

STUDY ON NEW SHRIMP DISEASE “Internal mortality syndrome” OF EXOTIC SPECIES *litopenaeus vannamei*

Anil Kumar Moola

Abstract— Aquaculture is the fastest growing food sector globally and is established itself as high protein resource to fulfil the food demand since the natural resources exhibits over exploitation. But presently various biological and non-biological agents causes's mortality of cultured shrimp. Unusual shrimp mortality was reported in Nellore region of Andhrapradesh. The signs and symptoms of the disease are not matching with any of the disease reported so far for cultured shrimp species. Due to its symptoms like Internal mortality with no moribund/ surfacing of the shrimp and white to yellowish faecal matter it was named as “Internal mortality syndrome” A study was conducted to found out the etiology by using microbiological, molecular, histological, immunological disease diagnostic and water quality analysis techniques. The study shows there is correlation between water quality parameters like hydrogen sulphide(> 0.03 ppm) dissolved oxygen(< 2.5 ppm) and vibrio bacteria count found in the hepatopancreas(>5.0 X 10⁶) and mid gut of the shrimp. The study reveals the there is no virus involved.

Index Terms— Exotic species, internal mortality syndrome, shrimp diseases, Histological studies, Non-biological agents.

1. Introduction:

Shrimp farming has a long and colorful history whose development can be divided into three distinct periods; start up era (1982-87), hatchery era (1988-96) and breeding era (1997-present). The breeding era 1997 to the present, saw a resumption of growth, which jumped to more than 20% year for more than 10 years. This rapid industry growth was primarily driven by the domestication, breeding and world wise spreading of *L.vannamei* from the west Indonesia. World shrimp production using *L.vannamei* expanded from only 10% total production in 1998-75% of total world production in 2006. [1]

In India commercial shrimp farming started gaining roots only during the mid eighties. It was relatively late start in India by this time shrimp farming had reached peak in most of the neighboring Asian countries .the boom period of commercial scale shrimp culture in India started in 1900 and the best came in 1995-96 with the outbreak of viral disease (sept 2002 aquaculture authority).

According to aquaculture, national portal of India, Andhrapradesh ranked in coastal aquaculture, fresh water aquaculture. Andhrapradesh contributes nearly 40% of the total marine exports of the country.

1.1 Specific problem:

In Andhrapradesh region Nellore the species *L.vannamei* and *p.monodon* (black tiger) has been cultured .In present study we have targeted on the predominant problems of high mortality and white feces in both species. But majorly high mortality rate observed in *vannamei* and white feces Morley seen in black tiger shrimp .so we have diagnosed for the virus diseases like (YHV, TSV, WSBV and GAV) and bacterial diseases like VIBRIOSIS .We have targeted on the major organs for white feces (Shown in figure 1 and 2).

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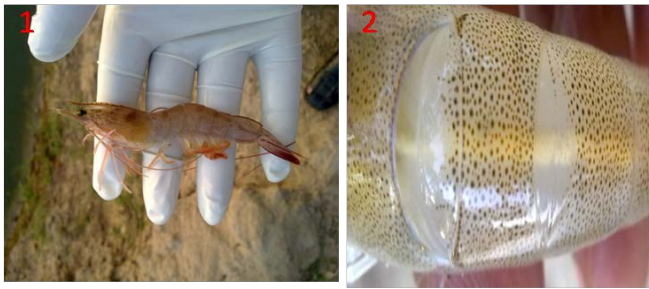


Fig (1) High mortality rate species observed in *L.vannamei* (Nellore dist gangapatnam farm) and in fig (2) white gut observed in the species *p.monodon*

with feulgen positive inclusions and occluded lumens.

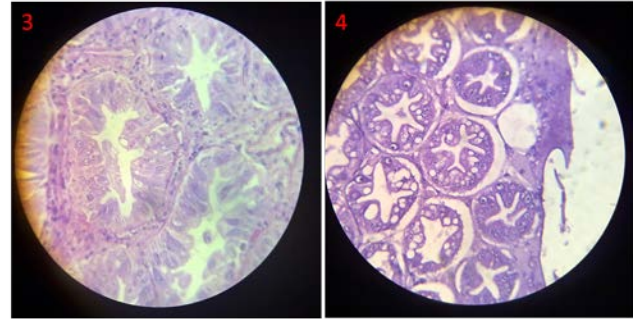


Fig (3) Destruction of tubular cells in Hepatopancreatic tissue in Black tiger shrimp(*p.monodon*) and Fig (4) Clear tubular structure observed in Hepatopancreas of White shrimp

2. Materials and methods:

2.1 Histopathology:

For the study of presumably diseased shrimp, select those that shrimp are moribund, discolored, displaying abnormal behavior, or otherwise abnormal, except in the case of intentional random sampling for estimation of diseased prevalence. Have ready an adequate supply of fixative. A general rule is that a minimum of 10 volumes of fixative should be used for one volume of tissue sample (i.e. a 10 g sample of shrimp would require 100 ml of fixative).

