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Training Manual on

MANAGEMENT OF EMERGING DISEASES OF SHRIMP WITH SPECIAL REFERENCE TO PACIFIC WHITE SHRIMP, *LITOPENAEUS VANNAMEI*

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Sponsored by
National Fisheries Development Board, Hyderabad

AQUATIC ANIMAL HEALTH AND ENVIRONMENT DIVISION
CENTRAL INSTITUTE OF BRACKISHWATER AQUACULTURE
(Indian Council of Agricultural Research)
75, Santhome High Road, R.A.Puram, Chennai - 600 028.

Training Programme on

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10th – 14th December, 2012

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Forward

Pacific white shrimp, *Litopenaeus vannamei* culture has started gaining popularity in India as seen from the increased shrimp production in 2012. Based on the risk assessment carried out by CIBA, Govt. of India permitted its culture based on Coastal Aquaculture Authority guidelines for seed production and farming. This was well accepted by the farmers as an alternate species to tiger shrimp, *Penaeus monodon*. Till the late of 90's, tiger shrimp culture had gained immense popularity amongst the farmers of India. However, appearance of white spot syndrome virus (WSSV) resulted in repeated loss of crop. Due to difficulties in domestication of tiger shrimp, developing Specific Pathogen Free (SPF) stocks has been limited to few players. On the other hand, there are number of sources for Pacific white shrimp, *Litopenaeus vannamei*. In addition, Pacific white shrimp has higher growth rate and suitable for high density culture system. All these favourable attributes has led to increased number of farmers opting for vannamei culture.

Success of any cultured species to a large extent depends on the disease prevalence. The devastating role of many shrimp viruses such as WSSV, Yellow head virus (YHV), Taura syndrome virus (TSV) and Infectious Myonecrosis virus (IMNV) is well documented. Therefore, it is highly essential to create a better environment and take all necessary precautions to avoid the disease in the culture system. At the same time, it is also absolutely essential to take extra precautionary measures for the disease detection in vannamei considering the fact that seed production is based on imported stock. Introduction of any exotic pathogens will have high negative impact on the native species that are generally cultured in India. Therefore, along with developing culture system, awareness on the disease risk is very much necessary on the part of farmers and technical persons involved in vannamei culture.

I am extremely happy to know that the Aquatic Animal Health and Environmental Division is conducting a 5 day training programme on "Management of emerging diseases of shrimp with special reference to Pacific white shrimp, *Litopenaeus vannamei*" from 10th to 14th December, 2012. A manual is also being released on this occasion. I am grateful to the National Fisheries Development Board (NFDB), Hyderabad for sponsoring this programme. This training is the need of the hour and I am quite hopeful that it will provide immense benefit to the participants. My best wishes for all the participants and compliments to both the convenors, Dr. S.K. Otta and Dr. P.K. Patil for conducting this training programme.

(A.G.Ponniah)

Preface

Culture of Pacific white shrimp, *Litopenaeus vannamei*, has been taken up by the farmers of India on an extensive manner. Though, Specific Pathogen Free brooders are used to produce the larvae, the culture environment remains the same. Therefore, there is every possibility of disease outbreak by the existing pathogens like WSSV. There is also the likelihood of introducing transboundary pathogens considering the non-native status of vannamei. A number of infectious viral pathogens, have been documented in vannamei. In this regards, special attention by all the personnel involved with vannamei culture practice with respect to disease is highly essential. The necessity for rapid, sensitive and accurate diagnosis as well as biosecurity measures to prevent the disease outbreak should be well understood. The present training programme has therefore been organised for the benefit of participants. Fortunately, this training programme has been proposed in a time when vannamei culture is gaining popularity.

We are very much grateful to NFDB for their support and encouragement to conduct this training for the benefit of people who are interested in vannamei culture. We have received overwhelming response from several parts of the country regarding the participation in this programme.

The programme has been planned for a period of 5 days from 10th to 14th of December and consists of both theory and practical classes by the scientists from CIBA as well as eminent scientists from outside. Materials pertaining to all these classes have been compiled and put in the form of this manual. All the lecture notes and the protocols for practical have been designed to be very specific and relevant to the disease management in vannamei culture system. We are very much hopeful that this manual will provide sufficient information and guidance to all the participants of this programme.

We are very much grateful to Dr. A. G. Ponniah, Director for his encouragement and support to conduct the training programme. Our sincere gratitude to Dr. K. P. Jithendran, Scientist In-Charge, Aquatic Animal Health and Environment Division, who has been with us from the very beginning and provided all possible support for the improvement of this manual. We thank all the scientists for their prompt contribution to bring out this manual in time. A special thanks to all the scientists of Aquatic Animal Health section for their unconditional support.

We are sure that the manual to be a helpful guide to all the participants for a successful and sustainable vannamei culture.

Convenors

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OVERVIEW OF *Litopenaeus vannamei* CULTURE PRACTICE AND IMPORTANCE OF GOVT. REGULATION MEASURES

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Introduction

Shrimp farming in India, till 2009, was synonymous with the monoculture of tiger shrimp, *Penaeus monodon.* About 1,90,000 ha brackishwater area have been developed for shrimp culture in the country. Since 1995 culture of *P. monodon* is affected by White Spot Syndrome Virus (WSSV) and the development of shrimp farming has become stagnant. Most of the Southeast Asian countries like Thailand, Vietnam, Indonesia were also culturing *P. monodon* and since 2001-02 onwards most of them have shifted to culture of exotic Whiteleg shrimp, *Litopenaeus vannamei* because of the availability of Specific Pathogen Free (SPF) and Specific Pathogen Resistant (SPR) broodstock. In India, Pilot-scale introduction of *L. vannamei* was initiated in 2003 and after a risk analysis study large-scale introduction has been permitted in 2009. The biology of both the species differs in minor aspects and accordingly the culture practices are also different.

Biology

L. vannamei is native of pacific coast of Mexico and Central and South America as far south as Peru. It is mainly found on mud bottoms, down to a depth of 75 m. It is commonly known as white legged shrimp or Mexican white shrimp. It is greyish-white in color. The maximum weight of females in the wild is about 120 g. The males are smaller at 60-80g. It lives in the column and prefers clayey loam soil.

For *L. vannamei* the growth at 30°C is much higher than at 25°C. The optimal range of temperature for the species is between 30 and 34°C. At 20°C growth virtually stops. It can tolerate salinity levels of 0 to 50 ppt. Growth is uniform within 10-40 ppt They can grow in freshwater also but the growth is slower below 10 ppt. pH range of 7 to 9 is tolerated with optimal growth at pH 8.0. Dissolved oxygen levels above 4.5 ppm is required for optimal growth. Turbid water with flocculated particles of more than 0.5 micron resulted in better growth than clean water mainly because of the presence of algae and bacteria. Ammonia –N and Nitrite – N levels should be less than 0.1 ppm and 1 ppm respectively.

L. vannamei is an omnivorous scavenger and is less aggressive and less carnivorous than *P. monodon.* Food intake is more during evening and night. Retention time of food in the gut is 2.2 to 5 hours. Food is digested at modest acidities of pH 5.5-7. Growth of *L. vannamei,* under confined culture conditions was similar to *P. monodon* till they attain 20g size. Beyond that the growth rate was poor. The shrimps attained the size of 20g within a period of 100-120 days depending on the stocking density.

Susceptibility to viruses and Specific Pathogen Free stock

L. vannamei is highly susceptible to a number of viral pathogens. White Spot Syndrome Virus (WSSV), Taura Syndrome Virus (TSV), Yellow Head Virus (YHV), Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV), Lymphoid Organ Vacuolization Virus (LOVV), Reo like Viruses (REO) are some of the viruses reported in the species. In order to eliminate the presence of the virus in the seed, Specific Pathogen Free (SPF) stock has been developed by producing a number of generations in highly bio-secure facility with continued surveillance of pathogen presence. Although SPF shrimp are, by definition, free of all specifically listed pathogens, SPF shrimp may be infected with a known

pathogen that is not included on the SPF list of the shrimp supplier, or with an un-known pathogen that has not yet been described. Offspring of SPF shrimp are not considered SPF unless they are produced and maintained at an SPF facility. SPF status changes with the pathogen condition of the shrimp, as well as the type of environment within which they are cultured (i.e. the level of biosecurity). One of the main advantages of culturing *L. vannamei* is commercially available as high health animals from Specific Pathogen Free (SPF) stocks while *P. monodon* have very limited availability from SPF stocks.

Bio-security requirements of shrimp farms

Stocking pathogen free post larvae alone doesn't guarantee a disease free culture since the pathogens could still enter the culture environment horizontally and infect the shrimps during the culture. Viral pathogens can still enter the culture environment through the following means and a better understanding of these can help in prevention of horizontal transmission

- By persisting in the soil
- Intake water
- Aquatic vectors introduced through intake water, by crabs and other animals.
- Besides the above mentioned carriers, viral particles can also enter the farming system by mechanical carriers like:
    - o Contaminated land animals and birds
    - o Contaminated farm inputs – through live feed, semi-moist feed
    - o Contaminated farm implements, nets and vehicles etc.,
    - o Contaminated personnel

Crabs are one of the carriers of viral pathogens and providing crab fencing in shrimp farms is considered as one of the important biosecurity requirements. Carriers like crabs could also move from pond to pond over land barriers. To prevent such movements fencing made of 0.5 m plastic sheet should be put around the culture pond.

Feed ingredients of aquatic origin used in aquaculture can be a source of pathogens (viruses, bacteria and parasites) to shrimp species. Pathogens in feed can infect the animals directly by means of consumption of feed or indirectly via environmental sources. Live feed and moist feed are more likely to contain pathogens because their ingredients are either in a raw state or subject to insufficient processing.

Birds such as crow/water crow pick up the dead and moribund shrimps affected with viral disease from ponds and may drop in unaffected ponds, thereby transmitting the virus mechanically. This could be avoided by using bird scares and bird fencing over the pond. Similarly land animals like dogs, cats and cattle can mechanically carry the virus from one pond to another. Preventing entry of stray animals and unauthorized personnel into the farming area through fencing is the only way to address this problem

Use of tyre bath with disinfecting solution of calcium hypochlorite (200 ppm) and foot bath/ hand wash for the disinfection of farm personnel is essentially required to avoid contamination.

Pond to pond transmission of virus within a farm could easily occur through the use of farm implements and farm workers. Providing an independent set of implements for each of the ponds will be the best solution. Routine disinfection of the implements before every use should also be made a part of the SOP so that it becomes the routine practice with farm personnel. Similarly disinfection of hands and feet of the farm personnel before entry into any pond should be made mandatory.

Workers move from pond to pond attending to their work. So, restriction on the movement of farm workers from pond to pond is necessary. Personal disinfection has to be instituted among the farm workers before they enter the pond and after they come out of the pond.

Farm Design requirements

L. vannamei lives in the column and hence increasing the depth of the pond will help in increasing the stocking density. Generally shrimp farms which were culturing *P. monodon* had a water depth of about 1 m. But it is advisable to have a depth of 1.5 to 1.8 m water column for culturing *L. vannamei.*

Since mechanical aeration is one of the major requirements for *L. vannamei* culture, constant circulation water is expected in the pond. This will lead to the erosion of the soil in the dyke and bottom. To avoid this compacting of the pond bottom and the dykes is essential. In intensive culture ponds total lining of the pond HDPE sheets is done to avoid any erosion. In high density cultures, accumulation of sludge in the bottom is a major problem and provisions of central drainage or use of sludge pumps is essential. Positioning of paddle wheel aerators should aid in bringing the sludge to the centre of the pond from where they can be removed.

Bio-security requirements like reservoir ponds, fencing, crab fencing, bird fencing, and disinfection facilities are incorporated in the design. To avoid disease in most cases zero-water exchange system of farming is practiced with recirculation facilities. In such cases more than 40% of the water area in the farm is allocated for reservoirs and waste sedimentation ponds.

Management of the farm

Drying and Liming

The sludge left in the pond, which might have had viral disease outbreak during the previous culture, may contain high organic load, bacteria, viral particles and DNA as well as many other viral carriers. All these should be removed to prevent the persistence of viral disease. This could be achieved by the application of burnt lime (CaO) @100 ppm, followed by exposure of the pond bottom to sunlight until it dries and cracks, removal of the top soil and compacting the bottom soil.

Water Management

White spot virus has been reported to survive as a free living form in water up to seven days. Direct use of creek or sea water carries the risk of introducing the virus into the system. Most of the aquatic crustaceans including the planktonic forms are reported to be carriers of WSSV virus. A number of other aquatic organisms could be mechanical carriers because of their filter-feeding habit. There is a need to eliminate these from water before use in culture ponds. Use of filter nets of 60 micron mesh/ cm^2 in the delivery pipes/ inlet sluice should be strictly followed. Water should be taken in reservoir ponds and treated with calcium hypochlorite @ 30 ppm and aged up to seven days, to eliminate the viral pathogens.

Farmers should ensure that only treated water be used in the culture ponds for compensating the evaporation losses. Regular water exchange is not advised to avoid cross contamination of pathogens from source water.

Fertilization and addition of carbon source

Culture of *L. vannamei* can be done under two systems - with plankton as natural feed or with bacterial floc. The fertilization schedule with urea and superphosphate is followed for plankton method while provision of carbon source in the form of molasses and dolomite is used for the development of bacterial floc. The volume of bio-floc was controlled at 15 ml/ liter.

Stocking

SPF shrimp seed from a reputed hatchery is used for stocking. PL8-PL9 is normally selected after ensuring the pathogen free status of the seed. The seed acclimatization is a very important requirement before stocking. Temperature, salinity and pH of the transportation water should be gradually brought to the level of pond water by gradual mixing of both over a period of 6-12 hours depending on the difference. Stocking densities of 40 to 60 no/m^2 is preferred. Higher stocking densities above 60 no./m^2 is not permitted.

Feed Management

Protein requirement varied between 25 to 40% depending on the density. Marine source of protein was more effective than plant source. Lipid requirement was around 6-8% with 2% marine unsaturated fatty acids and 0.25 to 0.4% of cholesterol. Feeding rate was between 6.6 to 16% for 1 g shrimp which was reduced to 2% for 15g shrimp. Optimal feeding frequency was between 2 and 6 times in a day with maximum percentage of feed distributed in the evening and night rations. Check trays are used to monitor the feed consumption and the feeding ration is adjusted accordingly. The FCR levels of 1.1 to 1.3 are expected.

