrophoresis with appropriate DNA ladder to find out the exact size of the amplified PCR amplicon in post-PCR room.

The test reliability is assured by analyzing the results of the controls. If the controls did not yield the expected results the causative reason must be investigated and corrective must be taken,

Controls	Expected results	Corrective action to be taken
Positive control	Positive, there must be an amplicon of 941bp length.	Repeat the test from the nucleic acids extraction and check the positive control stock
Negative control	Negative, there should be any amplification	Repeat the test from the nucleic acids extraction and check all the nucleic acid extraction reagents and disinfect the nucleic acid extraction, template addition chambers.
Method control	Negative, there should be any amplification	Repeat the test from the nucleic acids extraction, check or change all the PCR reagents.

6.10.1 Interpretation of Results

The following criteria must be followed for data interpretation of diagnostic samples.

Results	Interpretation
Clear DNA band of 941bp length.	Positive
Absence of any clear band at around 941bp length.	Negative
Weak DNA band at the range of around 941bp length	Inconclusive, repeat the test from the nucleic acids extraction

7. INFECTIOUS MYONECROSIS VIRUS (IMNV)

7.1 Objective

The aim of this document is to detect Infectious Myonecrosis Virus in aquatic animals (Crustacean, Polychaetes, Faecal, Mollusc, Algae, Copepod, Artemia and Artemia Products, Post Larvae, Live/Frozen Shrimp and Crab, Feed and Feed additives for Aquaculture) using **PCR.**

7.2 Definitions

A. Positive control (PC) – The PC typically consists of the plasmid construct with respective target sequence. The successful performance of this control (Ct value falls within pre-defined ranges) set indicates that the PCR reaction was properly performed and all components of master mix are working properly

B. No template control (NTC) – The successful performance of this control (Ct value undetermined or zero) indicates that contamination did not occur during the PCR master mix setup protocol. Nuclease-free water serves as the NTC, as the NTC does not contain template

C. Method control (MC)- Successful performance of this control (Ct value undetermined or zero) indicates that contamination did not occur during the PCR template and positive control addition protocol. Nuclease-free water serves as the NTC, as it does not contain template

7.3 Equipment / Instruments

- Autoclave
- Micropipettes (2.5, 10, 100, 200, 1000 µL)
- Spectrophotometer
- Refrigerated centrifuge
- Thermal cycler
- Gel electrophoresis system
- Gel doc or UV transilluminator with desk top PC

7.4 Reagents

- 2x PCR master Mix (Taq DNA Reaction Mix)
- primers
- IMNV positive control
- Ladder - 100 bp
- Agarose
- 6x gel loading dye
- Green R DNA Gel stain
- 1x TAE buffer (04M Tris-acetate, 1 mM EDTA)

7.5 Storage of Kit Components

- 2x PCR master Mix, Primers & DNA ladder – store -20°C
- Positive control – store at -20°C in a separate refrigerator.

7.6 Primers

Oligo nuclotide	Sequence (5'- 3')	Amplicon size	Reference
IMNV 1F	CGA CGC TGC TAA CCA TAC AA	328 bp	WOAH, 2023
IMNV 1R	ACT CGG CTG TTC GAT CAA GT		
IMNV 2F	GGC ACA TGC TCA GAG ACA	139 bp	
IMNV 2R	AGC GCT GAG TCC AGT CTT G		

7.7 Samples

- Specimens for testing for infection with IHHNV are aquatic animals (Crustacean, Polychaetes, Faecal, Mollusc, Algae, Copepod, Artemia and Artemia Products, Post Larvae, Live/Frozen Shrimp and Crab, Feed and Feed additives for Aquaculture)
- Samples such as pleopods, gills, haemolymph, stomach and abdominal muscle of crustacean are recommended for submission
- Haemolymph, faeces or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary

7.8 Procedure

7.8.1 RNA extraction

- Perform RNA extraction of pathogenic samples according to RNA extraction protocol in document SOP (NRLBAAD/SOP/22) in nucleic acid extraction room.
- Total RNA is first transcribed into complementary DNA (cDNA) by reverse transcription using cDNA Synthesis Kit according to manufacturer's instructions.

