Training Manual on

DISEASE DIAGNOSTIC METHODS IN SHRIMP AQUACULTURE – WITH A SPECIAL REFERENCE TO SHRIMP EARLY MORTALITY SYNDROME (EMS)

Edited by

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FORWARD

Starting from the beginning and till late 90's, tiger shrimp, Penaeusmonodon was widely cultured in India and was popular among the shrimp farmers. However, the industry was then totally shaken by the outbreak of disease, particularly by that of white spot syndrome virus (WSSV). Subsequently, Pacific white shrimp, Litopenaeusvannamei culture started gaining popularity. At present this is well accepted by the farmers as an alternate species to tiger shrimp, Penaeusmonodon. Pacific white shrimp has several added advantages such as wide salinity tolerance, higher growth rate and suitable for high density culture system. This has become added advantage for the farmers to their specific preference for vannamei as an alternate species.

A successful shrimp culture practice is always hampered by the disease prevalence. The loss created by some of the shrimp viruses such as WSSV, Yellow head virus (YHV), Taura syndrome virus (TSV) and Infectious Myonecrosis virus (IMNV) has well been felt by the farmers throughout the world. Similarly, the recent outbreak of the emerging disease, EMS/AHPND has created havoc in a way nothing less than that any of the above viruses. Considering this, the adoption of better management practice to create a suitable environment for disease prevention is well felt. Even after taking all the necessary precautions, sometimes it becomes difficult to avoid the outbreak of diseases. Therefore, it is also absolutely essential to take extra precautionary measures for the disease detection in shrimp culture practice.

I am very happy to know that the Aquatic Animal Health and Environmental Division is conducting a training programme on "Disease diagnostic methods in shrimp aquaculture – with a special reference to shrimp Early Mortality Syndrome (EMS)''during 19th May to 8th June, 2014 and planning to bring out a manual on this occasion. Considering the importance of shrimp aquaculture practice, this training is very much essential and I am quite hopeful that it will provide immense benefit to the participant. My best wishes to the participants and AAHED for conducting this training programme.

(C. Gopal)
Director

PREFACE

Shrimp culture has evolved from its traditional form and come a long way to its present developed form. Whether it is the earlier cultured tiger shrimp or the presently adopted Pacific white shrimp, the industry has gained popularity for the high earned export value. Many a times the industry is struck by disease problems and suffers severe loss. Similarly, the exotic status of vannamei makes it more vulnerable for the introduction of several exotic pathogens. Therefore, disease diagnosis has become an important aspect for a successful shrimp culture practice. Timely and accurate disease diagnosis can provide valuable information to take necessary steps in adopting control or preventive measures.

Aquatic Animal Health section of AAHED has been actively engaged in disease diagnosis research. The section has well developed facilities and trained personnel to provide necessary training for the people who want this.

This particular training programme has been planned for a period of 15 days starting from 19thMay to 6th of June and contained both theory and practical classes.The necessary materials have been compiled to prepare this manual. The theory and practical chapters included in this manual have been specifically designed based on the need of the candidate.

I am very much grateful to the former director Dr. A.G.Ponniah, for initiating this programme. I am also grateful to the present director, Dr. C. Gopal for his encouragement and support. My sincere thanks to Dr. S.K. Otta, Senior Scientist, Dr. P. Ezhil Praveena, Scientist, Dr. T. Bhuvaneswari, Scientist and Mr. Jagan Mohan Raj, Technical Officer of AAHED for smoothly conducting the theory and practical classes and preparing the material for this manual.

I am sure that this manual will benefit the candidate in continuing his effort for shrimp disease diagnosis. My best wishes to the candidate.

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CONTENT

SHRIMP DISEASES AND THEIR DIAGNOSTICS

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Introduction

Shrimp aquaculture has gained immense popularity within a very short span of time. The already existing traditional culture practices were further modified by various innovative scientific methods to make it more productive. The high export value of this commodity and thereby more profitability within a short time period has attracted a large chunk of entrepreneurs throughout the world to jump into this novel practice. Now it has been possible to establish genetically improved stocks for some species and thereby avoid the traditional practice of wild captured broodstock use for seed production. This way, it has been possible bring significant reduction in biosecurity risks. This kind of species diversification has made the industry move at a rapid pace and makes all its effort to meet the growing demand of rapidly increasing population. At present, with the depletion of natural stock, aquaculture seems to remain the sole hope to meet the need of the people. It is perhaps this reason for which production from aquaculture accounts a major share in the total shrimp production.

Invariably, disease often comes as a major hurdle on the way to success of many aquaculture practices and shrimp farming has not been an exception. Loss due to disease has been enormous and the same is witnessed throughout the world. This has been primarily because of the high stocking density, poor management practices, unethical approach about the discharge water protocol and inadequate biosecurity practices. Bad quality seeds lead to under developed immunity of shrimp and this has also been another important factor for which this animal becomes an easy target for many of the infectious agents. Presence of various kinds of stress further complicates this process. As a result of all, shrimps suffer various kinds of diseases leading to severe mortality and thereby farmers suffer huge loss.

All kinds of infectious agents have been found to be associated with shrimp disease. Initially, the problem was more with bacteria, fungi and parasites. Subsequently, virus came into picture and made the situation more complicated as mass mortality was reported due to several of such viruses. While comparing all kinds of microorganisms, it has been observed that about 60% of the losses that occur in shrimp industry are due to viral diseases. Due to this, the marginal losses caused by other infectious agents remain unnoticed. However, sometimes gain of external virulence factors, particularly by bacteria can also make the situation very complicated.

Viral diseases

After the first virus in shrimp being reported in 1981, new shrimp viruses are being continuously added to the list. By now more than 20 shrimp viruses have already been reported.

Countable numbers of these reported viruses have been highly virulent to shrimp and thereby have got special status by the OIE. A list of all these viruses has been listed in Table 1. As shrimp is an arthropod, some of the viruses belong to the same family as reported for many of the insect viruses. However, some also come exclusively under a new classification. While some of the shrimp viruses are host specific, multiple occurrence of several viruses in the same host has also been reported.

Table 1: List of viruses considered important for shrimps

Virus name and acronym	Disease caused by	Family	Nucleic acid type	OIE List(2012)
White spot syndrome virus (WSSV)	White spot disease	Nimaviridae	dsDNA	Yes
Infectious hypodermal and hematopoietic necrosis virus (IHHNV)	Infectious hypodermal and hematopoietic necrosis	Parvoviridae	ssDNA	Yes
Monodon baculovirus (MBV)	MBV disease	Baculoviridae	dsDNA	No
Hepatopancreatic parvovirus (HPV)	Hepatopancreatic disease	Parvoviridae	ssDNA	No
Yellow head/Gill associated/Lymphoid organ virus (YHV/GAV/LOV)	Yellow head disease	Roniviridae	ssRNA	Yes
Taura syndrome virus (TSV)	Taura syndrome	Dicistrovirida e	ssRNA	Yes
Infectious myonecrosis virus (IMNV)	Infectious myonecrosis	Totiviridae	dsRNA	Yes
Laem-Singh virus (LSNV)	Monodon Slow Growth Syndrome	Luteoviridae?	ssRNA	No
Penaeus vannamei nodavirus (PvNV)	Muscle necrosis disease	Nodaviridae	ssRNA	No

Several viral pandemics had taken place in different parts of world for which the shrimp industry was virtually shaken up. Every now and then new viruses emerge and threat to the status of this industry.

Bacterial diseases

Bacteria are the natural habitat of shrimp aquaculture environment. However, depending on the stress and immune status of the shrimp, they can bring disease and thereby considered as opportunistic pathogens. Sometimes at hatchery level, they can become the main pathogen while during culture conditions these cause secondary infection and thereby increase the severity of the problem. As has been observed during the recent times, bacteria can also become a major pathogen for cultured shrimps.

Bacterial disease can cause antennal cut, shell necrosis, reddening of body part, blisters on antenna and telson, black gill etc.

Vibriosis has been considered as the major bacterial disease in shrimp. This is caused by different Vibrio species. A number of important Vibrio species those have been responsible for shrimp disease include *Vibrio harveyi, V. prahemolyticus, V. mimicus, V. campbelli, V. alginolyticus, V. anguillarum,V. penaeicida* etc.

Various kinds of filamentous bacteria such as *Leucothrixsp* and *Thiothrixsp* are also considered as fouling organisms and settle in large number on shrimp body which leads to stress and thereby facilitate secondary infection by other microorganisms.

Fungal and parasitic diseases

Different types of fungi and parasites have been reported to cause disease and in many cases this leads to secondary infection by bacteria and other microorganisms. These infections are generally occurred due to bad water quality and a combination of high stocking density with bad pond management practice.

Some of the fungal pathogens such as *Saprolegniasp., Fusariumsp. Lagenidiumsp.*and *Sirolpidiumsp.* have been responsible to cause either larval mycosis or black gill disease in cultured shrimps. At the same time, some of the fungal species such as Microsporidia can be a parasite to shrimp and cause milk or cotton shrimp disease.

Protozoans have been reported to be major parasites to shrimp. These include Zoothamnium *sp., Epistylis sp., Acineta sp., Lagenophrys sp., Ephelota sp.* and *Apostomesp.* Due to severe gill infestation, these parasites can cause respiratory problem particularly during the early hours of the day and thereby bring mortality.

Disease diagnosis

For a successful aquaculture practice, disease diagnosis remains an important part of management practices. It is essential to have rapid, sensitive and accurate diagnosis in order to suggest proper management practices both to control and prevent the spread of diseases. A number of diagnostic methods are available for preliminary and final detection of respective pathogens in shrimp.

Field observation for clinical signs

It is essential to note various clinical symptoms such as body colour, appendage deformity, exoskeleton marks, feeding and swimming activity etc. while sampling to provide initial information for a suspected agent and effort can be made accordingly for rapid detection of that particular agent (Example: White spot on carapace and body surface – WSSV suspected, white discolouration near tail region – IMNV suspected, rostrum deformity – IHHNV suspected, Black gill – either fungus or bacteria etc.). Similarly, the detail history of the particular pond will also be helpful for proper identification.

Light microscopy

This is important for the detection of larger pathogens such as parasites and fungus. A direct microscopy of the body scrape, gill or the affected part will indicate the presence of any pathogen. Many times for emergency purpose the presence of virus can also be detected by tissue squash preparation and staining. This can then be observed under microscope for particular viral infection (Example: Hepatopancreas preparation with malachite green for detection of MBV, Gill tissue preparation with eosin and other stains for WSSV)

Electron microscopy

For electron microscopy, shrimp tissues to be fixed in a special way and processed. This is mainly done for the detection of virus particles in internal organs or even to get clear pathological changes due to other pathogens.