Maintenance of water quality

Regular monitoring of water quality is very essential. Water quality parameters like temperature, salinity, pH and alkalinity are monitored on daily basis. DO levels are recorded at least 2 times a day. Other parameters like Ammonia, Nitrite, Ca, Mg are monitored on weekly basis. DO levels should be maintained above 4 ppm although and operation of paddle wheel aerators should be done to maintain the level. The number of aerators required is about 1 HP per every 300 kg of biomass. The location of the aerators should be adjusted in such way the sedimentation occurs at the centre of the pond which will aid in its easy removal.

Most of the culture systems avoid regular water change for fear of introduction of viral pathogens into the pond system. In case water exchange is necessitated during extreme water quality conditions, treated water from the reservoirs only should be used for filling the pond. In high density zero water exchange systems bioremediators in the form of water probiotics is used to maintain the water quality. Calcium and Magnesium levels in the water play an important role in the moulting process of the shrimps. The desired levels are 200ppm of Ca and 200 ppm of Mg. Whenever there is a drop in this, regular supplementation in the water should be done.

Removal of sludge from the pond bottom during culture is essential in case of high density cultures. Aerators are positioned in such a way that the sludge is accumulated in the centre of the pond and from there it could be removed through central drainage or using sludge pumps. To aid in the process,

sludge settled at other places should be disturbed regularly. This is achieved through dragging of chains at the bottom at regular intervals on all sides of the pond.

Health Management

Weekly monitoring of shrimps for their growth and well being is essential. *L. vannamei* normally grows at the rate of 0.2g / day after the first 30 days. Weekly growth rate will range between 1.5 to 2.0g, depending on the stocking density. At 60 nos./m^2, the shrimps attain 20g size within 100 to 120 days.

Harvest and post-harvest

L. vannamei is a column living shrimp and hence maximum stock can be harvested by either cast netting or drag netting and this will help in harvesting them without much overcrowding and stress. Final harvesting by draining the water should be done within 6 hours. Compared to *P. monodon*, *L. vannamei* discolourizes faster if there is any delay in icing down of the harvested stock. Hence the stock should be 'ice killed' immediately on exposure and stored in ice.

Cost of production

The cost of production of *L. vannamei* in Indian conditions considering the industrial rate for electricity might work out to Rs. 120 to 160 for production levels of 8 to 10 tons per ha. The average size at harvest ranges from 18 to 22 g and the sale price is more or less same for both *P. monodon and L. vannamei* of similar size at Rs. 200 to 250. The profit margin is very high and even though only 50% of the area will be utilized for grow-out, it is beneficial than *P. monodon* culture.

Government Regulatory measures

Impacts of introduced species have been assessed under two broad categories: ecological, which includes biological and genetic effects and socio-economical. However, these two categories are inter-dependent one influencing the other. Under the biological impacts, the biodiversity could be affected through competition, hybridization, predation and disease transmission. The change in stock composition and alteration in the habitat by the introduced species might affect the fishery management seriously. Human communities may also be impacted through change in fishing patterns due to a newly-established fishery or through changes in land use and resource access when high valued species are introduced into an area. While positive impacts of introductions are visible in the short term and quantifiable, there is time lag before negative impacts are observed and it is more difficult to quantify the impacts. In the absence of data for modelling risks, the present approach has been using expert judgment to quantify risks and develop management or mitigation measures.

Risk Analysis

The scope of the Risk analysis for introduction of exotic aquatic organisms is well defined. The major hazards normally considered while evaluating introduction of exotic aquatic organisms are:

a) introduction of exotic pathogens

b) its effect on bio-diversity,

c) ecological impacts and

d) environmental impacts of culture.

However it is also necessary that the following social and economic risks are also evaluated:

a) impact on existing market and

b) overall impact on the socio-economic conditions of the small farmers and other stakeholders.

Conclusion

Introduction of exotic fishes and crustaceans might become a necessity since aquaculture production is one of the important livelihood option of coastal poor and aquaculture to great extent provide food and nutritional security for the developing nations. But the introductions should be done through careful planning after undertaking a risk analysis study as per the guidelines of OIE as indicated in the present document.

Table 1. Risks and Guidelines to mitigate the risks

RISKS	GUIDELINES
Health Risk- Introduction of exotic viruses which might affect the native shrimp species.	• Short listing of the SPF Vannamei suppliers based on the genetic programme and the status of SPF facility. • Legal empowerment under Livestock importation Act, 1898 and establishment of Aquatic quarantine facility at Chennai, the port of entry. • Formulation of SOP and monitoring by a committee headed by CAA with members from AQ&CS, CIBA, Ministry, NFDB, MPEDA, RGCA
Illegal production of non-SPF stock may affect the sustainability of *L. vannamei* culture	• Only hatcheries and farms registered with CAA will be permitted to import and culture *L. vannamei.* • A separate permission should be obtained for the purpose from CAA • Hatcheries can sell seed only to farmers who have been permitted to culture • Farmers should buy only from hatcheries permitted to rear vannamei seed • Processors should buy *L. vannamei* only from farmers permitted to culture vannamei • A movement document will be maintained which should contain the copies of the permission granted to the source hatchery, the farm where it was cultured and the processor who is exporting
Ecological risk of escape into natural environment and establishment thereby affecting the biodiversity	• No direct release of wastewater from Quarantine, Hatcheries and Farms permitted. • Effluent treatment system is mandatory for all the three stages. • Effluent treatment to include complete cholorination and dechlorination during quarantine and hatchery stages to prevent the escape of smaller larvae and also the pathogens

Environmental risk- Globally *L. vannamei* culture is practiced in intensive systems which will lead to high nutrient loading in the system.	• Stocking density of up to 60 no/m2 permitted • Strict compliance to waste water standards prescribed by CAA • Effluent Treatment System mandatory for all the farms culturing *L. vannamei* irrespective of the size of the farm and at no time water should be directly released into the open source water. • ETS should have a minimum size of the largest pond in the farm or 10% area whichever is higher. • Regular inspection of the farms with the collection of samples of waste water will be done by a committee constituted for the purpose by CAA with State Fisheries Departments taking active role
Market risk- Globally the supply level will increase and result in further reduction in prices and we have to compete with established players in the field.	• Information on market and other related information should be collected from the major *L. vannamei* producing countries and made available to all the stakeholders. • A mechanism to collect and analyse the economic data should be put in place once the culture of *L. vannamei* is taken up in large scale • Stakeholders' meeting at the end of one year to review the results of economic analyses of the farming • The information on the number of shrimps imported, results of pathogen testing, number of seed sold, number of farmers permitted and the production levels should be made public

IMPORTANCE OF BEST MANAGEMENT PRACTICES TO DEVELOP DISEASE FREE ENVIRONMENT FOR *Litopenaeus vannamei* CULTURE

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Crop related risk factors in an aquaculture system either increases or decreases the probability of occurrence of an adverse event during a specified time period. Presence of the necessary cause alone like white spot disease (WSD) will not lead to an outbreak in a pond. In a farm situation, a number of "component causes" (risk factors) along with the "necessary cause" might become "sufficient cause" to produce WSD outbreaks. These risk factors occur throughout the shrimp cropping cycle and can be categorized as shown below during the different stages of the crop cycle:

- Stocking season
- Pond water preparation
- Water management
- Feed management
- Pond preparation
- Seed quality and screening
- Pond bottom management
- Disease treatments

Effective management of these factors can be essentially the core aspect in controlling the environmental factors and thereby reduce the risks of disease occurrence in the pond.

Risk management

The risk management is to develop practical measures for containing/preventing shrimp disease outbreaks that should specifically cover identification of disease risk factors, diagnosis and management strategies to control disease in farms. Eventually two key areas are identified:

- Better management practices (BMPs) that are practical farm-level interventions to address the key "risk factors".
- Farmer organization/self-help groups/clusters to address social and financial risks associated with farming and allow effective dissemination of the BMPs among group members.

In order to improve the sustainability of shrimp farming by increasing the consistency of production many organizations focus on the implementation of **Best Management Practices (BMPs)**. Recently, number of stakeholders insists on Good Aquaculture Practices (GAPs) during certification programmes and these two practices can be differentiated as:

- BMPs tend to be farm management practices prepared to minimize the potential for farm-raised fishery products to be contaminated with pathogens, chemicals, or unapproved or misused animal drugs.
- GAP can be defined as those practices necessary to address food safety concerns.

BMPs are based on the International Principles for Responsible Shrimp Farming which covers farm selection, farm design, water use, broodstock and post-larvae, feed management, health management, food safety and social responsibility.

The BMPs have to be simple and practical but science based their adoption requires an understanding of the farmers and their culture systems. Once implemented BMPs are analyzed both

to understand how they were developed, how they work, and what further needed for adoption by other producers. Based on the adoption analysis of BMP for shrimp culture, responsible shrimp farming in countries like Indonesia, Vietnam, Thailand and India is being rigorously practiced. The goal is to constantly seek out better practices, not just because they reduce impacts, but also because they are more efficient and more profitable. The experiences in different countries have shown that well designed and implemented BMPs can support producers to:

- Increase efficiency and productivity by reducing the risk of shrimp health problems.
- Reduce or mitigate the impacts of farming on the environment.
- Improve food safety and quality of shrimp farm product; and
- Improve the social benefits from shrimp farming and its social acceptability and sustainability.

BMPs can be country specific and are developed for a particular location, taking account of local farming systems, social and economic issues, markets and environments. In India experiences of NACA have shown that although principles are widely applicable there is considerable local variation in BMPs. Thus, implementation programmes of BMPs in various countries give boost to farmer societies and through these societies the traceability and certification programmes and access to premium international/domestic markets can be achieved. So, BMPs are often voluntary practices for local regulations but can form an important tool for certification programmes.

Pre-stocking preparations

Generally in unprepared ponds that are not well dried, the organic matter from previous crops accumulated in pond bottom cause crop losses. Even if the bottom is partially dried, the rich blackish organic muddy soils are removed and just put inside of the dike which again is the cause of poor pond preparation. Thus, for shrimp culture to be successful much depends on good bottom soil condition and its pre- preparation before shrimp stocking.

However, there is a diversity of soil which plays a vital role in shrimp culture systems. Among different soils, some soils may have undesirable properties like potential acid sulphate acidity, high organic matter content or excessive porosity. On the other hand, even if the site is good, the problems may still crop up due to the large quantity of inputs used during culture like feed and fertilizers, which lead to excessive phytoplankton production, low dissolved oxygen, high ammonia, poor bottom soil condition and other problems. Most of these problems can be avoided by adopting proper best management practices during pond preparation and culture period.

1. POND PREPARATION

The main objectives of pond preparation are to provide the shrimp with a clean pond base and appropriate stable water quality. Pond preparation has to be done for both virgin and newly constructed and in already existing ponds.

1.1 Newly constructed ponds

In newly dug out ponds, the characteristics of the soil has to be analysed first before adopting the various measures to prepare the pond. Soil deficiencies should be identified and treated in new ponds before stocking. If the soil of a new pond is acidic, it should be limed before initiation of aquaculture.

1.2 Post harvest pond preparation

Before initiating a second crop, the pond has to be prepared by drying, removing the earlier organic matter etc. However, after every cropping, the soil conditions change tremendously and are never of the same characteristics as the virgin ponds or before the first crop initiation. The various pond preparation strategies include:

i) Cleaning

The considerable quantity of waste accumulated in the ponds depending upon the culture practices must be removed to ensure sustained production. Removal of waste by draining and drying of the pond bottom after the end of the production cycle is followed for keeping pond environment clean. Two systems are commonly used to clean the pond after a production cycle. One is to allow the pond to dry out and then remove the waste or the other is to wash away the waste before it dries off.

ii) Dry method

After the final drain harvest, the pond bottom is allowed to dry and crack, primarily to oxidize the accumulated organic components. The pond bottom should be dried for 7-10 days or the soil should crack to a depth of 25 - 50 mm. The waste can either be removed manually or with machines at least 5cm of the top soil. Drying and cracking of pond bottom enhances aeration and favours microbial decomposition of soil organic matter. Drying also enhances the mineralization of organic phosphorous which is dependent to the availability in the water column and pond soil. One of the main BMP components is the disposal of solid waste away from the pond site instead on the pond bunds which is cost driven and needs site for dumping.

iii) Wet method

Alternatively in ponds where complete drying is not possible organic, biodegradable, piscicides such as Mahua oil cake (100-150 ppm) and tea seed cake (15-20 ppm) can be used for eliminating unwanted organisms. Formalin, potassium permanganate, benzyl chromium chloride, provodone iodine etc can be used to kill bacteria and external parasites and these compounds are degraded within culture systems and usually do not causes water pollution. In this method, after the final drain harvest, the accumulated black material on the pond bottom is flushed in the form of thin slurry using a pump. In certain areas sodium metabisulfate is used as a post harvest treatment of shrimp and this substance is acidic and it reacts to remove dissolved oxygen from water. However, the disposal of used sodium metabisulfate solutions has to be treated before draining in natural waters as it can cause killing of localized fish. Use of other products to a lesser extent in aquaculture include zeolite, aluminum sulfate, ferrous chloride, sodium bicarbonate, MS-222, rotenone, chlorine compounds, herbicides, insecticides, bacterial cultures, enzyme preparations and possibly others which essentially improve the soil quality and convert the waste matter. Thus, removal of the slurry is a quick and more efficient process than the dry method, reducing the period between production cycles. The advantage of this method is that waste is removed in suspension. This method needs a settling pond where waste is removed from the water and treated repeatedly to avoid polluting the local environment.

iv) Pond maintenance

During pond preparation the weak dikes are strengthened with soil and the inner slope of the dike is consolidated with outside soil other than from the pond bottom. Tunnels and holes caused by burrowing organisms are plugged. Reconditioning of the bottom trench, levelling of pond bottom using tractors and repairs of sluice structures and sluice screens are also done.

v) Liming

Liming of aquaculture ponds is done to neutralize soil acidity and increase total alkalinity and total hardness concentrations in water. This enhances pond productivity of food organisms for higher aquatic animal production. Based on either the total alkalinity or soil pH, agricultural limestone dose is estimated. If both are available and values are not in agreement, use the variable that gives the greatest agricultural limestone dose. Brackishwater ponds with total alkalinity below 60 mg l^{-1} and any pond with soil pH below 7 usually will benefit from liming.