Davidson's AFA (Alcohol, Formalin, Acetic acid) fixative composition contains Strong formalin (37%) =500 ml, Alcohol =750 ml, Glacial acetic acid =250 ml and distilled water = 750 ml (Do not substitute other acids, such as HCL, for acetic acid. Histological sections prepared from HCL-Davidson's solution are not suitable for routine haematoxylin and eosin staining.)

The presence of spherical inclusion bodies, staining from eosinophilic to basophilic, in the cytoplasm of target cells (cuticular epithelium, gills, hind and fore gut, connective tissue, muscle etc.. The pathogenic lesions are associated with pyknotic, karyorrhectic and hypertrophied nuclei of tissue of ectodermal and endodermal in origin. Absence of haemocyte infiltration in characteristic of the acute phase, compared to the recovery chronic phase. [2] ,[3],[4] In case of YHV lymphoid organ tubules contain necrotic cells

2.2 Rapid gill staining:

The staining method involves application of hem alum, which is a complex formed from aluminium ions and oxidised haematoxylin, these colours nuclei of cells are blue. The nuclear staining is followed by counter staining with an aqueous or alcoholic solution of eosin, which colours eosinophilic other structures with various shades of red, pink, and orange.

The staining of nuclei by hem alum does not require the presence of DNA and is probably due to binding of the dye metal complex to arginine rich basic nucleoproteins such as histones. Feulgen –positive intranuclear inclusion bodies in hypertrophied nuclei of hepatopancreatic tubule epithelial cells.

Consequent lateral displacement and compression of the host cell nucleolus, which becomes hypertrophied inclusion bodies. Is a key morphological feature characteristic of an HPV infected nucleus chromatin marginating is also a prominent characteristic of an HPV infected nuclei.

Early in their development, HPV inclusion is small eosinophilic bodies centrally located within the nucleus and closely associated with the nucleolus. Later in virogenesis the inclusion body becomes intensely basophilic, affected cells are most common in the distal portion of tubules in F or E type epithelial cells.

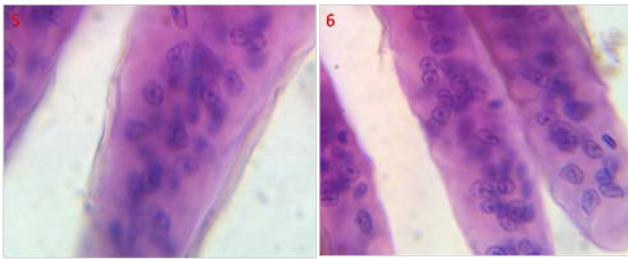


Fig (5&6) clear nucleus and nucleolus observed under light microscope by H&E staining procedure

2.3 Antibiotic sensitivity:

Antibiotic sensitivity is a term used to describe the susceptibility of bacteria to antibiotics. Antibiotic susceptibility testing (AST) is usually carried out to determine which antibiotic will be most successful in treating a bacterial infection (Table: 1). Testing for antibiotic sensitivity is often done by the Kirby-braver method. Small wafers containing antibiotics are placed on a plate upon which bacteria are growing. If the bacteria are sensitive to the antibiotics a clear ring or zone of inhibition, is seen around the wafer indicating poor growth.

| Sl.NO | Antimicrobial agent | Green colonies | | Yellow colonies | |
|-------|---------------------|----------------|---|-----------------|---|
| | | S | R | S | R |
| 1 | Amikacin | + | | + | |
| 2 | Amoxicillin | + | | + | |
| 3 | Cephalaxin | + | | + | |
| 4 | Cefotaxime | + | | + | + |
| 5 | Ciprofloxacin | + | | + | + |
| 6 | Doxycycline | + | | + | |
| 7 | Chloramphenicol | + | | + | |
| 8 | Netromycin | + | | + | |
| 9 | Azithromycin | + | | + | |
| 10 | Cifixime | + | | + | |
| 11 | Putnox | + | | + | |
| 12 | Clarithromycin | + | | | + |
| 13 | Co-trimoxazole | | + | | + |
| 14 | Cloxacillin | | + | | + |
| 15 | Gentamycin | | + | + | |
| 16 | Neomycin | | + | + | |
| 17 | Norfloxacin | | + | | + |
| 18 | Pencillin | | + | + | |
| 19 | Ofloxacin | | + | + | |
| 20 | Pefloxacin | | + | | + |