Histopathology

This is also a general technique for the observation of any pathological changes in the animal. Though, it will not be possible to observe directly the small size pathogens like virus, these can be predicted based on the typical structure they produce due to respective virus (Ex. Inclusion or occlusion bodies). For shrimp, particularly for hepatopancreas, propper fixation methods and processing by experienced personnel are very much essential for accurate diagnosis.

Molecular methods

These are considered as the most rapid and sensitive methods for detection of any kind of pathogens. Polymerase chain reaction (PCR) has extensively been used for detection of all the known pathogens. The PCR protocols have been modified in several ways (Example: direct and nested PCR) to make the diagnosis even more rapid and sensitive. Real time PCR can also be used for more accuracy and quantification. Based on the sequences of pathogens, probes can be designed for the detection of respective pathogens. However, some of the most commonly disadvantages of this method are false positive and false negative and therefore can misled the diagnostics.

Immunological methods

This is based on the antigen and antibody reaction and therefore very specific to a pathogen. Antibodies can be developed against antigens from the respective pathogens and the same can be used for the detection. One of the main advantages of this antibody based methods is that these can be designed to directly use at field condition (Example: Immunoblots, Immunosticks). Many times these are considered as most accurate diagnostic methods as these are protein based. Compared to molecular methods, these are considered to be less sensitive. Chances of getting false positive or negative is also possible by this method and therefore necessary precautions should be taken.

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APPLICATION OF HISTOPATHOLOGY FOR DISEASE DIAGNOSIS IN SHRIMP

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Introduction

Global shrimp culture is advancing steadily in the recent years, but is limited by various factors like diseases which play a central role. Prevention of the diseases at right time will curtail the loss to some extent. The knowledge about the commonly encountered shrimp diseases and their pathogenesis will lend a hand in preventing the disease incidence in shrimps. The commonly encountered shrimp diseases are

Bacterial Diseases

Vibriosis

Vibriosis is a bacterial disease caused by gram-negative, motile, facultative anaerobic bacteria ofthe family *Vibrionaceae*. It is pervasive throughout the world and all marine species including shrimp, are vulnerable when their natural defense mechanismsare suppressed. This disease is caused by a number of species *likeV. harveyi, V. vulnificus, V. parahaemolyticus, V. alginolyticusandV. penaeicida* and are commonly referred as black shell disease, tail rot, septic hepatopancreaticnecrosis, brown gill disease, swollen hindgut syndrome and luminous bacterial disease.

Clinical Signs

The commonly observed clinical signs are lethargy, loss of appetite, discoloured and necrotic hepatopancreas withred discolouration of the body, yellowing of the gill tissue and white patches in theabdominal muscle, melanisation, granulomatous encapsulation, necrosis andinflammation of organs (lymphoid organ, gills, heart etc.) and luminescence. Adult shrimps suffering from vibriosis may appear hypoxic, red colouration of the body with red to brown gills, reduced feed intake and seen lethargic swimming behaviourand seen at the edges and surface of ponds. *Vibrio* spp. also causes red-leg disease, characterized by red colouration of the pleopods, periopods and gills. Infected postlarvae may show symptoms like empty guts with reduced motility and phototaxis. Shrimps with lesions of bacterial shell disease the body cuticle,appendages and the gills will appear brown or black. While the postlarvae may display cloudy hepatopancreas, gills appear brown in colour. Septic hepatopancreatitis is characterized by atrophy of the hepatopancreas with multifocal necrosis and haemocytic inflammation.

Histopathology

Systemic vibriosis the changes noticed are formation of septic haemocytic nodules in the lymphoid organ, heart and connective tissues of the gills, hepatopancreas, antennal gland, nerve cord, telson and muscle. Infected hepatopancreocytes may appear poorly vacuolated, indicating low lipid and glycogen reserves. In external vibriosis the lesions observed are heavy cuticular bacterial colonization. In enteric vibriosis, the changes seen are colonization in the internal cuticle i.e. in the oral region, esophagus and stomach. Sloughing, necrosis, inflammation and melanisation are the changes observed in the hepatopancreas or midgut region.

Early Mortality Syndrome

Recently, a new emerging disease commonly referred to as ''earlymortality syndrome'' (EMS) or moretechnically known as ''acute hepatopancreaticnecrosis disease'' (AHPND) was reported from shrimp farms. It was first reported in China in 2009 and subsequently in Vietnam, Thailand,and Malaysia. It affects shrimp post larvaewithin 20–30 days after stocking andfrequently causes 100% mortality. The species which are commonly affected by this disease are *Penaeusvannamei* and *Penaeus monodon*. The causative agent hasbeen reported to be a bacteriumpresumably Vibrio parahaemolyticus.

Clinical signs

The clinical signs and mortality will be observed as early as 10 days post stocking. The affected shrimps will exhibit lesions like pale to white hepatopancreas with significant atrophy, soft shells and partially full to empty guts, Black spots or streaks within the hepatopancreas sometimes visible. Hepatopancreas (HP) does not squash easily between thumb and finger. Moribund shrimp will sink to bottom.

Histopathology

The major lesions are noticed in the hepatopancreas with acute progressive degeneration of the hepatopancreas with initial decrease of R, Band F-cells followed by a markedreduction of mitotic activity in E-cells. The development of lesion is noticed from proximaltodistal with dysfunction of R, B, F, andlastly E-cells, with affected HP tubulemucosal cells presenting prominentkaryomegaly (enlarged nuclei), and roundingand sloughing into the HP tubules. The sloughed HP cells provide a substrate for bacterial growth, resulting in massive secondary bacterial infection (putative *Vibriospp.*) and complete destruction of HP at the terminal phase of the disease. Intertubular haemocytic aggregation and haemocytes encapsulation of necrotic HP tubules and melanisation of the more proximal portions of HP tubules in some shrimp.

Necrotizing Hepatopancreatitis (NHP)

The NHP bacterium is a gram-negative, dimorphic, intracellular rickettsial like organism that occurs free within the cytoplasm of infected hepatopancreatic cells. This disease is seen in many penaeid species and mainly affects late larval stage, juvenile and adult stages of the animal. Mortality of about 90% is seen within 30 days of the outbreak of the disease.

Clinical signs

The affected shrimps are nonspecific in nature and characterized by lethargy, emaciation, soft shells, heavy fouling from external parasites, black gills, reduced growth and atrophied hepatopancreas.Infected shrimp display empty midgut with increased superficial epicommensal cuticular fouling and/or opportunistic infections (i.e. black spots) also present.Digestive gland (hepatopancreas) appears pale to white.The hepatopancreas is the target tissue for this disease. Microscopic examination of unstained tissue squashes from suspected hepatopancreas may show reduced lipid and dark melanized necrotic tubules.

Histopathology

Histologically, infected tubular epithelial cells will appear initially hypertrophied with a generalized basophilic intra-cytoplasmic granularity due to the presence of numerous pleomorphic intra-cytoplasmic rickettsial-like organisms. Three stages of infection have been described in histologic studies. In early NHP infection (stageI), intra-cytoplasmic rickettsial-like organisms can be detected free within the cytoplasm of resorptive, fibrillar B cells of scattered tubules with increasing levels of tubular epithelial cell hypertrophy or desquamation. Tubular necrosis, interstitial haemocytic infiltrates and lipid depletion occurring in stages II and III.

Viral Diseases

White Spot Disease (WSD)

It is considered as the single most serious shrimp pathogen worldwide and was first reported from farmed *Penaeusjaponicusin* Japan in 1993. White Spot Syndrome Virus (WSSV) belongs to a family of *Nimaviridaeunder* the genus *Whispoviridae*. The virions are ovoid or ellipsoid to bacilliform in shape, have a regular symmetry, and measure 120–150nm in diameter and 270–290nm in length. WSSV has an extremely wide host range. The virus can infect a wide range of aquatic crustaceans. All life stages are potentially susceptible. The best life stages for disease diagnosis are late PL stages, juveniles and adults. The major targets of WSSV infection are tissues of ectodermal and mesodermal origin, especially the cuticular epithelium and subcuticular connective tissues,especially samples from the pleopods, gills, haemolymph, stomach or abdominalmuscle are recommended for disease diagnosis. Although WSSV infects the underlying connective tissue in the shrimp hepatopancreas and midgut, the hepatopancreatic tubular epithelial cells of these two organs are of endodermal origin, and they do not become infectedand are notappropriate tissues for detection.It has wide range of vectors which includes rotifers, marine molluscs, polychaete worms and non-decapodal crustaceansincluding artemiaand copepods, as well as non-crustacean aquatic arthropods such as seaslatersand *Ephydridaeinsect* larvae. All these species can accumulate high concentrations ofviable WSSV. The infection can be transmitted vertically, horizontally and by water-borne routes. Dead and moribund animals are also a source of disease transmission.

Clinical signs

Based on the clinical manifestationWSD outbreaks in penaeid shrimps are divided into three types (Type I, II and III).

Type I outbreak (acute or sub-acute) moderate to high infection with significant mortalities observed within 7-10 days. The affected shrimps have a rapid reduction in feed intake, increased lethargyand have a loose cuticle with white spots (hence, the name "White spot" disease) of 0.5 to 3.0 mm in diameter, which are more apparent on the inside surface of the carapace, and they sometimes coalesce intolarger plates.The white spots represent abnormal deposits of calcium saltsby the cuticular epidermis.

Type II outbreak, the affected shrimp exhibits massive reddening, the tissue level severity of infection was very high and mass mortalities occurs within 2-3 days.

Type III outbreak (chronic) there is a low tissue level severity of infection, with absence of white spots and reddening of tissue, and the mortalities of shrimp were spread over period of 15-28 days.

Clinical pathology

WSSV-infected shrimp always has a delayed (or sometimes completelyabsent) clotting reaction.