The amount of usage of lime materials to raise the pH to 7 varies in different lime materials. Agricultural limestone is a safe product. However, burnt lime and hydrated lime can result in dangerously high pH if used in excessive amounts. So, agricultural limestone should be spread uniformly over the bottom of empty ponds up to the top of the dike and left for 10 - 15 days, or alternatively, it may be spread uniformly over water surfaces. A large proportion of the lime should be spread on the feeding areas and any part of the pond that has remained wet. Agricultural limestone will not react with dry soil, so when applying over the bottoms of empty ponds, it should be applied while soils are still visibly moist. Tilling after liming can improve the reaction of agricultural limestone with soil. Generally, for low pH 4.0 to 4.5 of about 11.5 to17.0 tons/ha is required of quick lime or agricultural lime or dolomite with lowest for quick lime. Similarly for different soil pH, the quantities have been assessed and applied accordingly.

vi) Tilling

Tilling bottom soils can enhance drying to increase aeration and accelerate organic matter decomposition and oxidation for reduction of compounds. Soil amendments such as agricultural limestone or burnt lime can be mixed into soil by tilling. Accumulations of organic matter of other substances in the surface layer of soil also can be mixed with deeper soils to reduce concentrations of the substances in the surface layer. Pond bottoms are not be tilled when they are too wet as the tillage machinery may not function to its capacity. Ruts caused by machinery will fill with soft sediment and be likely sites for anaerobic conditions. Ruts also interfere with draining and increase the difficulty of drying pond bottoms. Depth of tillage usually should be 5 to 10 cm, so mould board plows, often called turning plows, can be used to turn soil over. Tilling can be counterproductive in ponds where heavy mechanical aeration is used. Tilling will loosen the soil particles and aerator-induced water currents will cause severe erosion of the pond bottom. Thus, if bottoms of heavily aerated ponds are tilled, they should be compacted with a heavy roller before refilling.

vii) Fertilization

Decomposition in organic soils is slow because pH usually is low and the amount of carbon to nitrogen (C: N ratio) is high. Nevertheless, because of high organic matter content, such soil often becomes anaerobic during shrimp culture. Application of agricultural limestone and inorganic nitrogen fertilizers increases the pH and supply of nitrogen for faster soil organic matter degradation during fallow periods between crops. Urea can be spread over pond bottoms at 200 to 400 kg ha^{-1} at the beginning of the fallow period to accelerate decomposition of organic soil. Agricultural limestone should not be applied until a few days after urea is applied to prevent a high pH. Sodium nitrate can be applied @ 20 to 40 g m^{-2} to wet soil to encourage organic matter decomposition in wet areas. However, nitrate fertilizers are more expensive and are not recommended where soils can be adequately dried.

The rate of application of inorganic fertilizers ranges from 25 - 100 kg/ha as a basal dose during pond preparation with minimum water depth of 10 - 15 cm. When the shrimp culture progresses, depending upon the phytoplankton density as exemplified by turbidity of the pond water, required quantity of the fertilizers may be applied in split doses at short intervals for sustained plankton production. The main nutrient limiting phytoplankton production in brackishwater ponds is phosphorus. Hence both phosphorus and nitrogen should be applied in the ratio of 1:1. Excessive application of urea and ammonium fertilizers may cause ammonia toxicity to shrimp and also may lead to algal blooms reducing dissolved oxygen. During over blooming of phytoplankton copper sulfate can be used to "thin" phytoplankton blooms by applying the product at a safe concentration of one-one hundredth of the total alkalinity as it quickly precipitates to the pond bottom and does not remain in the water. However, accidental overdoses or spills can cause shrimp mortality in ponds which has to be monitored.

Shrimp being bottom dwellers, benthic organisms constitute their main food items. Hence fertilization of soil instead of water is more effective. Productivity of benthic organisms may be low in ponds with concentrations of organic carbon below 0.5 to 1.0%. Organic fertilizer can be applied to such soils to enhance organic matter concentration. Chicken and other animal manures have been applied at 1,000 to 2,000 kg ha^{-1} to pond bottoms during the fallow period. In brackishwater conditions decomposition of cattle dung is slow and hence application of chicken manure, if available, is advisable. The rate of chicken manure is one-third of cattle dung. However, application of a higher quality organic matter such as plant meals-e.g., rice bran, soybean meal, and crushed corn-or low-protein-content animal feed at 500 to 1,000 kg ha^{-1} are more efficient. When organic fertilization of pond bottoms is practiced, ponds should be filled with 10 to 20 cm of water and allowed to develop a dense plankton bloom. In shrimp farming, both organic manures and inorganic fertilizers are supplementary to each other and one cannot be exchanged for the other. It is always better to apply both organic and inorganic fertilizers together as a basal dose during pond preparation for optimum result.

viii) Nutrient exchange between soil and water

The two most important nutrients in pond aquaculture are nitrogen and phosphorus because these two nutrients often are present in short supply and limit phytoplankton growth. These two nutrients are added to ponds in fertilizers, manures, and feeds. Fertilizer nitrogen usually is in the form of urea or ammonium, and urea quickly hydrolyzes to ammonium in pond water. Ammonium may be absorbed by phytoplankton,

converted into organic nitrogen, and eventually transformed into nitrogen of shrimp protein via the food web. Ammonium may be oxidized to nitrate by nitrifying bacteria, and nitrate may be used by phytoplankton or denitrified by anaerobic microorganisms in the sediment. Nitrogen gas formed by denitrification diffuses from sediment to pond water to the atmosphere. Ammonium is in equilibrium with ammonia, and ammonia also can diffuse from pond waters to the atmosphere. A small amount of ammonium may be adsorbed on cation exchange sites in pond bottom soils. Uses of salt at concentrations up to 100 mg/L chloride are safe which can counteract nitrite toxicity. Organic nitrogen in plankton and in aquatic animal feces may settle to the bottom to become soil organic nitrogen. Nitrogen in soil organic matter may be mineralized to ammonia and recycled to the pond water, but the rate is slow.

Phosphorus usually is present in fertilizer as calcium or ammonium phosphate. Phytoplankton can rapidly remove phosphate from water, and phosphorus in phytoplankton may enter the food

web culminating in shrimp. Pond soil strongly adsorbs phosphorus, and the capacity of pond soil to adsorb phosphorus increases as a function of increasing clay content. Most of soil phosphorus was tightly bound, and only a small amount was water soluble. Pond soils are not a major source of phosphorus to water because soil-adsorbed phosphorus is highly insoluble. Phosphorus released by decomposition of organic matter in pond bottoms is rapidly adsorbed by soil and little of it enters the water. Soils that are near neutral in pH have less capacity to adsorb phosphorus and a greater tendency to release phosphorus than do acidic or alkaline soils. Nevertheless, even neutral soils remove phosphorus from the water and are a sink rather than a source of phosphorus. Once dissolved in the water, nitrogen and phosphorus originating from manures and feed also will enter the same pathways as nitrogen and phosphorus applied in chemical fertilizers.

ix) Application of microbial products for improvement of soil quality

A number of products are promoted to enhance beneficial chemical and biological processes and to improve soil quality. These products include cultures of living bacteria, enzyme preparations, composted or fermented residues, plant extracts, and other concoctions. There is no evidence from research that any of these products will improve soil quality. Nevertheless, they are not harmful to the culture species, surrounding environment, workers, or quality of aquaculture products.

x) Raising of water level

The pond is filled with brackish or seawater by pumping or by opening the sluice with proper screens to prevent entry of unwanted organisms into the pond. The water level is maintained to 30 - 40 cm and allowed to remain for 10 - 15 days. By this time, the colour of water may turn dark green with algal bloom and a layer of benthic algae along with associated food organisms will form at the bottom. Subsequently small doses of organic and inorganic fertilizers are applied based on the Secchi disc observations (transparency with 25-30 cm -optimal) of algal production. The water level is then raised to 100-125 cm. Now the pond is ready for stocking post larvae of shrimps.

xi) Monitoring of soil parameters during pond preparation

Monitoring of soil quality condition can be valuable in shrimp culture pond management. Major concerns in pond bottom soil management are low soil pH, high soil organic matter, loss of the oxidized layer, and accumulation of soft sediment. In older ponds with impaired soil quality, problems should be corrected and prevented from recurring. These materials have combined effect on the environment of the pond bottom. To understand the condition of the pond bottom, the following parameters are monitored :

a) Soil pH

This is one of the most important soil quality parameters since it affects the pond condition. Generally, soil pH ranging between 6.5 and 7.5 is the best suited where availability of nitrogen, phosphorus, potassium, calcium and magnesium is maximum. The micronutrient whose requirements are very small is also available in this pH range. The low pH of bottom sediment indicates unhygienic condition.

b) Organic matter - Redox-potential

The changes observed during culture period in the soil bottom in terms of increasing organic load forms a indicator of the soil quality conditions. Anaerobic condition developes in pond, when input of organic matter exceeds and the supply of oxygen needed for decomposition of organic matter

depletes. This reducing condition can be measured as the redox potential (E_h). Redox potential indicates whether the water or soil is in reduced condition or oxidized (E_h with '+' ve value) condition. Reduced or anaerobic sediments may occur at the pond bottom in heavily stocked pond with heavy organic load and poor water circulation. Under anaerobic condition in the pond bottom, reduced substances such as H_2S, NH_3, CH_4 etc. is formed which are toxic to benthic organisms. In shrimp ponds, development of highly reducing conditions at the inter phase layer of water and pond mud is highly undesirable. Water circulation by water exchange, wind or aeration helps to move water across mud surface and prevents the development of reduced condition. Draining at the centre of pond, as is being practiced by some farmers, is an ideal remedy for the prevention of formation of highly reducing condition especially during the last phase of culture period. Bottoms should be smoothened and sloped to facilitate draining of organic waste and toxic substances. The redox potential (E_h) of mud when exceed -200 mV, the pond condition is critical. So, during pond preparation, drying of the pond is essential as it changes anaerobic condition to aerobic condition of bottom soil.

Thus adoption of best management practices during pond preparation paves way for higher production, lesser incidence of disease outbreaks and crop losses provided further BMP procedures are strictly followed during stocking and culture phase also. One of the BMPs principles that governs a successful crop in an area is strict, uniformed and disciplined adoption of all principles by one and all in the shrimp culturists within the culture activity zone.

Shrimp seed selection

Best management practices in shrimp seed production can be defined as the practices or measures or methods adopted to secure a disease free environment in all production phases in the hatchery for improved seed quality. BMP is the ability to prevent losses to disease through effective elimination of pathogens and their carriers. The shrimp aquaculture industry has been experiencing severe setbacks due to the devastating viral diseases. These diseases are believed to be transferred between regions through the importation of hatchery broodstock, postlarvae and shrimp products. Once new pathogens are imported to an area, infection of wild stock appears to be inevitable, eliminating future possibilities of using uncontaminated wild stock to culture.

BMP encompasses policy, regulatory and programme frameworks in response to managing risks associated with diseases. The basic elements of a BMP programme in a shrimp hatchery include the physical, chemical and biological methods necessary to protect the hatchery from all diseases of high risk. Responsible hatchery operation must also consider the potential risk of disease introduction into the natural environment and its effects on neighbouring aquaculture operations and the natural fauna.

The BMP issues in shrimp hatcheries may be either **internal** concerning the introduction and transfer of pathogens within the facility or **external** concerning the introduction and transfer of pathogens from outside sources to the facility or vice versa. In case of disease outbreak within the aquaculture facilities the options available are either **treatment-** by application of methods that reduce the effects of the diseases, **containment-** by restriction of the disease from spreading to other tanks/facilities, or **elimination-** of the diseases from the vicinity. Implementation of BMP programme for a shrimp hatchery should include the following elements:

- Specific pathogen free (SPF) or high health (HH) shrimp stocks should be used.
- All the incoming stocks should be quarantined in the designated area.

- All incoming stock should be analyzed for diseases.
- All incoming water sources should be treated to eliminate pathogens.
- Equipment and materials should be sterilized and maintained clean.
- Personal hygiene measures like shower bath including washing of hands and feet and clothing.
- Knowledge of the potential pathogenic diseases and the sources of risk and methods and techniques for their control and /or eradication
- Specific pathogen resistant (SPR) stocks to be used.
- Maintenance of optimum environmental conditions.
- Immune enhancers and probiotics to be used in place of antibiotics.

Infrastructure requirements

Shrimp hatcheries should be designed (or modified, in the case of existing hatcheries) to ensure good biosecurity, efficiency, cost-effectiveness and the implementation of the hatchery standard operating procedures. A well-designed shrimp hatchery will consist of separate facilities for quarantine, acclimatization, maturation, spawning and hatching, larval and nursery rearing, indoor and outdoor algal culture, and for the hatching of *Artemia*. Additionally, there will be supporting infrastructure for handling water (facilities for abstraction, storage, filtration, aeration, heating and distribution), and feed (laboratories for analysis and preparation and storage facilities), as well as maintenance areas, packing areas for nauplii and PL, offices, storerooms and staff living quarters. The physical separation or isolation of the different production facilities is a feature of good hatchery design and should be incorporated into the construction of new hatcheries. In existing hatcheries with no physical separation, effective isolation may also be achieved through the construction of barriers and implementation of process and product flow controls. The hatchery facility should have a wall or fence around the periphery of the property, with enough height to stop the entrance of animals and unauthorized persons. This will help to reduce the risk of pathogen introduction by this route, as well as increase overall security.

Water quality and treatment

Water for the hatchery should be filtered and treated to prevent entry of vectors and any pathogens that may be present in the source water. This may be achieved by initial filtering through sub-sand well points, sand filters (gravity or pressure), or mesh bag filters into the first reservoir or settling tank. Following primary disinfection by chlorination, and after settlement, the water should be filtered again with a finer filter and then disinfected using ultraviolet light (UV) and/or ozone. The use of activated carbon filters, the addition of ethylene diamine tetra acetic acid (EDTA) and temperature and salinity regulation may also be features of the water supply system.