Table: 1 R-Resistant S- Sensitive

2.4 Reverse-transcription polymerase chain reaction:

Detection of viruses in carrier shrimp or other crustaceans cannot be realistically accomplished by histological methods, and more sophisticated techniques are required. The protocol mentioned here is adapted from Wongteerasupaya et.al[5]. An alternative assay for YHV is reported by Tang & Lightner [6]. A nested PCR method, which is more sensitive and will detect and discriminate both YHV & GAV.

Collect fresh shrimp sample (50 ul), mix immediately with 500 ul of TrizolT reagent and extract RNA according to the protocol. Resuspend RNA (2ul) in 20 ul of PCR buffer (10 mM Tris/HCL, Ph 8.3,50 mM Kcl) containing 2.5 U of M-MLV (moloney murine leukaemia virus) reverse transcriptase, 1.0 u of ribonuclease inhibitor, 0.75 um of antisense primer, 1mM each of dATP, dTTP, dCTP, and dGTP , and 5 mM OF Mgcl2 and incubate at 42⁰C for 15 minutes to synthesise cDNA .Incubate the mixture at 100⁰C for 5 minutes to inactivate the reverse transcriptase and allow the mixture to cool to 5^oc. Add the PCR mixture containing 2.5 U of Taq DNA polymerase (Perkin Elmer Cetus) 2mM Mgcl2 and 0.75 um of sense primer to give a final volume of 100 ul. Overlay the tubes with 100 ul of mineral oil and carry out PCR amplification for 40 cycles at 94⁰C for 30 seconds, 58⁰C for 30 seconds, and finishing at 72⁰C for 30 seconds. And finishing at 72⁰C for 30 seconds. Include a negative control containing a diethyl pyro-carbonate (DEPC) - treated distilled water instead of RNA extract, and a positive control containing YHV RNA. The sequences of the PCR primers are

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

The RT-PCR method of Wongteerasupaya et al. Will not detect GAV due to sequence difference from YHV at the PCR primer sites. The nested RT-PCR procedure that allows detection of both YHV and GAV in the first amplification and discrimination of the genotypes in the nested amplification.

The sequence of the nested PCR primers is
GY1 5'-GAC-ATC-ACT-CCA-GAC-AAC-ATC-TG-3'
GY2 5'-CAT-CTG-TCC-AGA-AGG-CGT-CTA-TGA-3'
GY4 5'-GTG-AAG-TCC-ATG-TGT-GTG-AGA-CG-3'
GY5 5'-GAG-CTG-GAA-TTC-AGT-GAG-AGA-ACA-3'
Y3 5'-ACG-CTC-TGT-GAC-AAG-CAT-GAA-GTT-3'
G6 5'-GTA-GTA-GAG-ACG-AGT-GAC-ACC-TAT-3'

The sequence of above RT-PCR primers generic for GAV and YHV (or) specific for GAV or YHV are as follows.

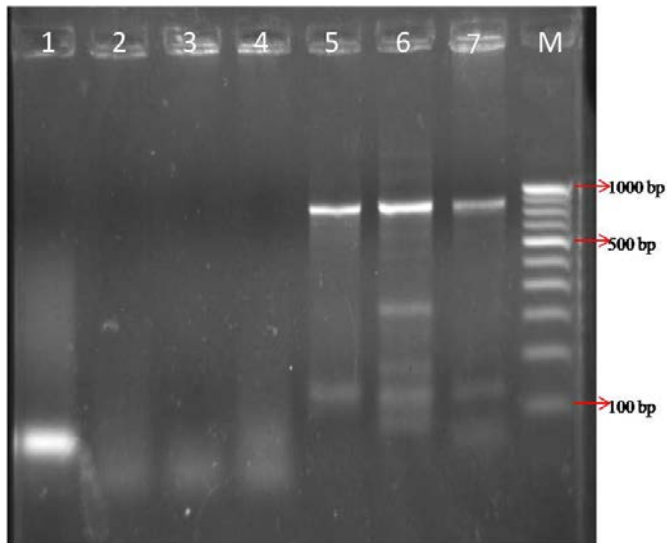


Fig (7) A agarose gel showing a specific DNA fragment showing YHV negative was observed by polymerase chain reaction (Lane 1 and 2) and nested PCR reaction (Lane 3 and 4) amplification using a hepatopancreas of *L.vannameli* (Lane 5,6 and 7 are internal control) and molecular weight marker 100bp ladder are shown in Lane M.