Histopathology

Presence of intranuclear inclusion bodies as prominent eosinophilic (in the early stages they are Cowdry type A) to large basophilic intranuclear inclusions with variable multifocal necrosis in most tissues of ectodermal and mesodermal origin. These tissues include the gills, haemocytes and haematopoietic tissue, lymphoid organ, connective tissues, subcuticular epidermis, stomach, foregut and hindgut epithelium, heart, striated muscle, midgut and ovary walls, antennal gland and the nervous tissues.Besides this the cells in the affected tissues will exhibitsevere nuclear hypertrophy,chromatin margination

Yellow Head Disease (YHD)

The causative agent of this disease is yellow head virus (YHV), a corona-like RNA virus in the genus *Okavirus*, family *Ronaviridae* and order *Nidovirales*. YHVvirions are enveloped, rod-shaped, positive, singlestrandedRNA genome. Thisdisease is highly infectious for most known cultivated penaeid species. Transmission occurs by horizontal, direct from the water column and through ingestion of infected material. YHV can infect cultured shrimp from late postlarval stages onwards,but mass mortality usually takes place in early to late juvenile stages.Yellow head virus (genotype 1) is one of six known genotypes in the yellow head complex of viruses andis the only known agent of YHD. Gill-associated virus (GAV) is designated as genotype 2. GAV and fourother known genotypes in the complex (genotypes 3–6) occur commonly in healthy *P. monodon* and are rarely or not at all associated with disease. Vectorsinclude asymptomatic

carrier crustaceans.YHV targets tissues of ectodermal and mesodermal origin including lymphoid organ, haemocytes, haematopoietic tissue, gill lamellae and spongy connective tissue of the subcutis, gut, antennal gland,gonads, nerve tracts and ganglia. The most appropriate tissues for diagnosing this disease are lymphoid organ and gills. YHV can induce up to 100% mortality in infected shrimps within 3 days ofthe first appearance of clinical signs.

Clinical signs

Clinical signs seen in an infected animal are white, yellow or brown gills, yellowing of the cephalothorax caused by the underlying yellow hepatopancreaswhich may be exceptionally soft when compared with the brown hepatopancreas of normal shrimp and general bleaching of body (from which the disease got its name), yellow and swollen digestive gland which makes head appear yellow.Infected prawns feeds at abnormally high rate for several days and then cease feeding entirely.Mass mortality observed three days after cessation of feeding. Moribund prawns aggregate near surface at pond edges. In many cases, totalcrop loss occurs within a few days of the first appearance of shrimp showing gross signs of YHD. Similarly, gross signs of GAV disease include swimming near the surface and at thepond edges, cessation of feeding, a reddening of body and appendages, and pink toyellow colouration of the gills. However, these signs occur commonly seen in diseasedshrimp and are not considered a reliable method for diagnosis of GAV disease. Shrimp chronically infected with YHV or GAV display normalappearance and behaviour.

Histopathology

Presenceof moderate to large numbers of deeply basophilic, evenly stained, spherical, cytoplasmic inclusionsapproximately 2μm in diameter or smaller in tissues of ectodermal and mesodermal origin is the characteristic lesion seen in this disease. The lymphoid organ, haemocytes, haemopoietic tissue, gill,heart, cuticular epithelium, midgut and connective tissues are the primary targettissues and organs for YHV infection. Systemic necrosis of ectodermal and mesodermal tissues with prominent nuclearpyknosis and karyorrhexis is also another feature of this disease. Cellular changes in early infections mayinclude nuclear hypertrophy, chromatin diminution and margination, and lateraldisplacement of the nucleolus. Loss of tissue structure within lymphoid organ,stromal matrix cells that comprise tubules become infected leading to loss of tubularstructure, tubules appear degenerate. Lymphoid organ spheroids (LOS) developduring infection, ectopic spheroids may lodge in constricted areas of the haemocoel(heart, gills, subcuticular connective tissues etc). Necrosis of the lymphoid organ (LO) inYHD infections can be used to distinguish YHD from acute Taura Syndrome (TS) inpenaeid shrimp.

Taura Syndrome (TS)

This disease is seen in many shrimp species but the infection is found to be very severe in *L.vannamei* farms. TS is best known as a disease of nursery or grow-out phase *P. vannamei* that occurs within ~14–40 days of stocking postlarvae (PL) into grow-out ponds or tanks. Larger shrimp may also be affected, especially if they are not exposed to the virus until they are larger juveniles or adults. It was first described in the year 1952 in Ecuador. Initially it was thought

be caused by some toxic agents but subsequently an infectious agent was found to be the cause and it was named as Taura Syndrome virus or TSV in the year 1995. TSV is a cytoplasmic, linear, positive sense ssRNA, non-enveloped icosahedral virus of 32nm in diameter belonging to the family *Dicistroviridae*. TSV is a systemic virus, and it does not replicate in enteric tissues (e.g. the hepatopancreas, the midgut, or its caeca) and tissues of ectodermal and mesodermal origin. Hence, enteric tissues are inappropriate samples for detection of infection by TSV. The principal target tissue in the acute phase of TS is the cuticular epithelium. In chronic infections LO is the principal target tissue. Haemolymph or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary. The vectors for this disease is found to be sea birds (wild or captive sea gulls and chickens),Aquatic insects (water boatman) and frozen TSV-infected products. Suitable specimens for diagnosis of disease include PL, juveniles and adults. TSV survivors can grow through adults as infected but grossly appear as normal animals and canproduce infected PL that can also appears to be normal.

Clinical signs

The disease occurs in three distinct phase i.e. acute, transition and chronic. Gross signs are obvious in the acute and transition phases.

Acute phase: Moribund shrimp will appear pale reddish in colour with tail and pleopods appearing distinctly red commonly referred as 'Red tail disease'. In such shrimp, close inspection of the cuticularepithelium in thin appendages (such as the edges of the uropods or pleopods) with hand lens reveals signs of focal epithelial necrosis. The affected shrimp also show signs of soft shells and empty guts and generally die during ecdysis.

Transition (recovery) phase: This phase is seen only for few days and is characterized by gross signs of random, multi-focal, irregularly shaped melanizedcuticular areas. These melanised spots are haemocyte accumulations indicating the sites resolving TS lesions in the cuticular epithelium. Such shrimp may or may not have soft cuticles and red-chromatophore expansion, and may be behaving and feeding normally. If shrimp with these black lesions survive the next molt, the lesions disappear and they appear grossly normal, despite the continuing presence of the virus, especially in the lymphoid organ.

Chronic phase: Shrimp in the transition phase move into the chronic phase in which no obvious signs of disease is seen.

Histopathology

Acute and chronic phases can be diagnosed consistently by using histological methods. In acute phase, the pathognomonic lesions are seen in the cuticular epithelium, while in the transition and chronic phases there are no pathognomonic lesions.

Acute phase of the disease is characterized by multifocal areas of necrosis in the cuticularepithelium of the general body surface, appendages, gills, hindgut, and foregut (the oesophagus,anterior and posterior chambers of the stomach). Cells of the subcuticular connective

tissues andadjacent striated muscle fibres are occasionally affected. In somesevere cases the antennal gland tubule epithelium is also destroyed. Cytoplasmic remnants of necroticcells are often extremely abundant and these are seen as spherical bodies (1–20μm in diameter) which are eosinophilic to pale basophilic.These structures, along with pyknotic and karyorrhectic nuclei, give acharacteristic 'peppered' or 'buckshot-riddled' appearance, which is considered to be pathognomonic forTS disease when there is no concurrent necrosis of the parenchymal cells of the LO tubules. Theabsence of necrosis of the LO in acute-phase TSV infections distinguishes TS disease from acutephaseyellowhead disease in which similar lesionis seen. There is absence of haemocytic infiltration or other host-inflammatoryresponse which distinguishes it from the transitional phase of the disease.

In the transitional phase typical acute-phase cuticular lesions decline in abundance and severityare replaced by conspicuous infiltration and accumulation of haemocytes at the sites of necrosis.The masses of haemocytes may become melanised giving rise to the irregular black spots thatcharacterize the transition phase of the disease.

In chronic phase there is no gross signs of infection, but the prominent histopathological lesion is the presence of an enlarged LO with numerous LOS, which may remain associated with the main body of the paired LO, or may seen detach and become ectopic LOS bodies that lodge in constricted areas of the haemocoel (i.e. the heart, gills, in the subcuticular connective tissues, etc.).

Infectious Hypodermal and Hematopoietic Necrosis (IHHN)

IHHN was first discovered in blue shrimp *Penaeusstylirostris* and white shrimp *L. vannamei* in the Americas in the early 1980's and IHHN virus (IHHNV) is the smallest of the known penaeid shrimp viruses. Thevirion is 20–22 nm in size, non-enveloped, icosahedral, contains linear single-stranded DNA. Based on its characteristics IHHNV placed within the family of *Parvoviridae*. Three distinct genotypes have been identified and they are Type 1, Type 2, Type 3Aand Type 3B. The first two genotypes are infectious to *L. vannamei* and *P. monodon*, while the latter two are not infectious to these species. Most penaeid species can be infected with IHHNV, including *P. monodon*; it has been reported to cause acute epizooticsand mass mortality in *P. stylirostris*. By contrast, it does notcause mortality in *L. vannamei*, but rather reduced,irregular growth and cuticular deformities, gross signs collectively referred to as "Runt-Deformity Syndrome" (RDS). In spite of no mortality, commercial losses, shrimps that survive IHHNV epizootics may carry the virus forlife and pass it on by vertical and horizontal transmission. The infected adult carriers show nosigns of disease. IHHNV infects and has been shown to replicate in tissues of ectodermal and mesodermal origin from the embryo. Thus, the principal target organs includes the gills, cuticular epithelium (or hypodermis), all connective tissues, the haematopoietictissues, haemocytes, the lymphoid organ, antennal gland, and the ventral nerve cord, its branches and its ganglia. Hence, whole shrimp(e.g. larvae or PLs) or tissue samples containing the aforementioned target tissues are suitable for most testsusing molecular methods.Haemolymph or excised pleopods may be collected and used for testing when non-lethal testing of valuable broodstock is necessary.IHHNV is a systemic virus, and it does not replicate in enteric tissues (e.g. the hepatopancreas, the midgut, orits caeca). Hence, they are inappropriate samples for detection of infection.

Clinical signs

RDS, a chronic form of IHHN disease, occurs in *L. vannamei*. RDS has also been reported in cultured stocks of *P. stylirostris* and *P. monodon*. Juvenile shrimp with RDS may display a bent (45° to 90° bend to left orright) or otherwise deformed rostrum, a deformed sixth abdominal segment, wrinkled antennal flagella,cuticular roughness, 'bubble-heads', and other cuticular deformities. Populations of juvenile shrimp withRDS display disparate growth with a wide distribution of sizes and many smaller than expected ('runted')shrimp.