Each functional unit of the hatchery should have independent water treatment facility and it should be isolated from other water supply systems for other areas. Separate recirculation systems may be used for part or the entire hatchery to reduce water usage and further enhance biosecurity, especially in high-risk areas. The discharge water from the hatchery, particularly that is known or suspected to be contaminated (for example, water originating from the quarantine areas) should be held temporarily and treated with hypochlorite solution (>20 ppm active chlorine for not less than 60 minutes) or another effective disinfectant prior to discharge. This is particularly crucial where the water is to be discharged to the same location as the abstraction point.

Broodstock selection and quarantine

Some viral diseases are believed to be transmitted vertically from parent to offspring and this may be eliminated by the use of SPF domesticated shrimp. If SPF shrimp are not available, broodstock should be tested for infection by an appropriate diagnostic test and any infected individuals should be destroyed. Shrimps testing negative for the disease or pathogen should still be considered a risk and placed in a quarantine facility until their health status is fully known.

Hatcheries normally prefer wild broodstocks since they produce more healthy nauplii. In recent years, however, the high risk of introducing viral pathogens with wild broodstock has changed this preference. Domesticated stocks either genetically improved or suspected to be resistant or tolerant to specific pathogens may be used as broodstock. SPF stocks are generally maintained in highly biosecure facilities and their offspring (designated "high health" rather than SPF) are supplied to the industry. SPR shrimp are those that are not susceptible to infection by one or several specific pathogens, and Specific Pathogen Tolerant (SPT) shrimp are those that are intentionally bred to develop resistance to the disease caused by one or several specific pathogens.

The quarantine facilities are essentially a closed holding area where shrimp are kept in individual tanks until the results of screening for viruses (and for bacteria, where applicable) are known. The broodstock quarantine unit should be physically isolated from the rest of the hatchery facilities. If this is not possible, the hatchery design should be altered so that there is no possibility of contamination from the quarantine or holding area into the other production areas. Particular care should be taken with waste disposal and effluent treatment. Staff working in this area should not be permitted to enter other production sections and should follow sanitary protocols at all times. The quarantine unit should have the following characteristics:

- Adequate isolation from all of the rearing and production areas to avoid any possible cross contamination.
- Should be in an enclosed and covered building with no direct access to the outside.
- There should be means provided for disinfection of feet (footbaths containing hypochlorite solution at >50 ppm active ingredient) and hands (bottles containing iodine-PVP (20 ppm and/ or 70% alcohol) to be used upon entering and exiting the unit.
- Entrance to the quarantine area should be restricted to the personnel assigned to work exclusively in this area.
- Quarantine unit staff should enter through a dressing room, where they remove their street clothes and take a shower before going to another dressing room to put on working clothes and boots. At the end of the working shift, the sequence should be reversed.
- An adequate number of plastic buckets should be available in the quarantine room to facilitate effective daily routine movement of shrimp in and out of the facility.
- The quarantine facility should have an independent supply of water and air with separate treatment and disinfection systems and a system for the treatment of effluents to prevent the potential escape of pathogens into the environment.
- The seawater to be used in the facility must enter a storage tank where it will be treated with hypochlorite solution (20 ppm active ingredient for not less than 30 minutes) before inactivating with sodium thiosulfate (1 ppm for every ppm of residual chlorine) and strong aeration.

- All wastewater must be collected into another tank for chlorination (20 ppm for not less than 60 minutes) and dechlorination before release to the environment.
- All mortalities or infected animals must be incinerated or disposed of in another approved manner.
- Used plastic containers and hoses must be washed and disinfected with hypochlorite solution (20 ppm) before reuse.
- All the implements used in the quarantine unit must be clearly marked and should remain in the quarantine area. Facilities for disinfection of all equipment at the end of each day should be available.

Broodstock health screening

When broodstock are large in numbers, the tests may be carried out on pools of 10 individuals from different broodstock groups. Although PCR testing should be conducted on broodstock upon arrival during their quarantine, it is worthwhile to conduct additional PCR testing (at least for WSSV) after spawning. This is because there is evidence that broodstock that tested PCR-negative for WSSV during quarantine may test positive if analyzed following exposure to a stress such as spawning. Infected animals should be disposed of by incineration or some other method (e.g. autoclaving and deep burial) that will prevent the potential spread of virus. Animals should be kept under observation in the quarantine facility until all tests are completed prior to transferring them to the acclimatization area. The equipment used for the transfer should be kept separate from that used in the quarantine room and disinfected before and after transport. All equipment used in the quarantine area should remain in the quarantine area and be disinfected at the end of each day in tanks specially designated for that purpose.

Broodstock nutrition

Fresh feeds such as squid, polychaetes, *Artemia*, mussels, oysters, clams etc., must be screened for contamination before use and it is always better to use fresh feeds. The feeds may be sterilized or pasteurized to inactivate any virus as long as this does not affect the acceptability or nutritional quality of the feed. Different types of frozen feeds should be stored in separate freezers to avoid cross contamination.

Spawning and hatching

Spawning should be done in a separate room from the maturation area in order to keep the spawning area clean and to be able to carry out daily washing and disinfection of tanks without disturbing the broodstock. Water-purification steps should be taken including UV light treatment and passage through activated carbon and cartridge filtration to <1 μm. The eggs should be collected and washed with adequately treated seawater (filtered and sterilized) and then disinfected using iodine-PVP (50–100 ppm/10–60 sec) before rinsing again with abundant clean seawater.

Hatching facility should also be a separate one. The nauplii should be collected and washed with adequately treated seawater (filtered and sterilized) and then disinfected using iodine-PVP (50–100 ppm/10–60 sec) before rinsing again with abundant clean seawater. While transporting nauplii the transport vehicle should first be disinfected before entering the hatchery facilities. After unpacking the nauplii, the packing material must be incinerated. The spawning and hatching tanks are to be washed daily with calcium (or sodium) hypochlorite solution (30 ppm active ingredient), and rinsed

with abundant treated water before being refilled. This disinfection will help to reduce the risk of disease transmission.

Larval rearing and maintenance

Entrance to the larval rearing areas should be restricted only to the personnel who work in these areas. Sanitary mats or footbaths containing a disinfectant solution must be placed at the entrance of each room of the hatchery. The disinfectant solution must be replaced as necessary. At each entrance to the larval rearing room, containers with iodine-PVP (20 ppm) and/or 70% alcohol should be available and all personnel must wash their hands in the disinfection solution on entry to, and exit from, the rooms.

Each room should have a complete complement of materials such as filters, meshes, buckets etc. for routine operation. A tank (500–600 litres) containing disinfectant (hypochlorite solution, 20 ppm active ingredient) should be provided to disinfect hoses, buckets, etc. Common-use equipment can be placed in this disinfecting tank at the end of every day and rinsed before re-use the following day. The disinfectant in this tank should be replaced daily or as required. Additionally, beakers, nets etc. used for each tank should be maintained in a bucket filled with sodium hypochlorite solution (20 ppm active ingredient) and dedicated to that one tank to prevent cross-contamination between tanks within the same unit. Samples of larvae and postlarvae for routine checking should be taken in disposable plastic containers that are disposed of once used. After the daily check is complete, the larvae or postlarvae should be discarded into a plastic container with sodium hypochlorite (20 ppm active ingredient) or another suitable disinfectant. Larvae and postlarvae used in the daily checks must never be returned to the larval rearing rooms or larval tanks.

Larval nutrition and feed management

All sources of live, fresh or frozen food should be considered from the point of view of pathogen risk. Staff from these areas should not be able to enter other production areas.

Algae

Appropriate sanitary and microbiological procedures should be used to ensure the quality of the culture. Contamination with protozoans that feed on algae, other species of algae, and bacteria (in particular harmful *Vibrio* spp.) should be avoided. Alternatively, pure starter cultures can be purchased from reputable algal culture laboratories and be on-grown in the hatchery's massive tanks using sanitary procedures. The procedure of buying one lot of pure algal culture and continuously sub-culturing it throughout each larval culture cycle is not recommended, as it can easily lead to contamination of the algae and eventually, of the larvae themselves. Following disinfection of the algal culture tanks with calcium (sodium) hypochlorite solution (10 ppm active ingredient), they should be rinsed with clean, treated water and washed with a 10% muriatic acid before being left to dry.

Artemia

Certification may be requested for freedom from TSV, WSSV and YHV viruses by PCR analysis for all *Artemia* cysts purchased. After harvest, the tanks used to hatch *Artemia* must be washed with detergent and water, and then disinfected using a sponge dipped in sodium hypochlorite solution (20 ppm active ingredient), rinsed with abundant treated (filtered and sterilized) water and washed again with a 10% solution of muriatic acid. Frozen *Artemia* nauplii or adults should be stored in a separate, exclusive freezer.

Use of Probiotics

Probiotic based hatchery managements are more and more accepted where good strain of probiotic bacteria or bioremediator are used to keep the pathogenic strain at bay.

Mode of application

Probiotics as a feed can be supplied from protozoea stage onwards along with microalgal feed. There are three widely used routes of administration of probiotics - either as a food supplement or as an additive to the water. But in hatcheries preferred mode of application is immersion method or bioencapsulation of probiotic components through live food organisms. Usually in hatchery systems, its application can start in the early stage of water treatment to prevent proliferation of pathogenic bacteria and as water conditioner agent via immersion method.

Protozoea being filter feeding organisms can ingest beneficial bacteria in the rearing systems. During mysis stage probiotic is fed through either bioencapsulation of live food organisms like Artemia and rotifer with probiotic bacteria or directly incorporated in to microencapsulated diets. In the later stage of larval life cycle mainly post larvae cell wall component of probiotic bacteria like peptidoglycan, LPS and yeast beta glucan are incorporated in to formulated diets

Larval condition and health assessment for qualty seed

Assessment of the larval health and general condition are carried out on regular basis. The most simple and widely used criterion is the visual observation of fry. Active fry with dark colour is considered to be best for stocking. The PL with clean carapace should be selected and it indicates the animal is growing fast and moulting frequently. Slow growth is indicated by the presence of pathogens and necrosis. Vibriosis is the most commonly occurring bacterial disease with variety of clinical signs such as necrosis of appendages; exuvial entrapment; reddening of the pleopods, pereiopods and gills; cessation of feeding; white intestine; excessive fouling; luminescence in the water and larval bodies. The diseases mostly encountered in hatchery includes Monodon baculo virus (MBV), White Spot Syndrome Virus (WSSV), Baculoviral midgut gland necrosis virus (BMNV) among the viral diseases, Vibriosis (bacterial) and larval mycosis (fungal) and other protozoan diseases.

Health assessment in the hatchery at three levels helps producing high health larvae.

- Level I: Examination of health condition, deformity, feeding behaviors, activity and stress tolerance;
- Level II: Bacterial load of the system and animal, microscopic observation;
- Level III: Screening through PCR and other high tech tools

Data collection and record keeping regarding day to day operations, larval health, treatments/ chemicals used, water quality and other relevant information are to be performed and monitored.

Muscle gut ratio

It was reported that the wild fry has a tail muscle generally exceeds their hind gut diameter by a ratio of at least 4:1. Based on this, muscle gut ratio is used widely to assess the PL quality in many hatcheries. The measurement is taken half way between the telson and last abdominal segment. The muscle should completely fill the shell form the gut down to the ventral side. Poor quality fry will often have muscle gut ratio less than 4:1. This method is proved to be very successful. However, it

should be noted that this procedure is limited to stages before PL_{20}, as in older fry (>22 days) it is hard to measure due to the prominent pigmentation.

Postlarva size

Increased growth and reduced variability in size during post larval stage is proved to be related to further growth to juvenile stage (Castle et al 1993). Uniformity of length of hatchery reared PL is widely used as an early indicator of PL quality. Studying the production characteristics of *Litopenaeus vannamei* Clifford (1999) proposed a scale. Length uniformity was evaluated using the coefficient of variation (C. V.), which is calculated as the standard deviation divided by the mean. If the C. V is lesser than 10% the population is considered to be excellent for stocking, and if C. V. is greater than 15% the population may have been infected.

Screening of postlarvae

Larval screening for pathogens has become a pivotal part of the larval quality evaluation. Testing for virus using molecular diagnostics such as Polymerase Chain Reaction (PCR) has become increasingly common. The Principle of the PCR is of amplifying DNA of the target organisms (in this case white spot syndrome virus and detected by electrophoresis. If band in the gel correspond to a specific disease primer (eg. WSSV), the virus is present.

Stocking period

Each separate unit of larval rearing tanks within a hatchery or, preferably, the whole hatchery should be stocked with nauplii in as short a time period as possible, usually limited to three to four days. Prolonging this stocking period often results in increased incidence of disease for the later-stocked larvae, presumably through bacterial contamination from the older to the younger tanks. This phenomenon is often associated with the so-called "zoea-2 syndrome", where late zoea 1 and early zoea 2 stage larvae refuse to eat and suffer high mortality with associated bacterial problems. This problem may be controlled through restricting the time of stocking to less than four days, using probiotics and maintaining good cleanliness in all areas of the hatchery at all times.

Shipping and transfer of postlarvae

All shipping containers and equipments (nets, air stones, air lines etc.) should be disinfected before and after use. If plastic bags are used, they should be incinerated after use, they should not be re-used for shipping postlarvae or broodstock shrimp. The vehicles that deliver the postlarvae are a potential source of contamination, as they may visit several farms and hatcheries in the course of making deliveries. If possible, postlarva packing should take place at a point isolated from the production facilities, and the transport trucks (at least the wheels and tires) should be disinfected before entry to the hatchery.