2.5 Phytoplankton analysis:

In your beautiful pond has turned into a green eyesore, or if you want to prevent your pond from turning green, you have to increase plankton and stop weed from growing with a little pond care. Fertilising your pond is an absolute aquatic weed.

Fertilising your pond increases plankton, the plankton will grow and cover the bottom of your pond, which prevent sunlight from penetrating through to the soil bottom of the pond and stops weed growth. In the early spring, feeds input to aquatic ponds usually are low, and there is not enough nitrogen and phosphorous concentration for the (purpose of promoting plant growth) in ponds water to promote growth of microscopic plant known as 'phytoplankton'.

As water temperature increases, feed input increases due to increased fish activity. Phytoplankton becomes more abundant as a result of increased food input and warm temperature an abundance of phytoplankton is called phytoplankton bloom. Fertilisation encourages excessive phytoplankton and a greater oxygen demand. Unnecessary fertilisation is detrimental to water. Quality in ponds may lead to a higher concentration of nitrogen and phosphorous effluents [7]. In good quality and toxic ponds three types of blue green algae were identified that is micocystis, nostoc,

and phormediom.

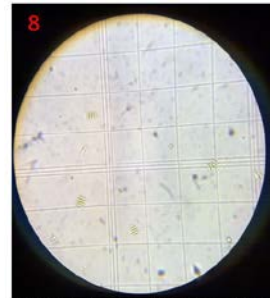


Fig (8) Calculating the number of cells present per ml in a good quality pond.

Total number = Number of cells × 10,000
Number of cells = 3
3 × 10,000 = 30,000/ml

2.6 Antibiotic sensitivity:

Antibiotic sensitivity is a term used to describe the susceptibility testing (AST) is usually carried out to determine which antibiotic will be most successfully in treating a bacterial infection. Testing for antibiotic sensitivity is often done by the Kirby-bauer method. Small wafers containing antibiotic are placed on a plate upon which bacteria are growing. If the bacteria are sensitive to the antibiotics a clear ring or zone of inhibition, is seen around the wafer indicating poor growth. Agar and broth dilution methods for minimum inhibition concentration. From our results for polymerase chain reaction and nested PCR for all viruses and bacterial diseases are showing negative result for YHV.

In our challenge test, culture pond mass mortality rates were gradually increasing after affecting the infection and mortalities mostly occurs at the regions of the aerator. According to couch, 1974. Lightener et.al, 1983[9],[10]., baculovirus associate with the diseases infected the Hepatopancreas. And all bacterial and viral diseases targeted the major organs like Hepatopancreas, gills and lymphoid organ. Therefore the Hepatopancreas is thought to be the target organ for these viruses.

In present study sign and symptoms are not matching with any of these diseases reported so far for cultured shrimp species, the symptoms like no moribund and white to yellowish faecal matter it was named as "Internal mortality syndrome". And microbiological, molecular and histological study de-

picts/reveals there is no virus involved. Still there may be an approach to study on prevalence of new shrimp diseases of exotic species.

3. Results and discussion:

One of the typical external signs of infected shrimp was yellow colour observed inside surface of the carapace (fig. 1). There was a dark lesions observed under the light microscope. And cephalothorax region is yellowish colour in some infected shrimp of the *L.vannamei*. Light yellow decolourisation of the cephalothorax region and bleached appearance was not observed in naturally affected shrimp [8].

From our light microscopic observations for two effected culture shrimps i.e. *L.vannamei* and *p.monodon* by the process of histopathology diagnosis, In case of *vannamei* species hepatopancreas was affected (Fig 3 and 4). Our study depicts for bacterial and viral diseases in rapid gill staining process a very clear nucleus was observed under light microscope (Fig 5 and 6). However there are pyknotic and karyorrhectic nuclei was seen

However in water quality analysis huge number of yellow colonies developed by streak plate method with in 8hrs in the infected pond water. After 8-12 hrs fermented bacteria converted into non fermented bacteria. In case of plankton analysis three types of diatoms were found. Still there may be an important approach to study on prevalence of new shrimp disease of exotic species.

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