Histopathology

Chronic IHHNV infections and RDS are difficult to diagnose using routine histological methods and molecular methods are recommended for detection. Histological demonstration of prominent intranuclear, Cowdry type A inclusion bodies provides aprovisional diagnosis of IHHNV infection. The characteristic IHHN inclusion bodies are eosinophilic andoften haloed, intranuclear inclusion bodies within chromatin-marginated,hypertrophied nuclei of cells in tissues of ectodermal (epidermis, hypodermal epithelium of fore- andhindgut, nerve cord and nerve ganglia) and mesodermal origin (haematopoietic organs, antennal gland,gonads, lymphoid organ, and connective tissue). The inclusion bodies caused by this infection may beeasily confused with inclusion bodies seen in WSSV infection.

Infectious Myonecrosis (IMN)

Infectious myonecrosis (IMN) is a recently identified disease in cultured *L. vannamei.* IMN virusfound to be most closely related to *Giardia lamblia* virus, a member of the family Totiviridae. The viral genome consists of a single, double-stranded (ds) RNA molecule, icosahedral in shape and 40 nm in diameter. IMN causes significant disease and mortalities in juvenile and subadult *L. vannamei*. IMNV has been demonstrated to be transmitted from shrimp to shrimp by cannibalismand vertical transmission from broodstock to progeny probably occurs. Mortalities range from 40 to 70% in cultivated P. vannamei, and food conversion ratios (FCR) of infected populations increase from normal values of ~ 1.5 to 4.0 or higher. This disease in *L. vannamei* occurs with an acute onset of gross signs and elevated mortalities, but it progresses with a more chronic course accompanied by persistent low level mortalities. IMNV infects tissues of mesodermal origin. The principal target tissues for IMN include the striated muscles (skeletal and less often cardiac), connective tissues, haemocytes, and the lymphoid organ parenchymal cells. In chronic infections, the LOmay be the principal target tissue. Haemolymph or excised pleopods may be collected and used when non-lethal testing of valuable broodstock is necessary.

Clinical signs

Affected shrimp present extensive white necrotic areas in the striated muscle, especially in the distal abdominal segments and tail fan which may become necrotic and reddened in some individual shrimp.

Histopathology

In acute phase the lesions noticed are coagulative necrosis ofmuscle, often with edema. In chronic phaseliquefactive necrosis of the muscle is seen which is accompanied by haemocytic infiltration and fibrosis. Significant LOS formation is seen and ectopic LOSare often found in the haemocoel and loose connective tissues, especially in the heart lumen and adjacent to antennal gland tubules. In some cases, perinuclear pale basophilic to darkly basophilic inclusion bodies are evident in muscle cells, connective tissue cells, haemocytes, and in cells that comprise LOS.

***Penaeusvannamei* Nodavirus (PvNV)**

PvNV is a new pathogen first reported from Belize in 2004 and caused by a nodavirus called *Penaeusvannamei* nodavirus (PvNV). This disease causes 50% reduction in production. The gross and histologicalsign mimics IMNV, i.e., whitened abdominal muscles, coagulativemuscle necrosis with haemocytic aggregation, and basophilic inclusions.

MonodonBaculoVirus (MBV)

The monodonbaculovirus (MBV) wasfirst recognized in shrimp in the year 1977 and is also known as *Penaeusmonodon*-type singly enveloped nuclear polyhedrosis virus (PmSNPV). It can cause serious disease in hatchery-reared larvae, postlarvae and early juvenilestages of *P. monodon*.

Clinical signs

Protozoea, mysis and early PL stages were affected severely by this disease and may present a whitish midgut (due to the presence of occlusion bodies and cell debris in the faecal material). The gross signs will not be apparent in juveniles and adults.

Histopathology

Direct staining of the hepatopancreatic cells (squash preparation of cells) withmalachite green and conventional histopathology wasused to detect MBV. By histopathology this disease is diagnosed by the presence of a characteristic spherical occlusion bodies in hepatopancreatocytes, gut epithelial cells, or gut lumen where in the cells will be stained bright redwith H&E stains. Further, the infected hepatopancreatic (or occasionally midgut) cellswill have distinctly hypertrophied nuclei with single or, more often, multiple eosinophilic occlusion bodies along with chromatin diminution and margination.

Hepatopancreatic Parvovirus Disease (HPV)

This disease is caused by the virus Hepatopancreatic parvovirus of penaeid shrimp (HPV) and was first reported by Lightner and Redman in postlarvae of *Penaeuschinensis* in the year 1985. This virus infects severalpenaeid species and is widely distributed in manyparts of the world. HPV particles are in average 22 nm in diameter,icosahedral, and the genome consists of a negative singlestranded DNA molecule of approximately 5to 6Kbp.

Clinical signs

Affected shrimps will exhibit signs like atrophy of the hepatopancreas,anorexia, poor growth rate, reduced preening activities which are usually non-specific and no specific clinical signs for this disease as such. The target organ for this disease is the tubular epithelial cells of thedigestive gland (hepatopancreas). While the anterior midgut caecum andmidgut mucosal epithelium cells were affected less common. In cultured shrimp chronic mortalities during the earlylarval or postlarval stages has been associated with thisdisease and may resultin stunted growth during the early juvenilestages. However its effect on adultshrimp is not clear.

Histopathology

In this disease characteristic histopathological lesion observed is the presence of an intranuclear inclusion body within E- and F-cells, mostly in the distalportion of the hepatopancreatic tubules. Typically, the nucleolus of affected cells alsoincreases in size and appears as a "cap" onthe developing inclusion body.

Conclusions

Disease diagnosis is important in augmenting shrimp production. So many diagnostic techniques are available nowadays. One has to select correct diagnostic technique to arrive at a definite diagnosis of the disease. Histopathology is one such technique where in it helps to make definite diagnosis.

BACTERIAL DISEASES OF SHRIMPAND ITS DIAGNOSIS

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Shrimps are highly susceptible to bacterial infection when the environmental conditions are encouraging for the bacteria to multiply in the host system. The shrimp immune system is not as developed as mammals to recognise and to protect against the bacterial antigens which they encounter normally in the environment. The bacterial problem in shrimp ponds seems to be multifactorial and recurrent in the affected ponds. The virulence of bacterial pathogens depends on various factors such as species, pathogen specificity, host defence and environment parameters etc. Among the diseases of shrimp, bacterial diseases is not a highly infectious or contagious as shrimp viruses, but the impact is detrimental to FCR and lowers growth rate incurring loss to sector. The bacterial disease of shrimp follows.

1. Vibriosis

Vibriosis is a major problem in shrimp hatcheries and culture ponds. The main cause of the disease is Gram-negative bacteria belonging to the genus *Vibrio*. The possible four main species are *V. parahemolyticus, V. vulnificus, V. alginolyticus* and *V. harveyi*. However, *V. harveyi*, a luminescent species is considered as the most devastating that causes extreme losses in both the hatcheries and shrimp-rearing farms. All larval substages of *L. vannamei* were reported to be susceptible to *V. harveyi* and *V. parahaemolyticus* with high mortality rates. This bacterial disease is mainly caused by pathogenic vibrio species affecting various larval stages of shrimps resulting in about 80% mortality. Further, the developmental stage from naupli to protozoea-3 exhibited greater susceptibility to these pathogens when compared to mysis to postlarval stages.Gross signs of vibriosis are light or dark brown focal lesions and necrosis of appendage tips. The change of color is the result of melanin produces by host hemocytes involved in the inflammatory process. Large number of motile bacteria is visible in the hemolymph and hepatopancreas of moribund shrimp. Affected shrimp exhibit decreased appetite. Some also had a darker, larger or shrunken hepatopancreas.

2. Bacterial necrosis in larval stages

This disease will appear as localized necrosis or discoloration on any appendage, causing high mortality of zoea and mysis stages, but affects post larvae to a lesser extent. In zoea, it often starts with a liquefaction of the gut contents. If the necrosis starts at zoea-1 stage, the entire tank is collapsed by zoea-2 or zoea-3 within a matter of 24 hours. However, if necrosis starts at zoea-3 stage, only part of them will moult to mysis and mortality prolongs for a few days.

3. Bolitasnegricans

Robertson et al. (1998) reported that infection of *L. vannamei* larvae with *V. harveyi* at 105 cfu /ml produced a larval disease called Bolitasnegricans and bioluminescence. The highest mortalities were recorded during the transition from zoea-3 to mysis. "Bolitas" is the Spanish name given to a syndrome involving the detachment of epithelial cells from the intestine and hepatopancreas, which appear as small spheres within the digestive tract. Development of bioluminescence reduced feeding and retarded development, sluggish swimming, reduced escape mechanisms, degeneration and formation of bundles of necrotic tissues within the hepatopancreas leading to death of infected larvae.

4. Red spot syndrome

The disease is more common in Zoea-1 to post larval stages of *L. vannamei* where the affected larvae exhibit acute necrosis of the entire body and lysis of all the tissue cells. The level of mortality observed in this disease can go up to 100%. Further, in this syndrome the hatchery water of the tanks stocked with the infected larvae turns red to pink colour. This is most commonly seen in *L. vannamei* hatcheries. It was reported to be caused by the bacterium belonging to the genus *Pseudomonas* which was identical to *P. mesophilica* and *P. anguilliseptica* (Soltani et al., 2010).

5. White gut syndrome

The etiology of whitegut syndrome cannot be pinpointed to a single agent in provocating symptoms like stunted growth with reduced feed intake. The gut was found to be empty and appeared opaque. Gut filled with gas and feed intermittently. Signs are more visible when the shrimp reaches 3 gms and above. If the environmental conditions are favourable to infectious agent causing white gut, the shrimp will stop feeding and die with the development of loose shell. The white gut in many occasions is correlated with increase in pathogenic vibrios in the cultured pond. In some instances the mortality has been recorded with the symptoms of stunted growth and opaque white gut visible through the transparent cuticle as a white streak. The feed consumed was released as white fluid material. Large number of motile bacteria is visible in the hemolymph and hepatopancreas of moribund shrimp. The possible main vibrio species are *V. parahemolyticus, V. vulnificus, V. alginolyticus* and *V. harveyi. However, V. harveyi* is highly pathogenic luminescent species that causes extreme losses in all stages shrimp farming. It has been reported that white gut affected samples had 0.2 x 105cfu/mL of vibrio load in hemolymph (Jayashreeet.al. 2006).