Facility maintenance

After each cycle, sanitary dry out (for larval rearing) for at least every three to four months (for maturation facilities), with a minimum dry period following cleaning of seven days should be practiced. This will help prevent the transmission of disease agents from one cycle to the next. Concrete tanks painted with marine epoxy or plastic-lined tanks are easier to clean and maintain than bare cement tanks. Tanks used for broodstock spawning, egg hatching, and holding of nauplii and postlarvae should be thoroughly cleaned after each use. The procedures used for cleaning and disinfection are basically the same for all tanks and equipment. They include scrubbing with clean water and detergent

to loosen all dirt and debris, disinfecting with hypochlorite solution (20–30 ppm active ingredient) and/or a 10% solution of muriatic acid (pH 2–3), rinsing with abundant clean water to remove all traces of chlorine and/or acid, and then drying. The walls of tanks may also be wiped down with muriatic acid; outdoor tanks and small tanks can be sterilized by sun drying. All equipment and other material used in the room (filters, hoses, beakers, water and air lines etc.) can be placed in one of the tanks containing hypochlorite solution after first cleaning with a 10% muriatic acid solution. All hatchery buildings (floors and walls) should be periodically disinfected. Before stocking tanks for a new cycle, they should once again be washed with detergent, rinsed with clean water, wiped down with 10% muriatic acid and once more rinsed with treated water before filling. Disinfection procedures may require adjustment according to the special needs of the facility. Appropriate safety measures must be taken when handling the chemicals used for disinfection.

Stocking

Selection of good quality seed for stocking into a pond is the first important step of the shrimp grow-out management. The farmer must ensure that he or she gets healthy seed by purchasing them from reliable hatchery or hatcheries. It may not always be possible to obtain the desired shrimp seed due to limitations in availability and quantity. The following parameters should be taken into consideration in purchasing shrimp seed for stocking which forms one of the BMP.

(i) Size

Seeds of PL 15-20, indicated by the appearance of 4-6 spines on the rostrum, are recommended for stocking in a pond. The healthy PL should have the muscle-to-gut ratio in the sixth abdominal segment of about 4:1 or the thickness of the gut should be about the thickness of the muscle. Practically, seed from the first and second spawning of a broodstock with uniform size can be used.

(ii) Morphology

The postlarvae should have normal appearance of trunk, appendages and rostrum. The abdominal muscle must be clear, no discoloration or erosion on any parts of the body, the gut should be full of food, and the muscle should fill the carapace.

(iii) Colour

Post larvae with the presence of pigment cells in the uropods should be used since this indicates the stage of development. PL that will have high survival and growth rates will be light gray, brown to dark brown and black in colour. Signs of red or pink coloration are normally related to stress.

(iv) Behaviour

Healthy seed swim straight, respond rapidly to external stimuli such as a tap on the side of the basin, actively swim against the current when the water is stirred, and cling to the sides rather than aggregate or be swept down into the center of the container when the current has subsided.

(v) External Fouling

Seeds should be free from external parasites, bacteria and other fouling organisms. The presence of these organisms indicates unhealthy conditions, which will affect growth and survival of the PL. It is recommended that before purchasing, the farmer should visit the hatchery to check the seed once or twice either in the early morning or late afternoon, especially one day prior to stocking. However, healthy seed with some fouling may be used when the animals are in good condition after treatment.

(vi) Pathogen Free

Seed should be checked for the presence of viral occlusion bodies. Seed with large numbers of occlusions indicate stress conditions and will not so vigorous in the pond.

Stocking density

Stocking the pond with hatchery reared disease free and high quality shrimp seed will ensure a successful culture. When a farm is ready for operation, the optimum stocking density of PL in a pond should be determined in accordance with the production capacity of the farm and the culture system, which include the soil and water quality, food availability, seasonal variations, target production, and farmer's experience. It is recommended that farmers should start a new crop with a low stocking density to access the production capacity of the pond. If production is successful, then the stocking density could be increased for subsequent crops. Overstocking should be avoided since it may result in management problems and loss of entire production.

The stocking density between 6- 10 no./m^2 is for improved traditional, 10-20 PL/m2 is usually practiced in a semi-intensive culture. In an intensive culture, a well-managed pond with consistent good water quality can stock up to 25-30 PL/m^2 at 1.2 m water depth and up to 40-50 PL/m2 at 1.5 m water depth or deeper. However, it must be emphasized that intensive cultures involve high densities and can only be sustained in well-managed farms under an experienced farmer.

Technique of stocking

Proper stocking techniques will prevent unnecessary mortality of seed. The following methods have shown excellent results.

(i) Transportation

Seed are normally transported in plastic bags. The bags are usually filled up to 1/3 with water, oxygenated and then placed inside styrofoam boxes. If the transportation is longer than 6 hours, small bags of ice should be added into the boxes to reduce the water temperature and maintain it at 20-22ºC. The densities of PL in a bag are 1,000-2,000 seed/l for PL 15 and 500-1,000 PL/l for PL 20. The ideal time for transportation is in the early morning or evening to avoid excessively high temperatures during the day, unless a covered vehicle is used.

(ii) Acclimation

To eliminate stress, the seed should be maintained in water of constant salinity for at least 1-week prior to transfer. The adjustment of salinity by about 3 ppt daily is advisable. Acclimation of seed to the water pH and temperature of the pond must be rendered upon arrival. Two common techniques are used for gradual acclimation of seed to the water conditions in the pond. The first method is accomplished by placing the seed and water from the transported bag into a tank at the side of a pond containing an equal volume of well-aerated pond water. The seed will be kept for 0.5-1 hr before being siphoned into the pond. The second method, the most favorable one, is to float the plastic bag in the pond until it has reached equilibrium. The bags are opened one by one and pond water is added gradually to an equal volume. After a further 30 min of acclimatization, the seed are released directly into the pond by distributing them throughout the area of the pond or into a nursing system. The actual numbers of seed at stocking can be estimated by counting the PL individually in 3-5 bags with a spoon or small net to attain the average number in each bag and multiplied by the total number of bags.

(iii) Nursing of shrimp postlarvae

To ensure high survival and adequate feeding of seed during the first 2-3 weeks, some farms may stock the PL in a separate nursing pond or a small impoundment, usually 5-10 % of the total pond area, within the culture pond. The nursing system will help in concentrating the seed in a limited area until they reach PL 30-40 and in more accurate monitoring for survival and feeding of the PL. However, it appears that the separate nursing pond system may lead to some unfavorable results in that the size of the PL varies widely, ('broken sizes'), and the seed difficult to harvest and would experience stress during harvest and transport to the culture pond. As a result, a farmer prefers to nurse the seed in an impoundment installed inside the pond, rather than in a separate pond. Recently, some farmers employ a system in which high densities of seed (100-200 PL/m^2) are stocked into a pond for 1-2 months, and then approximately half of the juveniles are transferred to another pond by large lift nets. The same acclimation process should be performed during seed and juvenile stocking.

In a very intensive pond (stocking density greater than 30 PL/m^2) where the nursing impoundment is not available, a survival pen may be installed to estimate the survival of the seed during the first 2 weeks after stocking to allow accurate feeding management. The survival pen may be a small net pen or hapa of approximately 1 m^2 containing 100 seed or a large net pen of usually 10 m^2 at 100 PL/m^2 stocking density. In the small pen, the seed can be counted accurately while the seed in the large pen may be counted by using a 1 m^2 lift net placed with 10% of the feed. In this method, seed should appear in the lift net at 3-4 days after stocking and the number of shrimp in the net should be counted at 2 hours after feeding once daily. The survival number of shrimp can then be estimated.

If the survival rate during the nursing period is less than 50%, the problems that cause this initial mortality must be identified and rectified and the addition of more seed should be considered. Seed can be added up to 30 days post-stocking without causing a variation in size at harvest. If the survival is less than 30%, the pond should be drained and prepared for a new crop. Some farmers release seed directly into the pond. In this direct stocking method, the survival number of seed during the first 2 weeks post stocking may not be accurately estimated, since the shrimp will not approach the feeding trays during this period.

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WATER AND SOIL STRESS PARAMETERS AND THEIR MANAGEMENT FOR PREVENTION AND CONTROL OF DISEASES IN INTENSIVE *Litopenaeus vannamei* FARMING

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The water and soil quality variables affecting shrimp survival and growth are determining factors for disease outbreaks. Disease is an expression of a complex interaction of host, pathogen and environment. Darkish hepatopancreas in shrimp is disastrous as consumers will not accept and it is more significant in White legged pacific shrimp, *Litopenaeus vannamei* where the contrast between the dark areas and the pink body of the shrimp is more noticeable. This problem occurs when shrimp are farmed under poor pond conditions and stressful harvest procedures. Generally disease will not occur when the culture environment (water and soil parameters) is maintained at optimum and balanced condition. Bacterial and fungal diseases can be usually controlled by good management. Adverse water quality conditions compromise management and increase shrimp stress level, thus, making them more susceptible to diseases.

1. Sea, brackish and freshwater based *L.vannamei* culture

Vannamei can be cultured in sea, brackish and fresh waters. Culture with seawater has to be developed in farms along the coastlines where wastewater and disease pathogens can be completely drained out to open sea. Though high salinity and clear water with less plankton always causes shrimp stunt, but this high salinity water affects shrimp only at juvenile stage when they mainly consume zooplankton. Bacterial infection and pond bottom deterioration generally caused by over blooming of phytoplankton as in brackishwater ponds are not observed in seawater based culture ponds. Culture in freshwater (inland areas) requires closed system to avoid viral diseases as virus carriers grow very fast in freshwater. Groundwater may differ significantly in terms of its relative ionic composition compared to seawater. Most saline groundwater is deficient in potassium although other key ions such as sodium, chloride, calcium and magnesium can also vary considerably depending on the aquifer. Low salinity water can also react with bottom soils, significantly affecting the ionic composition of water held in open ponds. Major ion deficiencies can have serious physiological consequences ranging from stunted or poor growth through to asphyxiation, oedema and death. Potassium has an essential role in regulating sodium and therefore fluid balance within the hemolymph. Hence there is a need to supplement potassium as and when required.

2. Water and soil stress parameters

The maintenance of good water quality in ponds is essential in providing a low stress rearing environment for *L.vannamei*. It is impossible to determine all the variables to evaluate the water quality in ponds and is very important to prevent the shrimp experience stress that can accelerate the shrimp to various diseases. The important water and soil stress parameters that requires management are detailed below.

A. Transparency and water colour

It reflects the type and density of plankton. The more intense the color of water signifies the more number of existing plankton. Too high plankton density may affect fluctuations in dissolved oxygen and pH in the pond. On a sunny day, the amount of dissolved oxygen will be very high and the pH tends to lower, while the evening will be very high pH and DO can decrease to less than 2 ppm.

Transparency must be maintained at a level of 30-40 cm. Flocculation and turning water milkfish colour with little or no primary productivity and excessive amount of foam are the causes for slow shrimp growth.

B. pH (Potential hydrogen)

The shrimp should not experience stress in adjusting pH of the body to its environment. pH in pond waters should be maintained in the range of 7.5-8.5. The influence of pH is harmful to the shrimp are usually caused by the mechanism of increasing the concentration of toxic or poisonous substances, such as an increase in anionic ammonia (NH_3) at pH above 7. Whereas in waters with low pH will cause an increase in the fraction of anionic sulfide (H_2S) and the toxicity of nitrite, as well as physiological disorders in shrimp. In the long term, low pH conditions would result in the release of sodium into the water body.

C. DO (Dissolved Oxygen)

DO is a key factor for the success of shrimp culture. DO content in the morning should be above 4 ppm and above 6 ppm during the day. DO concentrations below 4 ppm make shrimp difficulty capturing oxygen, and the shrimp will rise to the surface of the water to get oxygen. If this goes on for a long time, the shrimp will suffocate. Concentration of DO in the pond waters affects the physiology of the shrimp. Shrimp growth will be slow because the rate of feed consumption decreases with decrease in DO concentration.

D. Salinity

Optimal salinity is required for shrimp to establish the metabolic processes properly. If the salinity in the shrimp body fluids is higher than the environment, the water in the environment will enter into the shrimp body so that the cell will swell. On the contrary, if the environmental salinity is higher than the salinity of shrimp body fluids, the water in the shrimp body will come out so that the shrimp become thin. Optimal salinity for growth of shrimp is 15-30 ppt. Researchers indicated that a population of *L. vannamei* juveniles infected by IHHNV (Infectious Hypodermal and Hematopoietic Necrosis Virus) grew at a slower rate when reared in a high salinity (49 ppt) than in lower salinities (5-15 or 25 ppt).

E. *Temperature*

Temperature is one factor controlling the speed of biochemical reactions and regulating the activities of cultured animals. The optimum temperature for *L. vannamei* is 28 to 32^0 C. In brackishwater shallow ponds, where regular exchange between the tidal water and the pond water is not maintained during the hot dry months, the temperature of pond water may shoot up beyond the tolerance limit causing mortality of reared shrimps. The high rate of evaporation will also occur increasing the water salinity beyond the tolerance level. Similarly, during the winter season, the low temperature will have a chilling effect reducing metabolic and growth rates of cultured shrimps. Although it is common knowledge that stocking is best done when the temperature is higher and stable, many farmers continue to take risks by stocking early in the year to get better prices with a higher risk of failure if the temperature drops. Studies show that that the rate of mortality in shrimp infected with some viral diseases such as WSSV and TSV is affected by water temperature and had total crop failures unlike those who stocked later when the temperature was high and stable. If shrimp are infected, either as PL or older shrimp, they can survive reasonably well as long as the temperature remains above 30^0 C. However, if the temperature drops below around 27^0C, mortality rates increase.

F. Alkalinity

Alkalinity is the amount of carbonate, bicarbonate and hydroxide contained in the water. Alkalinity is important because of its ability to sustain the pH, because the addition of acid without lowering the pH value. Alkalinity should be equal to or greater than 80 ppm.