7.8.2 PCR Reaction

- Total RNA is first transcribed into complementary DNA (cDNA) by reverse transcription using Bio-Rad iScript ™ cDNA synthesis kit according to manufacturer's protocol
- Perform PCR reaction with the given primers for IMNV gene Determine the number of reactions (N) to set up per assay In addition include Negative control and Positive control in the test
- Prepare excess reaction cocktail to account for pipetting error If number of samples (n) including controls = 1 to 10, then N=n+1
- In the clean reagent preparation room prepare the master mix: Prepare the reaction mix with the following amount of each reagent and the primer set

Component	Volume (µl)
Nuclease free water	8.5
Taq DNA reaction mix	12.5
Forward primer	1.0
Reverse primer	1.0
Template cDNA	2.0
Total	25.0

- Mix reaction mixtures by pipetting up and down. Do not vortex
- Centrifuge briefly to collect contents at bottom of the tube, and then place the tube in cold rack
- Set up reaction tubes in PCR rack
- Dispense 23 µl of each master mix into each PCR tubes

- Before moving to nucleic acid handling area Add 2 µl of the nuclease free water in to NTC (No template control) tubes and Cap NTC tubes
- In the Pre PCR room, add 2 µl of each sample to respective tubes as per the set up
- Cap the PCR tubes to which the sample has been added
- Finally, pipette 2µl of positive plasmid template control into positive template control (PTC) tubes in positive control addition area Cap PTC wells Centrifuge the tubes for 10 seconds Make sure that bubbles are eliminated from the bottom of the reaction tubes
- The reaction volume is 25µl The cycling conditions as follows:

Cycling steps	Temperature	Time	No. of cycles	Amplicon Length
Step 1	60° C	60 min	1	379 bp
	95° C	2 min		
Step 3	95° C	45 sec	35	
	60° C	45 sec		
Step 3	60° C	7 min	1	
Step 4	4° C	Infinite hold		

- Prepare nested reaction mix as per I step with II step primer
- Add 2µl of I step amplicon as template for the nested reaction. Add each sample to respective wells as per the set up in I step. Centrifuge the tubes briefly. Make sure that bubbles are eliminated from the bottom of the reaction tubes
- After completion of the run, the PCR tubes and strips can be preserved in 4° C for further analysis
- Nested PCR thermal cycling conditions:

Cycling steps	Temperature	Time	No of cycles	Amplicon Length
Step 1	95° C	2 min	1	139 bp
Step 2	95° C	30 sec	35	
	65° C	30 sec		
	72° C	30 sec		
Step 3	72° C	2 min	1	
Step 4	4° C	Infinite hold		

- After completion of PCR, either it can be preserved in 4°C for further analysis

7.9 Data Analysis

The amplified product should be resolved through an agarose gel electrophoresis with appropriate DNA ladder to find out the exact size of the amplified PCR amplicon in post-PCR room

7.9.1 Acceptance Criteria

The test reliability is assured by analyzing the results of the controls. If the controls did not yield the expected results the causative reason must be investigated and corrective must be taken,

Controls	Expected results	Corrective action to be taken
Positive control	Positive, there must be an amplicon of 139 bp length.	Repeat the test from the nucleic acids extraction and check the positive control stock
Negative control	Negative, there should be any amplification	Repeat the test from the nucleic acids extraction and check all the nucleic acid extraction reagents and disinfect the nucleic acid extraction, template addition chambers.
Method control	Negative, there should be any amplification	Repeat the test from the nucleic acids extraction, check or change all the PCR reagents.

7.9.2 Interpretation of results

The following criteria must be followed for data interpretation of diagnostic samples.