6. Filamentous bacterial Infection

Leucothrixmucor,Thiothrixsp,Flexibactersp,lavobacterium,Cytophagaspare filamentous bacteriacause infection in penaeid shrimplarvae. Discolouration of gills, low growth and feeding,increased mortality and lethargy are common signsof the disease. The disease is associated with poorwater quality. Higher degree of infection may lead tonecrosis in gill tissue.

6. NecrotisingHepatopancreatitis (NHP)

NHP is causedby a bacterium that is relatively small, highly pleomorphic,Gram negative, and an apparent obligateintracellular pathogen. The NHP bacteriumhas two morphologically different forms: one is asmall pleomorphic rod and lacks flagella; whilethe other is a longer helical rod possessing eightflagella on the basal apex of the bacterrium, andan additional flagellum (or possibly two) on the crest of the helix. The NHP bacterium occupiesa new genus in the alpha Proteobacteria, and isclosely related to other bacterial endosymbiontsof protozoans (Lightner, 1996). NHP is also known as Texasnecrotizing hepatopancreatitis (TNHP), TexasPond Mortality Syndrome (TPMS) and Peru necrotizinghepatopancreatitis (PNHP).

7. Bacterial White Spot Syndrome

The bacterial white spot is similar to gross clinical signs of White spot disease (WSD) but PCR test for WSSV will be negative. This syndrome was reported in cultured *Penaeusmonodon*. Dull white spots are seen on the carapace and all over the body. Thewhite spots are rounded and not as dense asthose seen in WSD.The wet mountmicroscopy shows the spots as opaque brownishlichen-like lesions with a crenelated margin. The spot centeris often eroded and even perforated. During theearly stage of infection,shrimp are still active,feeding and able to moult – at which point thewhite spots may be lost. However, delayedmoulting, reduced growth and low mortalitieshave been reported in severely infected shrimp, *Bacillus subtilis* has been suggested as the causative agent of this syndrome (Wang et al., 2000).Vibrio Cholera was also often isolated in some bacterial white a spot case in Thailand has been reported.

Bacterial Diagnosis

Bacteriological sampling

- Nauplii, larvae and postlarvae - Use the whole animal after rinsing in sterile seawater or 2.5% NaCl saline solution. Pooled animal are homogenize, dilution made and streak on marine agar plates.
- Juveniles - Do surface disinfection (1% calcium hypochlorite, 1-2% povidone iodine and 70% ethyl alcohol) of the shrimp samples. Rinse in sterile seawater or 2.5% NaCl saline solution. Target tissue excise using flame sterilized dissecting tools and isolation made as follows:
  - Systemic infections: excise a block of abdominal muscle or the heart, touch it to the surface of the marine agar plate, steak and incubate
  - Enteric infections: excise the hepatopancreas, midgut and foregut and touch the exposed inner surfaces or contents of the excised organ to the surface of the agar plate, streak and incubate
- Sub-adult and adult - Preferred sample is the hemolymph. This can be removed either by using a syringe or cutting the tail (if animal is to be sacrificed). Place a drop of the hemolymph onto the agar plate and streak with sterile loop. If needed, dilutions can be made.

A. Gram Staining

This method is invaluable in demonstration of staining characteristic of the bacteria of concern. Gram negative bacteria are the primary concern in shrimp culture, mostly belonging to *Vibrio spp.*

B. Standard Culture Method

Zobell's Marine agar is the preferred general agar medium to obtain the greatest number and most variety of marine organisms present in the sample. Alternatively, the following general purpose media containing 2 to 2.5% NaCl can also be used:

a. Tryptic Soya Agar (TSA)

b. Beef Heart Infusion Agar (BHIA)

c. Nutrient Agar (NA)

These culture media will be used for primary isolation and purification of the bacteria. Likewise, Triple Citrate Bile Salts (TCBS) agar selective for *Vibrio spp.* can be used to make tentative diagnosis to involvement of potentially pathogenic *Vibrio spp.* to bacterial infection.

C. Culture and General Tests

1. Check plates at 12 to 18 hours for luminescent colonies as luminescence tends to fade within 24 hours after incubation
2. Purification is made in suitable media and primary identification can be employed in 24-hour culture using the following identification strategy:

   a. Rapid Identification Test Kits – Biolog, API NFT strips

   b. Classic methods – employing salt tolerance, biochemical reactions, fermentation of selected carbohydrates, growth using selected compounds as sole carbon sources

   c. Antibiotic sensitivity of the bacteria

D. Polymerase Chain Reaction

PCR methods were employed for the rapid detection of pathogenic *Vibrio* spp. They target specific regions which may be a gene or simply a stretch of nucleotide bases specific to that particular pathogenic *Vibrio* species. ln some cases multiplex PCR methods that can detect two or more genes of the same organism in a single PCR assay have been used.

EARLY MORTALITY SYNDROME (EMS) – AN EMERGING SHRIMP DISEASE

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From the past several years, an emerging disease popularly called as Early Mortality Syndrome (EMS) and scientifically called as Acute Hepatopancreatic Necrosis Disease (AHPND), has severely affected the shrimp aquaculture practice in many of the South East Asian countries. This disease has been assumed to be originated from the Hainan area of China in 2009 in the name of "covert mortality disease". Subsequently it has spread to Vietnam, Malaysia, Thailand and very recently to Mexico. Till now, this disease has not yet been reported from any other countries. Presence of EMS/AHPND has caused wide spread loss in these countries. Many of the farmers from EMS/AHPND affected area have reported losses to be as high as 80%.

Clinical signs of the disease

The disease appears in the pond as early as 10 days of post stocking and mass mortality may occur within 30-35 days of stocking. Hepatopancreas is the main target organ in EMS/AHPND where it brings several pathological changes. Clinical signs of HP include pale discoloration and atrophy of hepatopancreas(size reduction). In field level, the dissected HP will appear shrunken and when pressed between the fingers will appear granular. In some cases, the HP may also show black streaks. Pale and empty stomach, empty gut, reduced growth, loose shell and black discoloration are some of the other general clinical signs. Affected shrimps will also exhibit various behavioural changes such as lethargy, swimming sluggishly along the dikes, corkscrew swimming and reduced preening and feeding.

Species affected

So far, it has been reported that only 3 major penaied shrimps such as tiger shrimp, *Penaeusmonodon*, Pacific white shrimp, *Litopenaeusvannamei* and Chinese shrimp, *Penaeuschinensis* are the main species those are affected by EMS/AHPND. Further research work carried out in some parts of the world though considers tiger shrimp to be more resistance to this disease compared to others. Similarly, *Litopenaeusvannamei* has been considered to be the most susceptible shrimp species.

Causative agent

It took quite a long time before the discovery of the actual causative organism for this disease. In spite of a thorough and specific investigation of several suspected factors, it was not possible to identify the causative agent. Finally, it was narrowed down to a bacteria belonging to Vibrio group. It was found that EMS/AHPND is caused by a specific strain of *Vibrio parahaemolyticus*. Recent observations from the sequencing results show that EMS/AHPND strains have conserved sequences different from normal strains and it is located in plasmid.

Disease diagnosis

Initially at field level, the disease is suspected by the typical clinical signs. Subsequently, different laboratory protocols can be employed for accurate diagnosis. Since, HP is the main target organ for this disease, histopathological analysis of this organ is essential. Necrosis of B, F and R cells of HP, sloughing of cells to lumen, hemocytic infiltration and loss of mitotic E cells are some of the typical pathology. During the terminal phase, the HP is completely degraded by the secondary infection due to other bacteria.

Metagenomics and direct sequencing of the pathogen has revealed conserved regions based on which primers have been recently designed for identification by PCR. Based on this information, commercial kits are also now available for easy detection of the causative agent.

For the 1st time diagnosis of the disease, it is essential to combine both the histopathology and PCR method. It is also essential to isolate the specific strain of *V. parahaemolyticus* from the diseased animal and conduct laboratory infection to reproduce the disease and prove Koch's postulate.

Disease mechanism

Various experimental approach and field observations indicate that the pathogen has to enter through the oral route to cause the disease. Similarly, laboratory investigation has proved that the pathogen needs a specific number to produce the toxin and induce the specific pathology. It is assumed that the pathogen enters through the oral route, colonizes in the stomach and after reaching the specific number it produces the toxin by quorum sensing. The toxin is then transmitted to HP to cause necessary pathological changes.

Risk to human

It has been established that the causative organism of EMS/AHPND does not contain the necessary genetic material that can cause gastroenteritis which is otherwise caused by the human pathogenic *V.parahaemolyticus.* Moreover, the EMS/AHPND strain of *V. parahaemolyticus* is very sensitive to cold temperature and assumed not to survive during the transportation through the cold chain.

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PRACTICAL

GENERAL SAMPLING PROCEDURES FOR SHRIMP DISEASE DIAGNOSIS

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Sampling protocol for shrimp from farm

1. External observation

(a) Clinical symptoms

- Color change, overall
- Swimming pattern, any other abnormal activity
- Damage to exoskeleton and appendages
- Gut status – empty, partially filled, full
- Color change of organs – Gill, HP, Gut

(b)External fouling

- Slime deposit on body surface or appendages – Direct smear (site) for observation of parasites, Gram stain for presence of bacteria (lab)

2. Bacteriological analysis

A. Sampling

(a) Haemolymph

- Draw haemolymph in sterile syringe (site)
- Direct smear (site), Gram stain – Bacteria/Parasites (Lab)
- Spread on TCBS agar, ZMA (site)

(b) HP

- Direct smear (site), Gram stain – Bacteria/Parasite (Lab)
- Touch on TCBS and ZMA, streak (site)

B. Analysis(Lab)

- Total bacterial count, Vibrio count, Proportion of sucrose fermenter and non-fermenter (Green and yellow colonies)
- Species identification
- Virulence study of important isolates

3.Histopathology

- Dissect out organs (Gill, HP, Heart etc.) and putin Davidson's fixative (site) or inject Davidson's fixative to the whole shrimp, cut out the exoskeleton and put in Davidson's fixative (site)
- Processing, section cutting, staining, microscopy (lab)

4. Electron Microscopy

- Dissect different organs and fix in 2.5% glutaraldehyde (site)
- Process samples (lab)

5. Molecular biology

(a) Sampling

- Fix different organs/whole shrimp in 70%/95% ethanol (site)
- Fix organs in RNAlater (site)
- Put shrimps in dry ice (if processing has to be done latter)