G. *Metabolites*

Unfortunately a single metabolite may not be responsible for retarded growth or mortality of shrimp in ponds. It is essential to study at what level of toxicity shrimp can tolerate under combinations of two or more metabolites (ammonia, nitrite, sulphide).

i) *TAN (Total Ammonia Nitrogen)*

Ammonia is present in water in two forms, a toxic un-ionized ammonia (NH_4^+) form and a non-toxic ionized ammonia (NH_3) form, The relative amounts of these are dependent on the pH of water and to a lesser extent on water temperature. The percentage of the toxic form increases as pH and temperature rise during the day and can reach critical levels. Shrimp growth and survival can be reduced with long-term exposure to un-ionised ammonia at 0.1ppm and short term exposure to as low as 0.4 ppm. At pH levels below 8, un-ionised ammonia should be less than 10% of the total ammonia measured. It is necessary to know the pH of the pond water and use conversion tables to estimate the level of un-ionised ammonia in the pond. Due to high ammonia levels, the gills of shrimp will be turned yellow. A standard level of ammonia in the pond is not more than 0.01 ppm.

ii) *Hydrogen sulphide*

Under anaerobic condition, certain heterotrophic bacteria can use sulphate and other oxidized sulphur compounds as terminal electron acceptors in metabolism and excrete sulphide. Sulphide is an ionization product of hydrogen sulphide and pH regulates the distribution of total sulphide among its forms (H_2S, HS- and S_2-). Un-ionized hydrogen sulphide is toxic to aquatic organisms. Concentration of 0.01 to 0.05 mg/l of H_2S may be lethal to aquatic organisms and any detectable concentration is undesirable.

iii) *Nitrite*

Nitrite (NO_2) the intermediate product of bacteria mediated conversion of ammonia to nitrates more toxic in freshwater compared to brackish and seawater based culture ponds.

H. *Soil pH*

This is one of the most important soil quality parameters since it affects the pond condition. Generally, soil pH ranging between 6.5 and 7.5 is the best suited where availability of nitrogen, phosphorus, potassium, calcium and magnesium is maximum. The micronutrient whose requirements are very small is also available in this pH range. The low pH of bottom sediment indicates unhygienic condition and needs regular check up.

I. *Organic matter*

Unutilized feed, carbonaceous matter, dissolved solids, faecal matter, dead plankton etc. settle at the pond bottom and results in the accumulation of organic loads. The change in the bottom in terms of increasing organic matter load should be recorded regularly for the management of the pond bottom.

J. ***Redox potential***

Oxygen is required for the decomposition of organic waste settling at the pond bottom during culture operations. The quantity of organic load increases with the progress of the culture. When an input of organic waste exceeds the supply of oxygen, anaerobic condition develops. This reducing condition can be measured by redox meter. Redox-potential is represented as E_h, which indicates whether the bottom soil is in reduced or oxidized condition. Reduced or anaerobic sediments may occur at the pond bottom of heavily stocked pond with heavy organic load and poor water circulation. Under anaerobic condition of the pond bottom, reduced substances such as H_2S, NH_3 which are toxic to benthic organisms are liberated and diffused into water phase.

3. Management practices to maintain the stress parameters

Good pond management is critical as the water quality can deteriorate quickly due to the accumulation of organic matter from uneaten feed, faeces, dead shrimp and algal bloom crashes. Shrimp pond water quality is influenced by both environmental and management factors. Better control of water quality within the ponds became vital when farms reported incidences of shrimp coming up to the surface and problems of shrimp mortality. Water quality management is basically the management of water quality parameters daily to keep it in optimal conditions for growth of shrimp. Water management for the production of *L. vannamei* is to focus attention on measures to maintain color changes (plankton density) and increase DO concentration, use of chemical and biological technologies to improve water quality and sediment, and fed high-quality food to reduce water pollution.

A. ***Intake water treatment***

Polluted or self-polluted source water through aquaculture causes slow growth, disease outbreak and accelerated mortalities in shrimp. Reservoir has to be integral component and should be attached to grow-out ponds for sedimentation to settle organic loads and silt and chlorination treatment. Adding treated water from reservoir (approximately 30%) throughout the crop is essential to prevent excess salinity which may gradually increase through evaporation.

B. ***Water exchange***

Traditionally the management of water quality is through water exchange to reduce organic and to flush excess nutrients and plankton (cyanobacteria) out of the pond. Periodic partial removal of cyanobacterial and algal blooms by flushing or scooping out the scum facilitates optimum density and prevents sudden die-off of the bloom. However, due to increasing farm density, deteriorating intake water quality and rise in viral diseases, the use of water exchange as a method of pond water quality management is questionable. This practice increases the operating costs due to high water and energy consumption, and the lower retention time of nutrients within the culture systems, which would otherwise be available for biogeochemical recycling by bacteria and phytoplankton, thereby increasing the availability of natural food. Minimisation of water exchange will prevent viruses and carriers/bacterial pathogens from entering the ponds and reduce the possibility of disease transmission into shrimp ponds. This also led to the reduction of wastewater discharges and only the wastewater during harvest needs to be treated. But the reduction of water exchange requires closer control of water quality parameters such as pH and ammonia, effective sediment management, careful control of feeding and reduction of stocking density. However, improperly managed closed system increases the risk of stressful rearing conditions, bad water quality and diseases in ponds. Hence, the best water

management option available to farmers is limited water exchange from treated reservoir, which enables good water quality conditions in ponds, while reducing the potential of disease introduction to the farms through intake water. The potential of zero water exchange system will be greater if the nutrients generated within the system and further accumulated in the sediment could be removed.

C. ***Aeration***

In a typical black tiger shrimp pond, low rpm (revolution per minute) aerators may suffice but those with high rpm are required for *L. vannamei* culture. Paddle wheel aerators are commonly used and the newer ones such as the long arm aerators and spiral aerators can circulate oxygen to the pond bottom and apply more efficient aeration. In general, aeration to achieve more than 4 ppm of DO is related to production targets, stocking density, feed usage and salinity. Manage the concentration of DO in pond waters are very closely related to the amount and type of phytoplankton, the number and condition of the existing aerator, shrimp biomass, total organic matter content in the pond, and bacterial activity. Generally, one horsepower is suggested for 500 kg production and 50 PL/m. The placement of aerators is important to prevent localized deposition of sludge. Maintaining sufficient level of DO facilitates oxidation of ammonia to harmless nitrate by nitrifying bacteria.

D. ***Feed management***

The practice of providing food for the shrimp is trade-off between food source and water quality in the pond. It has been estimated that as much as 0.4 ppm ammonia can be added to the system for each 100 kg of feed used. Overfeeding, even in one feed can lead to sudden increases in ammonia, sometimes called ammonia spikes, a few hours later. These spikes can often be missed during daily or weekly sampling of water for ammonia levels. Thus, it is a prudent management strategy to reduce ammonia in ponds, even at lower pH. Feeding quantity should be strictly controlled, according to the weather, water quality, containing shrimp density and the actual flexibility to adjust food intake and other factors, so that smaller meals and scientific feeding.

E. ***Pond bottom management***

Pond bottom management is very important because most of the shrimp activities performed in the pond bottom. Pond bottom is a feeding area which is also where the accumulation of dirt as a result of the culture process. Keeping the pond bottom clean will indirectly protect water quality and shrimp health. Ponds with soft sludge give poorer yields. However, earthen pond bottoms can be improved with oxygenation by the tilling of the pond bottom and followed by sufficient drying and oxidation at least once a year. The accumulated materials on the pond bottom have combined effect on the pond environment. Water circulation by water exchange, wind or aeration helps to move water across mud surface and prevent the development of reduced condition. Bottom should be smoothened and sloped to facilitate draining of organic waste and toxic substances. Central drainage canal in the pond may also help in the removal of organic waste periodically. Negative (-) redox value shows reducing condition, whereas positive (+) value shows aerobic condition of the pond bottom mud. E_h of pond mud should not exceed -200 mV.

F. ***Use of chemicals, disinfectants and probiotics***

Various chemicals have been recommended for reducing the load of harmful bacteria in the pond. There is very little evidence for the efficiency of these compounds. Most of the recommended substances are broad-spectrum disinfectants including quaternary ammonium compounds (Benzalkonium chloride), buffered iodophores and calcium hypochlorite. External fouling is usually

associated with deterioration in the pond bottom or the water quality. Chemical treatment should be resorted only if the environment has been improved but the shrimp have not moulted.

If the pH in pond waters are under the range of standardized, it must be enhanced by the provision of lime. Zeolites, although widely used, have been shown in several studies to be ineffective in reducing ammonia at salinities above 1 ppt due to competition with other ions in salt water such as sodium, potassium, magnesium and calcium. Formalin (37 to 40% formaldehyde) @ 25 to 30 ppm is recommended to treat external fouling in shrimp ponds. The aerators should be allowed to run to help disperse formalin and maintain good amount of dissolved oxygen levels. Formalin is a reducing agent, which removes DO from the water, therefore if it is applied at night, DO levels must be very carefully monitored. Application of gas adsorbents or probiotics to adsorb or reduce ammonia and H_2S are being practiced. However, application of probiotics can give inconsistent results due to wide differences between bacteria counts and strains, differences in the environmental conditions in which they are used, and the slow growth of many probiotic bacteria strains in ponds.

4. Wastewater management

Coastal Aquaculture Authority has made wastewater (effluent) treatment system as mandatory for *L.vannamei* farming irrespective of the size of the farm. Shrimp farm wastewater after harvesting has to be treated and disinfected by chlorine before discharge to open water sources. The wastewater from the pond may be allowed into a settlement pond before letting it into the environment so that suspended solids may settle at the bottom and the sludge has to be removed periodically. Shrimp farm wastewater is rich in nutrients such as nitrogen and phosphorus and can be utilised by integration with other aquaculture production systems. Culture of finfish, molluscs and seaweeds in the wastewater from shrimp ponds can remove nutrients and particulate organic matter. To reuse the water, reservoir is required to ensure that water treated along the treatment system is within the standards acceptable for culture.

5. Conclusion

The two-pronged approach of combining pond management and health monitoring is the key for successful shrimp production. Sustainability of aquaculture depends on the maintenance of a good environment. The understanding of the ecological processes occurring in source water bodies and in *L.vannamei* shrimp culture ponds through regular monitoring will help us understand and solve some of the disease issues faced by shrimp farmers. It is important to know how much shrimp can be supported by the pond environment (carrying capacity of pond). Although the ideal carrying capacity can be low, higher production volumes can be achieved by partial harvesting more than once. The promotion of growth of natural planktonic or benthic microbial and microalgal communities (bioflocs and periphyton, respectively) present in the pond environment helps in the utilization of nutrients through autotrophic and heterotrophic processes accelerating the removal of organic and inorganic wastes, thus improving water quality in addition their biomass can be used as a source of food by the cultivated organisms.

BIOSECURITY AND THE ESSENTIAL PRINCIPLES SHRIMP HEALTH MANAGEMENT IN *Litopenaeus vannamei* HATCHERIES AND FARMS

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Introduction

Biosecurity in aquaculture has been defined as the sum of all procedures in place to protect living organisms from contracting, carrying and spreading diseases and other non-desirable health conditions (Moss *et al*., 1998). It is a set of standard scientific measures, adopted to exclude pathogens from the country (national level bio-security) and from culture environment and host (farm level biosecurity) and, more broadly, to limit pathogen establishment and spread. Worldwide shrimp farming activity is regressed by diseases and unsustainable practices which sometimes affecting the coastal ecosystem and livelihood. Approximately 80% of the disease losses in shrimp aquaculture (estimated to be US 15 billion $ globally) were attributed to only two pathogen groups, with viruses having approximately four times more negative impact on production than bacteria (Flegel, 2008, 2012). There is no known cure for viral diseases. By implementing biosecurity, the risk of pathological events will be reduced. Among the better management practices (BMPs), biosecurity measures are most important to prevent the transmission of diseases. The occurrence of disease is a combination of the health of the animal, the condition of the environment and the presence of a pathogen. The shrimp industry has to implement a biosecure production system to prevent the spread of infectious disease among farms. The recent successful improvement in shrimp aquaculture production in Asia from approximately 0.9 million metric tons in 2004 to 2.9 million metric tons (more than triple) in 2009 (FAO, Fish-Base Plus) is a testament of not only improved domestication and efficient breeding plans, but also GMP including biosecurity.

Once stocks leave the breeding center, they are considered high-health, which means they are free from certain pathogens to the best ability of the system to comply with the biosecurity.

Prevention of the horizontal transmission of the viral pathogens requires certain basic infrastructure, management of farm implements/ water intake and control in the entry of personnel. Twenty viruses has been identified as important to shrimp, and at times multiple viral infections by HPV, IHHNV and MBV or one (IHHNV infection) interfering with other (WSSV infection) makes it complex. Recently, disease status of exporting and importing countries in terms of past health history documentation and on-going monitoring program assume importance as per agreement of the world trade organizations. Adherence to biosecurity protocols to prevent horizontal transmission of pathogens is critical for successful farming.

Approach for implementation of Biosecurity in shrimp farm and hatchery

Pathogen exclusion Pathogen elimination

Lightner (2003) discussed ways of excluding pathogens from stock (i.e., post larvae and broodstock), especially through the use of quarantine and specific pathogen-free (SPF) certified stocks, and restricting imports of live and frozen shrimp. Excluding vectors and external sources of contamination and preventing internal cross contamination were suggested methods for excluding pathogens from hatcheries and farms. Physical, chemical, and biological precautionary measures along with the second line of defence in shrimp can be improved for pathogen exclusion and elimination.

Physical measures are implemented through prevention of intrusion of pathogen by:

Physical barriers

Water screening

Quarantine

Chemical measures include treatment to eliminate the potential pathogen risk:

Chlorination and Ozonation for water

Iodine and Chlorine for disinfecting tools

Biological measures through utilizing Specific pathogen free stock or fortifying the animal:

SPF stock

SPR stock

Immunostimulants or biomodulators to induce ability of shrimp to prevent disease

BIOSECURITY THREATS

Stocking pathogen free post larvae alone doesn't guarantee a disease free culture since the pathogens could still enter the culture environment horizontally and infect the shrimps during the culture. Viral pathogens can still enter the culture environment through the following means and a better understanding of these can help in prevention of horizontal transmission

- By persisting in the soil
- Intake water
- Aquatic vectors introduced through intake water, by crabs and other animals.
- Besides the above mentioned carriers, viral particles can also enter the farming system by mechanical carriers like:
  - Contaminated land animals and birds
  - Contaminated farm inputs–through live feed, semi-moist feed
  - Contaminated farm implements, nets and vehicles etc.,
  - Contaminated personnel

Crabs are one of the carriers of viral pathogens and providing crab fencing in shrimp farms is considered as one of the important biosecurity requirements. Carriers like crabs could also move from pond to pond over land barriers. To prevent such movements fencing made of 0.5 m plastic sheet should be put around the culture pond.