Results	Interpretation
Clear DNA band of 139bp length.	Positive
Absence of any clear band at around 139bp length.	Negative
Weak DNA band at the range of around 139bp length	Inconclusive, repeat the test from the nucleic acids extraction

8. FRACTIONATION OF NUCLEIC ACID BY GEL ELECTROPHORESIS

8.1 Agarose Electrophoresis

Agarose gel electrophoresis is a method of gel electrophoresis used in molecular biology to separate nucleic acids based on their size and charge. These gels are easy to cast and are widely used in laboratories. An agarose is a polysaccharide polymer material, generally extracted from seaweed. It is a linear polymer made up of the repeating unit of agarobiose, which is a disaccharide made up of D-galactose and 3,6-anhydro-L-galactopyranose. The melting temperature of agarose is 85-95 °C and gelling temperature of 35-42 °C. The nucleic acids have a net negative charge due to its phosphate back bone so they migrate towards the positive electrode in an electric field. The migration of nucleic acids affected by several factors like pore size of the gel, size of DNA being electrophoresed, the voltage used the ionic strength of the buffer, and the concentration of intercalating dye such as ethidium bromide.

8.2 Agarose Gel Preparations

8.2.1 To prepare a gel required quantities of agarose weighed and add in to the wide mouth glass conical flask with 1x TAE buffer and melt the mixture in the microwave oven, until it becomes clear without any gel particle.

8.2.2 Cool down the clear agarose gel under room temperature and add 1 µl ethidium bromide (10mg/ml) and slowly pour the gel into the gel mould. The volume of the gel varies from the size of the gel mould. The height of agarose gel only has to go above the bottom of the gel comb for about 0.3~0.5 cm, and thickness is suggested to be no less than 0.8 cm.

8.2.3 When agarose gel is completely solidified. Carefully remove blockers at both sides of the gel mould and place it in the gel tank containing 1x TAE buffer. After few minutes the comb will loosen up in the gel and can be carefully removed without damaging the wells. This agarose gel is ready for electrophoresis.

8.3 Electrophoresis

8.3.1 Add 1X TAE buffer over the gel box until the buffer level submerge the gel.

8.3.2 Load 5 µl each of the "PCR product-loading dye mixture" into each well. The mixture will sink to the bottom of the wells because its density is higher than buffer. This step should be carefully handled in order to avoid cross contamination between the adjacent wells.

8.3.3 5 ul of DNA marker is loaded at the extreme end of the gel. The DNA molecular weight marker is served as reference to predict the size of the PCR product.

8.3.4 After loading of all the samples, the gel was electrophoresed at constant voltage between 70V~100V.

8.3.5 The loading dye in the kit contains 2 colorants: Bromphenol Blue gives deep blue color; Xylene Cyanol gives light blue color. When the dark blue dye approaches 1/2 to 2/3 of the gel, stop the electrophoresis. Then, remove the gel from the gel box to proceed with the EtBr staining procedures.

8.3.6 To avoid contamination, DO NOT re-use the gel electrophoresis buffer unless several gels will be used in the same day. When the electrophoresis is finished, wash the gel box with plenty of water.

8.4 Staining and Visualization

The ethidium bromide intercalates into the major grooves of the DNA and fluoresces under UV light. So the gel can be viewed under trans-illuminator (254nm) to observe DNA bands. The exposure of DNA to UV radiation for as little as 45 seconds can produce damage to DNA and affect subsequent procedures such as cloning, *in vitro* transcription, and PCR. The exposure of the DNA to UV radiation therefore should be limited. The use of a higher wavelength of 365 nm UV light causes lesser damage to the DNA. The trans-illuminator apparatus fitted with image capture devices, such as a digital or polaroid camera allow an image of the gel to be stored in a computer or printed.

NOTES

Brackishwater Aquaculture for Food, Employment and Prosperity

National Referral Laboratory for Brackishwater Aquatic Animal Diseases
NABL Accreditated Laboratory
Aquatic Animal Health and Environment Division
ICAR – Central Institute of Brackishwater Aquaculture
(ISO 9001:2015 certified)
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