(b) Analysis

- Extract DNA/RNA from specific tissues (lab)
- PCR/RT-PCR for specific virus/bacteria (lab)
- Sequencing for verification

6. Bio-assay

- Isolated suspected bacteria/parasite
- Tissue homogenate and filtrate from specific organs

  Injection or immersion with healthy animals

  Co-habitat of healthy and diseased animals

Sampling protocol for water samples from a farm

1. General observation

- Water color (plankton density), presence of harmful algae (red discoloration)
- Luminiscence during night (Presence of luminiscent bacteria)
- Microscopy for presence of toxic plankton

2. Water Quality

- pH, Ammonia

3. Bacteriology

- Total plate count, Vibrio count

Sampling protocol from a hatchery

1. Larvae

- Direct microscopy of larvae for parasites and fouling organisms (lab)
- Wash larvae in saline, crush aseptically and touch streak on ZMA and TCBS for bacteriology (site)
- Put larvae directly on Davidson's fixative (site) and process for histopathology (lab)
- Fix larvae with 70%ethanol/95% ethanol/RNAlater (Site) and do PCR/RT PCR for different virus

2. Water

- Spread plate water sample on TCBS/ZMA for total and vibrio count (site)
- Direct microscopy of water samples

POLYMERASE CHAIN REACTION (PCR) FOR THE DETECTION OF SHRIMP VIRUS

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Different steps in PCR

1. Sampling
2. Extraction of the nucleic acid
3. Preparation of cDNA in case of RNA pathogens
4. PCR mixture consisting of DNAse/RNAse free water, Buffer with required concentration of magnesium chloride, pair of primers (Forward and Reverse), dNTPs, DNA polymerase and the template (DNA or cDNA)
5. Run the PCR programme in a Thermocycler consisting of several cycles containing denaturation, primer annealing and extension
6. Separation of PCR product in a agarose gel
7. Documentation

PCR protocol for detection of shrimp virus

1. Sampling

Depending on the situation, both lethal and non-lethal sampling can be carried out. Non-lethal sampling is done for broodstocks or cultured adult shrimps where only a piece of pleopod is cut and used for PCR and therefore not necessary to sacrifice the animal. Whereas for lethal sampling, entire animal (in case of larvae) or part of tissue material (juveniles or adult) can be collected based on the type of viral pathogen required for detection. Different parts those can be used for nucleic acid extraction include hemolymph, gill, muscle, pleopod, lymphoid organ, hepatopancreas, eye stalk and faecal matter. While moribund shrimps are usually preferred to detect the actual disease status, samples can also be collected from healthy shrimps to find out whether a particular virus is present or not.

Table 1. List of important shrimp viruses

Virus	DNA/RNA	Sample
White Spot Syndrome Virus (WSSV)	DNA	Larvae, Hemolymph, **Gill**, Lymphoid organ (LO), **Pleopod**, other ecto/mesodermal tissues
Infectious Hypodermal Hematopoietic necrosis virus (IHHNV)	DNA	Larvae, Hemolymph, **Gill**, Lymphoid organ (LO), **Pleopod**, other ecto/mesodermal tissues

Monodon Baculovirus (MBV)	DNA	Larvae, **Hepatopancreas**, gut, Faecal matter
Hepatopancreatic Parvovirus (HPV)	DNA	Larvae, **Hepatopancreas**, gut, Faecal matter
Yellow Head Virus/Gill Associated Virus (YHV/GAV)	RNA	Larvae**, Gill**, LO, Pleopod
Taura Syndrome Virus (TSV)	RNA	Larvae, **Gill**, LO, Pleopod
Infectious Myonecrosis Virus (IMNV)	RNA	Larvae, **Telson**, **Pleopod**, Gill, Muscle, LO
Laem-Singh virus (LSNV)	RNA	Larvae, **Eye stalk**, Pleopod, Gill, Muscle, LO
Panaeus vannamei noda virus (PvNV)	RNA	Larvae, **Muscle**, Pleopod, LO, Gill

Letters in bold indicates preferred tissues for PCR

2. Extraction of nucleic acid

a. DNA

In case any kits are used, the instruction given by the manufacturer can be followed to extract DNA. There are several other published methods are also available by which DNA can be extracted.

Method I

50 mg tissue - Add 500 µl of buffer (6M Guanidinium Hydrochloride, 10mM Tris-Hcl pH 8.0, 0.1 M EDTA pH 8, 0.1 M Sodium acetate)- homogenize - 30 minutes incubation at room temperature (RT) - Centrifuge 5000 rpm 5 mins at 4^0 C - Take 300 µl supernatant - Add 300 µl of ice cold ethanol - Vertex well - Centrifuge at 14000 rpm for 10 mins at 4^0 C - Wash the pellet with 95% ethanol (10000 rpm for 3 mins) - Wash with 70% ethanol (8000 rpm 5 mins) - Air dry the pellet - Dissolve with 100 µl PCR grade water

Method II (OIE protocol)

To 100–200 mg shrimp tissue in a 1.5 ml microfuge tube, add 600 µllysis solution (100 mMNaCl, 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). Using a disposable stick, homogenise the tissue in the tube thoroughly and incubate at 65°C for 1 hour. Add 5 M NaCl to a final concentration of 0.7 M. Next, slowly add 1/10 volume of N-cetylN,N,Ntrimethylammonium bromide (CTAB)/NaCl solution (10% CTAB in 0.7 M NaCl) and mix thoroughly. Incubate at 65°C for 10 minutes, and then, at room temperature, add an equal volume of chloroform/isoamyl alcohol (24/1) and mix gently. Centrifuge at 13,000 gfor 5 minutes and then transfer the aqueous solution (upper layer) to a fresh 1.5 ml tube and add an equal volume of phenol. Mix gently and centrifuge at 13,000 gfor 5 minutes. Collect the upper layer solution andrepeat the phenol extraction process once or twice. Transfer the final upper layer to a new tube, mix gently with two volumes of chloroform/isoamyl alcohol (24/1) and

centrifuge at 13,000 gfor 5 minutes. Transfer the upper layer to a new tube and precipitate DNA by adding two volumes of 95% or absolute ethanol followed by standing at –20°C for 30 minutes or –80° C for 15 minutes. Centrifuge at 13,000 gfor 30 minutes and discard the ethanol. Wash the DNA pellet with 70% ethanol, dry and resuspend in 100 µlsterilised double-distilled water at 65°C for 15 minutes. Use 1 µl of this DNA solution for one PCR.

b. RNA

Kits can be used to extract RNA following manufacturer's instruction.

A general method for the extraction of RNA is given below:

Homogenise 50mg tissue with 1ml Trizol reagent - Centrifuge at 12000 g for 10 mins - Transfer the supernatant to a new tube and incubate at RT for 5 mins- Add 0.2 ml chloroform for 1 ml Trizol, shake vigorously for 15 secs - Incubate at RT for 2 to 3 mins - Centriguge at 12000 g for 15 mins at 4^0C - Take the aquous phase to new tube- Add 0.5 ml of isopropanol - Incubate at RT for 10 mins - Centrifuge at 12000g for 10 mins - Wash the pellet with 1 ml 75% ethanol (7500 g 5 mins) - Air dry pellet - Dissolve the pellet with 30 µl RNAse free water

Synthesis of cDNA: 1x buffer, Reverse transcriptase 1 µl, Nuclease free water 10 µl, RNA 5 µl - 25^0 C for 5 mins, 42^0C 30 mins, 85^0 C 5 mins

3. PCR reaction set up

All the necessary reagents and enzymes are added to a PCR tube. The amounts are calculated based on the total reaction volume. PCR enzymes and reagents are extremely temperature sensitive and therefore, care should be taken to keep it in ice or cooling box.

It is preferable to prepare master mixes if several samples are there to analyze at the same time. For each PCR reaction, a positive control and a negative control are included.

An example for a typical reaction of 50µl set up:

Buffer with MgCl2 (10x): 5 µl

Primer F (10 pm): 1 µl

Primer R (10 pm): 1 µl

dNTP (Mixture of 10mM each): 1 µl

Taq (2.5 unit/µl): 0.5 µl

DNA: 1 - 2 µl

Water: - µl (Make up to 50 µl)

For a nested PCR reaction, the product of the 1st step PCR is taken as template and the reaction is set up in same manner as that of the 1st step PCR.

4. Thermocycling

The tubes are arranged in the thermocycler. Care should be taken to close it properly to avoid evaporation.

A typical cyclic condition for the amplification of WSSV in shrimp:

94 ^{0}C 3 mins – 28 cycles of 94^0 C 30 secs, 58^{0}C 30 secs, 72^0 C 30 secs – Final extension at 72^0 C for 5 mins

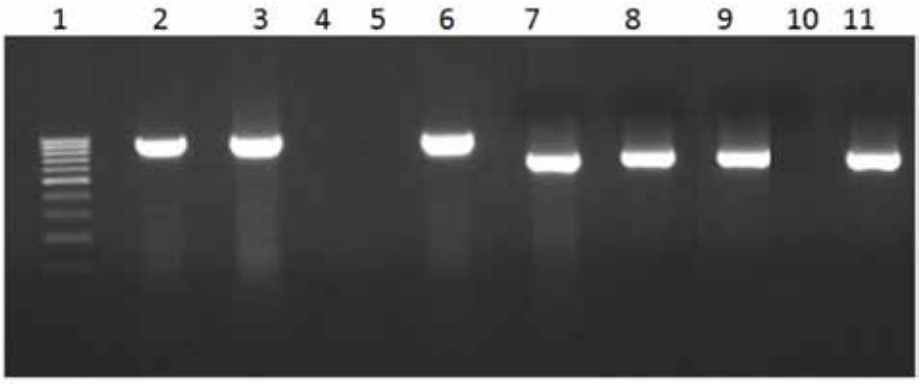
5. Gel separation of PCR products

Based on the size of the amplified product, 0.8 to 2% agarose gels are prepared either in 1x Tris-Acetate-EDTA buffer (1 litre 50x TAE – 242 g Tris base, 55 ml Glacial acetic acid and 37.2 g EDTA, pH 8) or 0.5 x Tris Boric acid EDTA buffer (1 litre 50x TBE – Tris base 540 g, Boric acid 275 g and EDTA 18.5g, pH 8.0). Ethidium bromide is added to the molten agarose (0.5 µg/ml final concentration) and then poured into the base. Once the gels are solidified, it is submerged in the tank with the same buffer. The amplified products are then mixed with 6x gel loading dye (For 100 ml – 30mg Bromo Phenol Blue, 30 mg Xylene cyanol, 12 ml of 0.5M EDTA pH8, 1ml of 1M Tris-Hcl pH8, 27 ml of distilled water and 60 ml of sterile glycerol). A total volume of 10 to 20 µl is added to each well. A molecular weight marker is also loaded to the gel to verify the size of the amplified product. After loading, the tank is connected to a power pack and electrophoresis is carried out. This starts with an initial voltage of 80 which is then increased to 120. Based on the gel size and voltage set, it may take 45 mins to 1 hour for the gel to complete the separation.