Feed ingredients of aquatic origin used in aquaculture can be a source of pathogens (viruses, bacteria and parasites) to shrimp species. Pathogens in feed can infect the animals directly by means of consumption of feed or indirectly via environmental sources. Live feed and moist feed are more likely to contain pathogens because their ingredients are either in a raw state or subject to insufficient processing.

Birds such as crow/water crow pick up the dead and moribund shrimps affected with viral disease from ponds and may drop in unaffected ponds, thereby transmitting the virus mechanically. This could be avoided by using bird scares and bird fencing over the pond. Similarly land animals like dogs, cats and cattle can mechanically carry the virus from one pond to another. Preventing entry of stray animals and unauthorized personnel into the farming area through fencing is the only way to address this problem. All vehicles must pass through a wheel bath with dimensions such as to assure complete washing of the wheels. The wheel bath must be regularly filled with an effective disinfectant solution [such as sodium (calcium) hypochlorite at >100 ppm active ingredient].

The entry of potential disease vectors into the hatchery facility must be controlled. Farm Bio-security requirements like reservoir ponds, fencing, crab fencing, bird fencing and disinfection facilities are incorporated in the design. To avoid disease in most cases zero-water exchange system of farming is practiced with recirculation facilities. In such cases more than 40% of the water area in the farm is allocated for reservoirs and waste sedimentation ponds.

Prevention of horizontal transmission of virus through strict bio-security protocol is essential. This includes

- Tyre-bath, foot-bath and hand wash are required to be provided
- Chlorination of intake water in reservoirs
- fencing around the farm
- crab fencing
- bird fencing
- use of independent implements and personnel for each pond

Pond to pond transmission of virus within a farm could easily occur through the use of farm implements and farm workers. Providing an independent set of implements for each of the ponds will be the best solution. Routine disinfection of the implements before every use should also be made a part of the SOP so that it becomes the routine practice with farm personnel. Similarly disinfection of hands and feet of the farm personnel before entry into any pond should be made mandatory.

Workers move from pond to pond attending to their work. So, restriction on the movement of farm workers from pond to pond is necessary. Personal disinfection has to be instituted among the farm workers before they enter the pond and after they come out of the pond.

HACCP in Biosecurity: Biosecurity, or "hazard reduction through environmental manipulation" (Plumb, 1992), is often defined as practices that reduce the number of pathogens that enter a facility. The HACCP approach is a preventive risk management system based upon a hazard analysis and has been widely used to identify and control risks to human health in food-processing systems. The critical control points (CCP) identified for the maturation and hatchery stages of shrimp production are the shrimp the feeds and the water. Maximum biosecurity in shrimp production facilities can be achieved through the isolation of breeding, hatchery and production phases. For different areas such as quarantine, maturation, hatchery, algal culture, *Artemia* production etc., it is necessary to identify critical control points.

Hatchery workers must be restricted to their specific area of work. The SOPs should address risks due to staff whose duties require them to pass through areas of the hatchery with different biosecurity

classifications. All staff must take adequate sanitary precautions when entering and leaving a production unit.

BIOSECURITY MEASURES DURING CULTURE

As preventive measures

Observing GMP or good management practices

Installing biosecurity measures

Monitor the presence of viruses through disease diagnostic laboratory

In case disease outbreak occurs

Containing contaminated pond water and not draining for at least one week after disinfection

Eradicating hosts stock before disinfection

Reporting disease outbreak in the area

After disease outbreak

Proper treatment and modifying culture system for ensuring successful crop

BIOSECURITY IN HATCHERY/POND MANAGEMENT

DESIGN AND CONSTRUCTION: Provision of buffer zones between aquaculture farm-to-farm, farm to agricultural land, farm to village and farm to other ecologically sensitive areas is also being made mandatory for obtaining license. The extent of buffer zones required will depend on the site characteristics, soil quality and tidal conditions. Though ETP has become mandatory for larger farms of above 5 ha, it is necessary that smaller farms that are located in close proximity to each other should consider setting up of common ETP to avoid self-pollution.

GOOD POND PREPARATION: It is one of the most important steps which determine the success of a shrimp farm. Following measures should be adapted during pond preparation.

a. **Removal of bottom sludge**: This involves the removal of black soil layer which contain high organic content.

b. **Ploughing of soil**: It exposes the black soil layer to sunlight and atmospheric oxygen which oxidize the organic waste.

Drying for at least two weeks to kill all disease causing organism such as fungi, protozoa, bacteria and viruses by oxidation

WATER INTAKE: Intake water must be filtered with fine mesh screen filter bag to prevent the entry of virus carriers such as crabs, wild shrimps and also to avoid entry of fish or crustacean, which may be predator or competitor for shrimp. Every drop of intake water must be disinfected with 60 ppm calcium hypochlorite and left for 3-4 days. It reduces the risk of pathogens and carrier like virus carriers such as wild shrimp, crabs, mysids, copepods and other crustaceans. Many animals like mudskippers, snakes, frogs could be out of farm by installing a fine net enclosure. Reservoir should be incorporated for any farm with even two ponds. Disinfection of water and fertilization for growth of plankton can performed in this reservoir before pumping in to grow out ponds.

SEED SELECTION AND STOCKING: Quality of post larvae plays a major role in success of shrimp farming. Before purchasing, shrimp post larvae should be checked for their general condition such as

activity, color, size, *etc*. If there is any dead and abnormal colored PL in the tank, the entire batch should be rejected. Before stocking at the pond, PL should be treated with formalin at 100 ppm concentration for 30 minutes in well aerated tanks to remove weak PL. The importance of disease free seed is fully understood by the farmers and presently most of the farmers undertake PCR testing of the seed to ensure virus free seed.

WATER QUALITY AND FEEDING MANAGEMENT

Disease outbreaks in shrimp grow out culture is directly related to pond bottom and water quality. Though water exchange at regular interval maintains the good quality pond water, zero water exchange (no water exchange) or minimal water exchange is advisable as it reduces the incidence of disease outbreak by ensuring the biosecurity. Ponds using aeration tend to have higher shrimp production and low disease outbreak. regular monitoring of soil and water quality parameter like temperature, salinity pH, total suspended solid etc. and chemical parameter such as ammonia, nitrate, nitrite, total organic carbon, dissolved oxygen, BOD, COD etc. Excess feeding and old feeds should be avoided; check tray and monitoring and feeding should be done as per the standing biomass and physiological condition of shrimp. Restricted feeding during molting should be practiced and live crustaceans or trash fish and its frozen product should never be fed.

HEALTH MANAGEMENT: It is well understood fact that control and prevention of diseases in aquaculture is a function of management. Disease problems arising in aquaculture can be attributed primarily to the environmental insult and most of the pathogens are facultative pathogenic in nature. Hence, management of pond or hatchery environment is of utmost importance for disease prevention and control. Time to time or at critical points shrimps should be checked for their general health conditions, like external appearance (body color, missing appendages, external / gill fouling, black gills or gill choking, *etc*), gut condition and growth in terms of weight or length. Shrimp behaviour and feeding trends should also be monitored. The gut content colour is a good indicator of the probable health status and corrective action to be taken. A black / brown / green gut content implies under feeding whereas a red or pink gut showed disease manifestation whereas a pale whitish gut showed gut infection. A normal gut will have a light or golden brown colour.

Probiotics, immunostimulants, bioremediating agents can be employed as prophylactic and disease treatment measures in grow out culture and hatchery system. Probiotics is a live microbial feed supplement which beneficially affects the shrimp by improving its intestinal microbial balance. Most commonly used probiotics in shrimp culture are Yeast, *Bacillus*, *Vibrio alginolyticus*, *Lactobacillus acidophilus* etc. Immunostimulant is a chemical or drug which enhances the non specific defense mechanism of the animals thereby impart better generalized protection. Many substances such as â-glucan, chitin, lipopolysacharide of gram negative bacteria, peptidoglycan of gram positive bacteria, lactoferrin, levamisole and many nutritional factors and cytokine are used as immunostimulants. There is a serious concern on the use of antibiotics and their use in shrimp farming should be avoided.

Biosecurity is never absolute because all the physical systems we can conceive in an economic system are imperfect. The practical role of ozone is diminishing the viral residual that escapes physical barriers.

Use of Level 1, 2 and 3 diagnostics in shrimp hatcheries is required for understanding any bio security lapses and take corrective measures before it is too late. Examination of broodstocks for their general health condition, removal of sick and moribund individuals, selection of quality nauplii and

larval stages by microscopic observation, PCR screening are required for checking the biosecurity efficacy of the system.

EFFLUENT TREATMENT

Pond effluents should be treated as per the norms set by coastal aquaculture authority of India Treatment of effluent is mandatory for bigger farms and collectively for smaller farms. This includes disinfection or biological filtration through cultivation of algae, sea weeds, clams and filter feeders or omnivorous fishes to reduce the excess organic matter and pathogenic microorganisms.

RECORD MAINTAINANCE

Records are necessary to identify problems in the pond environment and shrimp health and to rectify these problems at the earliest during the production cycle. Record keeping also helps the farmer to learn from past mistakes, thus reducing risk and costs of production in subsequent crops.

CONCLUSION

The virus can enter the shrimp and pond through different routes, including shrimp seed, water, carrier animals and transfer of infected animals and farm equipment from one farm to another. Evolving culture practices addressing biosecurity threats like that of closed systems and zero water exchange systems are preferred as WSD is widespread along the coastal waters. BMPs are to be followed strictly with biosecurity measures. Other problems faced by the farmers appears to be low farm-gate prices resulting from competition in the global market and losses due to disease can make things worse. These guidelines can never be considered as panacea for all the problems but at best can be taken as an indication of possible solutions since the environmental and social issues are all very site-specific. Biosecurity is relatively new approach, still in need of improved information on diagnostics, disease transmission, disinfection and eradication and should be further refined with nutrition, health, genetics and environmental science. With the adoption of domesticated, SPF stock and operating under obligatory biosecurity conditions follow global aquaculture practice (GAP), the future of penaeid shrimp aquaculture looks bright.

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Detection, Prevention and Management of diseases

MOLECULAR TECHNIQUES FOR THE DETECTION OF MAJOR VIRAL PATHOGENS OF *Litopenaeus vannamei*

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Shrimp farming is one of the fastest growing aquaculture sectors in many parts of the world. The world marine shrimp aquaculture industry has experienced rapid changes over the last few years. Culture of the introduced species, Pacific white leg shrimp, *Litopenaeus vannamei* has become very popular among shrimp farmers in S.E.Asian countries including India, as an alternative species to black tiger shrimp, *Penaeus monodon* which was affected by disease problems and syndromes leading to near collapse of the industry. In intensive farming, with the increase in stocking density, diseases are a natural consequence and damage due to them is very visible because of their negative impact. It is an established fact that viruses are the most important causative agents of several shrimp diseases. A quick response and damage control is required to prevent the spread of the disease. Early detection of sick/dying shrimp, use of pond side diagnostics and safe disposal of dying shrimp will help reduce the impact and spread of viral diseases. Some of the important viral diseases of *L.vannamei* listed by the World Animal Health Organization (the OIE – Office International des Epizooties) is given below.

Table 1 Major viral disease of penaeid shrimps proposed by OIE.

Disease	Infectious Agent
Taura syndrome	Taura syndrome virus (TSV)
Infectious hypodermal and haematopoietic necrosis	Infectious hypodermal and haematopoietic necrosis virus (IHHNV)
Yellow head syndrome	Yellow head virus (YHV)
White spot syndrome	White spot syndrome virus (WSSV)
Penaeus vannamei nodavirus disease	*Penaeus vannamei* Nodavirus (PvNV)
Infectious myonecrosis	Infectious myonecrosis virus (IMNV)
Hepatopancreatic parvovirus disease	Hepatopancreatic parvovirus (HPV)
Tetrahedral Baculoviriosis	Baculovirus penaei (BP)
Spherical Baculovirosis	Monodon Baculovirus (MBV)

Molecular methods

Due to the importance of shrimp culture, the availability of easy and rapid methods that allow early diagnosis is essential for routine monitoring of the animal health status and to restrain further disease outbreaks. Rapid detection of pathogens would be very essential for effective health management in aquaculture. While conventional microbiological isolation methods are used in case of bacterial pathogens, histopathology is widely used to detect viral infections. However, these methods are time consuming and lack sensitivities to detect latent pathogens. Efforts to overcome these problems have led to the development of immunoassay and DNA-based diagnostic methods. These molecular techniques facilitate the specific detection with high sensitivity, thus allowing rapid screening of viral pathogens.

1. Nucleic acid based diagnostic methods

1.1. Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a highly sensitive and robust technique for detection of shrimp pathogens. By definition, PCR is a nucleic acid amplification technique wherein a specific portion of nucleic acid from a target organism is amplified *in vitro*. Herein amplification is achieved using oligonucleotide primers that are specific for the portion of the DNA to be amplified. By designing oligonucleotide primers that are specific for an organism, it is possible to design PCR to amplify specifically DNA from any desired organism. The amplification requires the enzyme DNA polymerase, and the building blocks of DNA, the deoxyribonucleotides (dATP, dTTP, dGTP, dCTP). The reaction is performed in several cycles, each cycle consisting of three steps (a) DNA denaturation: this is the step in which the target DNA strands are separated by heating to about 95°C, (b) Primer annealing: this is the step in which the primer binds specifically to the target region. This step is carried out at 55-65°C, (c) Primer extension: this is the step in which the new DNA strand is synthesized by the DNA polymerase on the template strand. Normally about 30 cycles of reaction are performed. Since each cycle involves denaturation of DNA at 95°C, the DNA polymerase used in the reaction should be thermostable. The discovery of thermostable DNA polymerase from the thermophilic bacterium *Thermus aquaticus* led to rapid application of PCR in diagnostics.

PCR has been widely applied to the detection of shrimp viruses so that the risk of disease can be controlled. This includes screening of broodstock, larvae and post larvae in the hatchery and before stocking. PCR is also used for identifying carriers, checking water and sediment for viral contamination and monitoring health of shrimp in grow out ponds.