6. Observation and documentation

The gel is finally put in a gel-doc for complete analysis or on a UV-transilluminator for visualization. The positive result is read in the form of a band at the right position in the gel. Absence of band indicates negative reaction or absence of virus. Presence of band in the positive control and absence of band in the negative control indicates absence of technical error or contamination.

This is an example of a successive PCR reaction for a shrimp virus



1: Molecular Weight Marker
2: Sample 1-1st step
3: Sample 2 – 1st step
4: Sample 3 -1st step
5: Negative control-1st step
6: Positive control-1st step
7: Sample 1-nested
8: Sample 2 – nested
9: Sample 3- nested
10: Negative control – nested
11: Positive control - nested

7. Record maintenances

It is necessary to maintain a record regarding the results of each sampling. This will help to interpret the overall situation over a period of time.

HISTOPATHOLOGICAL ANALYSIS OF SHRIMP SAMPLES

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Histopathology plays an important role in disease diagnosis. This technique involves collection of suspected tissue samples from necropsy, fixation, preparation of sections, staining and finally microscopic interpretation. The various steps are

A. Collection of Materials

Collect sufficient number of moribund and normal shrimp separately in containers and label it with all details. It is advisable to collect three to five shrimp showing morbid changes along with equal number of normal shrimps.

B. Fixation

It is a process by which the cells and tissue constituents are fixed in a physical and partly also in a chemical state so that they will withstand subsequent treatment with various reagents with minimum loss of tissue architecture. This is attained by exposing the tissue to various chemical compounds, called fixatives. The shrimp samples should remain in fixative at room temperature for 48-72, which mainly depends on the size of shrimps. Then they can be transferred to 50% alcohol which can be kept until further processing. If the shrimp is of larger size (>12 g) they should be transversely slit open at the abdomen/cephalothorax and then immerse it in the fixative and can be kept in the fixative for longer time. The volume of the fixative added should be 10 times more than the volume of the tissues. Thin pieces of various organs of shrimps of 3-5 mm thickness are dissected out from it and are processed.

Common fixatives used for collection of shrimp are:

1. Davidson Fixative

Ethyl alcohol 95%	330 ml
Formalin	220 ml
Glacial acetic acid	115 ml
Tap/Distilled water	335 ml

2. Formal Saline

Formalin	100 ml
Sodium Chloride	8.5g

Tap/Distilled water 900 ml

C. Dehydration

This is the process by which the water is removed from the tissues. This is done to prevent undue shrinkage to the tissues. The steps involved in this process are:

Ethyl alcohol 70% - 1 hour

Ethyl alcohol 90% - 1 hour

Absolute alcohol I - 1 hour

Absolute alcohol II - 1 hour

D. Clearing

It is process of removal of alcohol from the tissues and prepares it for paraffin penetration for embedding and the steps involved are

Xylene I - 1 hour

Xylene II - 1 hour

E. Embedding

This is the process by which impregnating the tissues completely with paraffin (54- 56°C). The steps involved are two changes of paraffin one hour each.

F. Blocking

Melted paraffin is poured into the moulds and the tissues are oriented in such a position that the cutting surface of the tissue faces down. The blocks are removed from the moulds and they are ready for sectioning.

G. Section cutting

The blocks are trimmed off the excess paraffin and the section is cut using a microtome. Then the sections are transferred from the microtome to a tissue flotation bath having warm water. Sections spread out uniformly are then taken on to a clean glass slides coated with Meyer's albumin-glycerin mixture.

H. Staining of sections

Haematoxylin and eosin method of staining (H&E) is the routinely used stain for tissue sections. The steps involved are

1. Deparaffinise the section in Xylene for 5-10 minutes, two changes.
2. Removal of xylene by treating with absolute alcohol for 5-10 minute, two changes.

3. Treat the sections in 90%, 70% and 50% alcohol each about 5-10 minutes and then wash it in tap water.
4. Stain the tissues with Haematoxylin for 4-8 minutes and wash it in running tap water for 5-10 minutes.
5. Blue the sections by treating with ammonia water (0.5% Ammonium hydroxide)
6. Wash in tap water.
7. Counter stain with eosin 0.5% until the section appears light pink (15-30 seconds)
8. Wash in tap water.
9. Blot it dry
10. Dehydrate in alcohol
11. Clear in xylene.
12. Mount in DPX mount, keep slides dry and remove air bubbles, if any.

The processed slides are ready for examination under microscope.

ISOLATION AND IDENTIFICATION OF BACTERIA FROM SHRIMP

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Sampling

- Hemolymph – Draw aseptically from the heart or from the base of the periopod
- From infected parts with necrosis/blisters – wash the area with sterile saline to get rid of external contamination. With the help of a sterile swab, collect sample from the infected area
- From internal organs – Dissect out shrimp carefully to avoid any contamination from nearby organs. Sampling can be done from Hepatopancreas with an inoculating loop or swab. Similarly, other organs can be taken aseptically, homogenized with 1% TSB and proceed for culture

Culture

- Sample can be put either on TCBS or ZMA plates (1 or 2 drops of hemolymph and spread, streak the swab on plates, inoculate 100 µl from the homogenized part of internal organ and then spread)
- Incubate at 30 ^{0}C for 18-24 hours for colonies to develop

Identification:

A. Biochemical characterization of bacterial isolates

Identification of bacterial isolates is done based on the colony characteristics, physiological and biochemical characteristics.

Amino Acid Decarboxylase Test

- Prepare Moeller's decarboxylase broth base with 1% arginine, Moeller's decarboxylase broth base with 1% ornithine and Moeller's decarboxylase broth base with 1% lysine and dispense in tubes before sterilizing.
- Inoculate freshly prepared bacterial culture into the above broth using sterile inoculation loop. Overlay with sterile mineral (paraffin) oil after inoculation.
- Incubate at 37oC and observe for four days.
- Development of purple colouris positive, yellow colourrepresents negative result.

Salt Tolerance

- Prepare Tryptone water broth by adding 0.5 gm of Tryptone, 0.2 gm of yeast extract with 0%, 3%, 8% and 11% of NaCl to 100 ml of distilled water and transfer the prepared contents to each test tube and keep for sterilization.
- Pick single colony from TCBS plate and inoculate in sterile ZMA broth and incubate at 30 ºC for 24 hrs.
- Innoculatefreshly prepared bacterial culture into test tubes with different salt concentration andincubate at 37oC for 24 hrs and observe the medium for turbidity.

Indole Production Test

- This test detects production of indole from tryptophan.
- Inoculate the tryptone water with bacterial culture and incubate for 24 hours.
- After incubation add 0.5 ml of Kovac's reagent.
- In positive cases a pink colour ring appears is positive, yellow ring indicate negative result.

Methyl Red

- This test is performed to identify bacteria which produce lactic, acetic or formic acid from glucose via mixed acid fermentation pathways.
- PrepareMRVP broth and inoculate with freshly prepared broth culture and incubate for 48-72 hrs
- Add a few drops of methyl red indicator solution.
- Development of strong red colour indicates positive reaction.

VogesProskauer's Test (MRVP)

- This test detects the production of acetyl methyl carbinol as chief end product of glucose metabolism.
- PrepareMRVP broth and inoculate with freshly prepared broth culture and incubate for 48-72 hrs
- After incubation, add drops of alpha-naphthol and 40% sodium hydroxide along the walls of the test tubes carefully.
- Shake the test tubes at intervals to ensure maximum aeration. Appearance of red color indicate positive result for VP, whereas yellow color indicate negative result for VP.
- To above broth culture add Methyl red indicator

Fermentative Utilisation of Carbohydrates

- Prepare Phenol red broth base supplemented with 1% Salt and 1% Lactose, similarly prepare 1% Salicin, 1% Sucrose and 1% Mannitol, 1%Maltose and 1% Starch anddispense into tubes and sterilize.

- Inoculate the broth with freshly prepared bacterial culture and incubate at 37oC and observe for colour change at 24, 48 and 72 hr. intervals.
- Appearance of pink colour indicates positive result and yellow colour indicate negative result.

Oxidase Test

- Place an oxidase disc in a clean glass slide and moisten with distilled water.
- Pick single colony and place on moistened surface of oxidase disc.
- Observe for colour change immediately, purple colour as positive and yellow colour as negative.

Citrate Test

- Prepare Simmon Citrate Agar slants and inoculate by streaking the slants using sterile inoculation loop with freshly prepared bacterial culture and incubate at 30 ºC for 24 to 48 hr and observe for growth and colour change.
- Colour change of slants to blue is positive and green colourslantrepresents negative.

Antibiotic sensitivity test

- The organisms were grown for 24 hrs in ZMA broth and 0.1 ml of culture was uniformly spreaded on the ZMA plates.
- The antibiotic discs were gently pressed and placed at equal distance and the plates were incubated at 37 ºC for 24 hrs.
- The following nine antibiotic discs namely Ampicillin, Streptomycin, Neomycin, Tetracycline, Oxytetracycline, Ciprofloxacin, Chloromphenicol, were used and observed for the zone of inhibition.

Nitrate Reduction Test

- Nitrate broth is used to determine the ability of an organism to reduce nitrate (NO3) to nitrite (NO2) using the enzyme nitrate reductase. It also tests the ability of organisms to perform nitrification on nitrate and nitrite to produce molecular nitrogen.
- Prepare Nitrate broth and incubate after inoculation with bacterial culture
- After incubating the nitrate broth, add a dropperful of sulfanilic acid and a-naphthylamine. If the organism has reduced nitrate to nitrite, the nitrites in the medium will form a red-colored compound. Therefore, if the medium turns red after the addition of the nitrate reagents, it is considered a positive result for nitrate reduction. If the medium does not turn red after the addition of the reagents, it can mean that the organism was unable to reduce the nitrate. Therefore, another step is needed in the test. If the medium does not turn red after the addition of the nitrate reagents, add a small amount of powdered zinc. If the tube turns red after the addition of the zinc, it means that unreduced nitrate was present. Therefore, a red color on the

second step is a negative result. If the medium does not turn red after the addition of the zinc powder, then the result is called a positive complete.

A typical chart is presented below for the identification. Several of such charts can be found in the published literature based on which, the bacterial can be identified.

	V.alginolyticuss	*V.anguillarum*	*V.carchariae*	*V.cholerae*	*V.cincinnatiensis*	*V.damsela*	*V.fluvialis*	*V.furnissii*	*V.harveyi*	*V.metschnikovii*	*V.mimicus*	*V.parahaemolyticus*	*V.vulnificus*	*A.hydrophila*	*P.shigelloides*
Growth in TCBS	Y	Y	Y	Y	Y	G	Y	Y	Y/G	Y	G	G	G	Y	G
Oxidase	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Growth in :															
0% NaCl	-	-	-	+	-	-	-	-	-	-	+	-	-	+	+
6% NaCl	+	+	+	-	+	V	+	+	+	+	-	+	+	+	-
ONPG	-	+	-	+	+	-	+	+	V	+	+	-	+	+	-
Voges-Proskauer	+	+	-	V	+	+	-	-	-	+	-	-	-	+	-
Lysine decarboxylase	+	-	+	+	+	V	-	-	+	+	+	+	-	V	+
Acid from:															
D-cellobiose	-	+	+	-	+	+	+	-	nd	-	-	V	+	+	-

[a] Y = yellow, G = green, V = variable, nd = not determined, + = positive and - = negative

B. Identification based on 16s rRNA amplification and sequencing

16S ribosomal RNA is essential to the viability of bacterial cells and hence, the genes coding for them is highly conserved. The genes also contain short variable sequences which are highly useful in characterization and discrimination of microbes to the level of family, genus and species. The combination of conserved and variable sites makes these molecules ideal taxonomic markers to identify vibrios by PCR amplification and gene sequencing.

DNA Extraction

Bacterial isolates grown overnight at 30 oC in TSB containing 1% sodium chloride is used for DNA extraction on turbid tubes using a boiled cell method. Centrifuge 1ml of broth cultureat 12000 rpm for 2 min and discard the supernatant, resuspend the pellet with 500 µl of sterile distilled water and boil at 100 oC for 10 min. Cool the boiled cell lysate at -20 oC for 10 min for rapid release of DNA and centrifuge at 13,000 rpm for 3 min. The boiled cell lysate should be used as the DNA template for PCR detection of 16S rDNA for identification of Vibrio species.

Primer sequence for PCR amplification

S.No	Primer Sequences	Product length	Reference
1.	fD1[5′-AGAGTTTGATCCTGGCTCAG-3′]	~1,500 bp	(Weisburg *et al.*,1991)
2.	rP2[5′-ACGGCTACCTTGTTACGACTT-3′]		

Perform the PCR reaction for 50 µl volume using ready-to-use 2X Red dye master mix which includes Taq DNA Polymerase enzyme, 10 mM d NTP's (d ATP, d TTP, d CTP, d GTP), 5X PCR reaction buffer with Mgcl2 and Red dye (Gene Technologies).

PCR requirements

S.NO	Components	Volume in µl
1	2X Red dye master mix	25.0
2	Forward primer (10pmol)	1.0
3	Reverse primer (10pmol)	1.0
4	DNA template	1.0
5	Nuclease free water	22.0
	Total	50.0

The sample should be added to the prepared master mix except for the negative control and gently vortex the contents then spun down the PCR tubes by brief centrifugation. Amplify in a thermal cycler with the following thermal profile and cycling condition.

PCR program

Step	Program for	No. of cycles
Initial Denaturation	94ºC for 4.15 minutes	1
Denaturation	95ºC for 0.45 seconds	
Annealing	57ºC for 1 minute	30

Elongation	72ºC for 1.30 minutes	
Final Extension	72ºC for 5 minutes	1
Final Maintenance	22ºC for ∞	

The confirmation of PCR products must be done by electrophoresis in 1.5% agarose gel containing ethidium bromide (0.5µg/ml). PCR products have to be stored in -20 ºC for confirmation by sequencing and blast analysis. On comparing the sequence results with percentage similarity to available databases the species of the bacteria can be identified.

Agarose Gel Electrophoresis of PCR products

- The confirmation of PCR products was done by electrophoresis in 1.5% agarose gel containing ethidium bromide (0.5µg/ml).
- The agarose was weighed in an Ehrlenmeyer Flask to which 1X TBE was added
- The agarose was boiled to dissolve by placing the flask in microwave oven for about 5 minutes. Once the agarose had completely dissolved, the flask was removed from oven and was left at room temperature to cool.
- The gel casting tray was washed with distilled water, wiped dry with tissue paper and the open ends were sealed securely with cello tape.
- The comb was placed 1cm from the top end and was made sure that the tooth of the comb did not touch the surface of the platform; i.e. 1-2mm gap was maintained so that the DNA samples did not leak into the platform.
- The gel casting tray was placed on a smooth horizontal surface. Once the agarose solution was cooled to about 50oC , 0.1 µl ethidium bromide was added, mixed well, then the solution was poured gently to cover the entire surface of the platform without air bubbles and left undisturbed for about 15 minutes.
- Once the gel was formed, the comb was removed gently by pulling up and the cello tape was also removed from both the ends.
- The gel was placed along with the gel casting tray inside the electrophoretic gel tank with wells near cathode and electrophoresis buffer 1X TBE was poured through one side of the tank to cover the gel surface.
- The PCR product of sample was directly loaded into the well carefully using a micropipette.
- The master mix added initially during PCR amplification consists of an inert red dye and a stabilizer to allow direct loading of the final products on to the gel for analysis. DNA marker ladder was mixed with gel loading dye and loaded next to sample wells.
- Once the samples and ladder were loaded into the well, the cathode was connected towards the top end of the gel (wells) and the anode was connected towards the bottom of the gel. The electrophoresis unit was set run at approximately 5V/cm.

- As the tracking dye had moved 1cm above the bottom end, the power supply was disconnected and the gel was placed onto the UV transilluminator. The UV light was switched on and the DNA bands were seen and photographed using Bio Rad Gel documentation apparatus.

PCR Product Purification (Spin Column Based - Bio Basic – BS664)

- The PCR reaction mixture was transferred to a 1.5ml microfuge tube and three volume of binding buffer 1 was added to it.
- The above mixture was transferred to the bio basic spin column and was allowed to stand for 2min at room temperature and was centrifuged at 10,000 rpm for 2min.
- The flow in the tube was removed and 750 µl of wash solution was added to the coloum and was centrifuge at 10,000 rpm for 2min.
- The washing procedure was repeated in step 3.
- To remove the residual wash solution, it was spined at 10,000 rpm for additional 1min.
- The column was transferred to a clean 1.5ml tube and 30-50µl elution buffer was added and incubated at room temperature for 2min.
- It was centrifuged at 10,000 rpm for 2min to elute product
- It resulted in a 40 µl purified product.
- 1µl was used to calibrate the nanogram/µl in nanodrop

The purified PCR products were stored in -20 ºC for confirmation by sequencing and blast analysis of 16 S rDNA. On comparing the sequencing results with that of morphological and physiological characteristics the species of the bacteria can be identified.

PROTOCOLS FOR DETECTION OF SHRIMP EMS/AHPND

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Samples

Collect shrimps with typical clinical sign. Healthy post larvae or faecal strings of brood stocks can also be sued for detection

Step I: Bacterial isolation

From the suspected samples, aseptically dissect out stomach and HP and put in a 1.5 ml microfuge tube containing 500 µl 1% Tryptic Soy Broth (TSB) . TSB is available commercially. The composition is given below:

(Enzymatic Digest of Casein ..... 17.0 g

Enzymatic Digest of Soybean Meal ..... 3.0 g

Sodium Chloride ..... 10.0 g

Dipotassium Phosphate ..... 2.5 g

Dextrose ..... 2.5 g

Final pH: 7.3 ± 0.2 at 25°C)

Homogenize with a pestle. From this, add 100 µl to a fresh 2 ml TSB. Take a loop full and streak on TCBS plate.

Direct touch from the HP can also be taken and streaked on TCBS plates.

Incubate the plates and the broth at 30 ^{0}C for 18 – 24 hours. Bacteria grown on the broth is further purified on TCBS plates.

The green colonies grown can be taken for identification by biochemical reaction or for gene specific PCR for V. parahaemolyticus. Part of this colony can directly be taken for DNA extraction and PCR reaction (Adopted from Dr. T. W. Flegel, Thailand).

PCR primers for the detection of EMS/AHPND specific V. parahaemolyticus strain

Primers:

AP1F, 5'- CCT TGG GTG TGC TTA GAG GAT G -3';

AP1R, 5'- GCA AAC TAT CGC GCA GAA CAC C -3';

AP2F, 5'- TCA CCC GAA TGC TCG CTT GTG G -3';

AP2R, 5'- CGT CGC TAC TGT CTA GCT GAA G -3'.

PCR conditions

Pre-heat: 94°C, 5min

PCR 25~30 cycles with:

Denaturation: 94°C, 30 sec

Annealing: 60°C, 30 sec

Extension: 72°C, 60 sec

Final extension: 72°C, 10 min

When separated in an agarose gel, both the primer pairs yield products of ~ 700 bp.

Rapid method for detection of EMS/AHPND V.parahaemolyticus strain

Fecal string from broodstock

Stomach/HP/Gut from juveniles

Whole macerated larvae (15-20 no.)

Incubate in Vibrio enrichment media (Ex. 1% Alkaline Peptone Water (APW) at 1:9 ration) for 4-6 hours at 30 ^{0}C

Select green colonies from TCBS streak, extract DNA and do PCR

Confirm the PCR positive isolates by infection experiment

Histopathology

The target organ here is Hepatopancreas. Fix only moribund samples for histopathology. Process the HP sample for histopathology following the protocol described in this manual. Observe for typical pathology.

Bioassay

Grow bacteria in 1% TSB for 12-18 hours. Take healthy juvenile shrimps in a container. Based on the quantity of water, add the bacterial inoculum (Estimate the count in the broth either by plating method or by measuring the OD in a spectrophotometer) to have final bacterial count of 108/ml. With aeration, keep the shrimps for 1 hour. Thereafter release the shrimps to the original tanks with aeration. Observe for mortality. Confirm the mortality by PCR and histopathology.