1.2. Nested PCR

The sensitivity and specificity are the most important parameters of a detection method; nested PCR has been developed for this purpose, in which two sets of PCR primers are sequentially used. The first primer set amplifies a target sequence, which then serves as the template for a second amplification. The second primer set lies internal to the first amplicon. This secondary amplification will not occur if the primary amplification did not happen. A major shortcoming of nested PCR is that the reaction vessel needs to be opened toad the second primer set which increases the contamination probability from the laboratory environments.

1.3. Multiplex PCR

Multiplex PCR has been successfully applied in many areas of nucleic acid diagnostics. The cost and limited volume of test samples are the key points for the pathogen detection. The process is termed multiplex PCR, since multiple sets of primers are included in a single reaction tube. In this procedure, more than one target sequence is amplified in a single reaction system by including more than one pair of primers. A key point in the development of a multiplex PCR assay is the design of the primers. All of the primers must be designed with very close annealing temperature, and the amplification products need to be of markedly different sizes so as to be easily differentiated by agarose gel electrophoresis. In addition, the multiplex primers might cause interference in the amplification process, which often makes it difficult for optimization of the reaction, especially when the number of primer pairs in the reaction system increases.

1.4. Reverse-transcription PCR

In reverse-transcription PCR, the RNA target as of a RNA virus is first converted into a complementary DNA (cDNA) by the reverse transcriptase enzyme. This cDNA is used as template and amplified by standard PCR methods. Reverse-transcription PCR is used not only to detect pathogens, but also to detect the specific expression of certain genes during the course of growth or infection since they are amplified at a much higher number of messenger or ribosomal RNA than the number of DNA copies. In contrast to the detection of DNA from nonviable organisms using standard PCR, the detection of cDNA from messenger RNA encoded by a pathogen using reverse-transcription PCR could be evidence of active infection.

1.5. Real-time PCR

Real-time PCR which is used to amplify and simultaneously quantify a targeted DNA molecule enables detection and quantification of the viral pathogen in the tissues of infected shrimp. It offers continuous monitoring of PCR product formation throughout the reaction and eliminates post-PCR analysis process. Thus, it shortens detection time compared to standard PCR, and reduces the risk of amplicon contamination by frequent handling during various steps of conventional PCR. By using this technique the viral load in infected shrimp can be accurately determined which in turn helps in risk assessment as well as disease monitoring during culture. Four types of indicators have been used most frequently in real-time PCR methods for pathogen detection: TaqMan probes, SYBR Green dyes, molecular beacons, fluorescence resonance energy transfer (FRET) hybridization probes.

In TaqMan probe, a single stranded oligonucleotide probe complementary to a segment of 20 to 60 nucleotides with in DNA template and located between the two primers is used. In this assay a fluorescent reporter and quencher are covalently attached to the 5′ and 3′ ends of the probe, respectively. The single stranded probe does not show fluorescence due to close proximity of fluorochrome and quencher. During PCR the 5′ to 3′ exonuclease activity of *Taq* polymerase degrades the portion of the probe that has annealed to the template, releasing the fluorochrome from proximity to the quencher. Thus fluorescence is directly proportional to the fluorophore released and amount of DNA template present in the PCR product.

SYBR Green chemistry is an alternate method used to perform real-time PCR analysis. SYBR Green is a dye that binds to the minor groove of double stranded DNA. Here the intensity of the fluorescence emission increases with the amount of SYBR Green dye that binds to double stranded DNA. As the synthesis of double stranded amplicons continues in an exponential manner, SYBR Green dye signal increases.

In real time PCR assay, the exponential increase in the fluorescence is used to determine the cycle threshold (Ct), which is the number of PCR cycles at which significant exponential increase in fluorescence is detected. Using a standard curve for Ct values at different DNA concentrations, quantitation of target DNA in any sample can be made. Real time PCR assays have been successively applied for detection and quantification of IHHNV, TSV, WSSV, YHV, HPV etc. The real time multiplex PCR for the detection of more than two viral pathogens has been developed.

1.6. Loop-Mediated Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method using single temperature incubation. It allows amplification of DNA with high specificity, sensitivity and rapidity.

This technique can amplify target nucleic acid to 10^9 copies at 60–65 æ%C within 1 h. The amplification of nucleic acid is based on the principle of strand displacement DNA synthesis by the *Bst* DNA polymerase large fragment. The specificity, sensitivity and rapidity of LAMP are due to the high strand displacement activity of the *Bst* polymerase and a set of two inner primers and two outer primers. LAMP is highly specific for the target sequence because of the recognition of the target sequence by six independent sequences in the initial stage and by four independent sequences in the later stages of the LAMP reaction. The amount of amplicons generated can be quantified in real-time either by measuring the turbidity or by the signals produced by fluorescent dyes that intercalate the DNA. As the reaction is conducted under isothermal conditions, it can be carried out with a simple and inexpensive water bath so that a thermal cycler is not required. In addition to being inexpensive, isothermal amplification technique is further simplified by the use of chromatographic, lateral flow dipstick. Rapid detection of viruses by LAMP of genomic material with high specificity and sensitivity can be applied for diagnosis, monitoring and control of diseases in shrimp aquaculture. LAMP has been developed for the detection of major shrimp viruses including TSV, YHV, WSSV, IMNV, IHHNV, MBV, and HPV.

1.7. PCR–Enzyme Linked Immunosorbent Assay (PCR–ELISA)

The PCR–ELISA is an alternative method for the detection of nucleic acids which mimic enzyme linked immunosorbant assays. The technique mainly involves amplification of viral DNA by PCR followed by hybridization of the PCR product with a specific probe and finally the detection of the hybridized product by ELISA technique. In this assay, the PCR products will be hybridized to an immobilized capture probe with sequences internal to the PCR product. Thus, it is an alternative and less expensive technique than real-time PCR. PCR–ELISA, a promising diagnostic tool has been developed for detection of major shrimp viruses. This technique could detect up to three viral particles. Hence, PCR–ELISA is more sensitive than conventional PCR and histological examination and can be used for field level applications where large numbers of samples can be analyzed simultaneously.

1.8. Probe Techniques

The development of non-radioactive labeling of nucleic acid frgments has made gene probe technology readily available in shrimp diagnosis. This technology was first developed for the diagnosis of IHHNV and now it is being used for other shrimp viruses. Non-radioactively labeled digoxigenin (DIG) DNA probe has been used in dot blot, *in situ* hybridization and southern blot hybridization for detection and analysis of major viral pathogens of *L. vannamei viz* IHHNV, TSV, YHV, WSSV, HPV, MBV etc.

2. Immunological based diagnostic methods

A large number of immunological methods have been developed for the detection and characterization of shrimp viruses. Immunological methods such as immunohistochemistry (IHC), lateral flow chromatographic assay, enzyme linked immunosorbent assay (ELISA), agglutination (slide/latex), fluorescent antibody test (FAT/IFAT), and blot (dot-blot/dip-stick/western blot) enable rapid, specific detection of pathogens without the need to first isolate the pathogen. Both monoclonal and polyclonal antibodies (mAbs) provide ideal standardized reagents for such tests and many are now commercially available against a variety of shrimp pathogens. As an alternative to PCR, the progress in the use of immuno-based detection methods for shrimp viruses has been rather slow. This is primarily because of the difficulties associated with the availability of a purified antigen for immunization,

availability of purified antibodies and specificity and sensitivity that matches polymerase chain reaction based tests.

2.1. Enzyme Linked Immuno Sorbent Assay (ELISA)

The most widely used antibody based diagnostic technique is enzyme based immunoassays. In these techniques, the antibody molecules are linked to enzymes either directly or indirectly. In the direct method, the enzyme is conjugated to a portion of the antibody molecule that does not bind to the antigen. In the indirect method, a second step is required. Here, a carrier or second antibody is linked with the enzyme. The amount of enzyme is important in producing measurable signal when the primary antibody binds to its target. It detects specific substances in a complex mixture by binding them to antigen or antibody coated substances. Once binding has occurred, other reagents are added that allow the captured substances to be linked to indicators or enzymes, which can be quantified. The degree of colour change is proportional to the amount of antigen in the sample and thus the technique lends itself to quantitation.

2.2. Western blot

They are not used routinely as diagnostic methods, but their application can be useful in the characterization of pathogens. In western blotting, proteins are first separated by size, using sodium dodecyl sulphate polyacrylamide gel electrophoresis. The proteins in the gel are transferred to a membrane support nitrocellulose, and the membrane is then probed with pathogen-specific antibodies. Antibodies that bind to the proteins of interest can be visualized using chromogenic substrates, fluorescence, chemiluminescence or autoradiography.

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CLINICAL APPROACH TO DISEASE DIAGNOSIS AND CURRENT STATUS OF DISEASES IN *Litopenaeus vannamei* FARMS IN INDIA

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Introduction

In the recent years Indian shrimp farming is experiencing a rapid growth with the introduction of the exotic species *Litopenaeus vannamei* as an alternate candidate species for culture in the coastal states of India. The advantages of this species are the availability of Specific Pathogen Free (SPF) seed, fast growth in euryhaline conditions, maximum yield and high market demand in other countries. The culture of *L. vannamei* has been expanding very rapidly in India as most of the farmers switched over to culture *L. vannamei* and the prospects are very high to substitute *P. monodon* culture gradually. Despite the progress, there is a threat that *L. vannamei* culture system in India may be hit by viruses unless precautionary measures are adopted. As a consequence of the rapid growth and the current development of the penaeid aquaculture industry, many of the most significant shrimp pathogens were moved from the regions where they initially appeared to new regions even before the ''new" pathogen had been recognized, named, proven to cause the ''new" disease, and before reliable diagnostic methods were developed (Lightner, 2011). *L. vannamei* is known to be vulnerable to a wide range of viral diseases, and the reports of mass mortalities due to these infections and failure of the culture system have also been recorded. The social and economic impact of the pandemics caused by these diseases is profound in our country. This paper deals with clinical approach and current status of the diseases *L. vannamei* in India.

Clinical Approach

Farm history

It starts with getting the detailed history of the farm from the farmer which includes stocking density, seed source, feed, culture details, water source, salinity, dissolved oxygen, application of fetilizer if any, mortality pattern, symptoms exhibited by the morbid shrimp and previous disease outbreak, if any.

Gross observations

The disease investigator should observe the moribund and dead shrimps for any gross changes/ lesions exhibited by the animal.

Sample collection

This is the most important and crucial step in disease diagnosis wherein proper sample collection is essential for acurrate disease diagnosis. This includes collection of tissues from the moribund shrimps, feed from the farm for toxicologic analysis. These all will helps in arriving at a tentative diagnosis of the shrimps.

Bacterial disease

For diagnosing bacterial diseases haemolymph should be collected from the moribund shrimp and it can be plated on Zobell marine agar plates (ZMA) / Thiosulphate Citrate Bile Salt (TCBS) agar plates. Staining of the haemolymph smear will also give details about the infection.

Viral disease

The infected shrimps should be collected in 90% alcohol, RNA *later* or in ice for virus isolation and for molecular tests. For histopathological examination the tissues should be collected in Davidson's fixative or in 10% formal saline. Small shrimps can be collected as such in fixative but if it is of >12g size the abdomen can be slit open and then immersed into the fixative.

Miscellaneous diseases

For identifying parasitic and other diseases shrimps should be collected in physiological saline. On reaching the laboratory the squash preparation can be made from gills, hepatopancreas and other tissues which can be examined under microscope.

Haemocyte enumeration

Haemolymph will give an indication about the health status of the animal. Based on the clotting period and consistency of the hemolyph we can arrive at an conclusion whether the shrimp is affected by disease or not. Differential haemocyte count (DHC) will also give clear picture of the disease. Based on the morphological changes of the hemocyte, we can identify the nature of infection in shrimps. Determination of DHC, haemolymph clotting time, glucose, lactic acid, fatty acids, certain enzymes, etc are also used as an aid in disease diagnosis.

Disease diagnosis in shrimp culture

Diseases may be infectious, *i.e.*, caused by living organisms like viral, fungal, bacterial or parasitic organisms, or non-infectious, *i.e.*, caused by non-living factors, eg. nutritional deficiencies, behavioural problems and hostile environment or toxins. General principles of aquatic animal disease diagnosis may be applied in *L. vannamei* culture also. Asia Diagnostic Guide to Aquatic Animal Diseases (FAO, 2001) provides a broad outlines of general principles of diagnostics in aquatic animals for both farm and laboratory level diagnosis. This is built on a frame work of **three levels** of diagnostics and activities at each level are in fact, built on each other, contributing valuable data and information for optimum diagnosis.

Levels of diseases diagnostics

Level I Diagnostic activity include farm/production site observations, history and health management. A presumptive diagnosis based on clinical signs, necropsy, gross lesions etc. may be achieved at this stage. This forms the baseline for other diagnostic levels (II & III).

Level II Diagnostic activity includes parasitology, bacteriology, mycology and histopatholgy which require moderate capital and training and hence diagnostic site is at laboratory level. A definitive diagnosis can be reached at this stage.

Level III Diagnostic activity includes specialized areas like virology, immunology, molecular biology and electron microscopy etc. which require significant capital and training investment. Offers confirmatory diagnosis based on the principles of advanced diagnostic capabilities of the laboratory.

However, many of the level III diagnostics based on immunological / serological methods comes as field kits at farm site (Level I) eg. Immunochromatographic kits against WSSV indicating Level III

diagnosis accessible to the field. It is also important to know the diagnostic capabilities of the each laboratory for level II and III diagnosis before the dispatch of appropriate samples so that disease outbreaks are investigated readily and promptly. The three levels have wide range of application in disease diagnostics. Though, they all have their own limitations in terms of apt application, consistent sampling, testing protocols and elucidation of results and are also of limited value to newly emerging diseases where the etiology is unknown. In such cases, histology, a non-specific general technique focusing on the potential etiologies, is still the most suitable method to interpret diseases precisely. With the development of these technologies which has lead to enhanced rapid detection and diagnosis of disease crucial for early and effective disease control, there will be practical problems in their application.

Risks associated with vannamei culture

Being an exotic species, *L. vannamei* farming is associated with potential risks associated with diseases as shown below:

- *Risk associated with the existing pathogens in the country*–Even if SPF stocks are used, there is risk of getting these stocks infected with deadly existing pathogens in the system such as WSSV. Risk is also associated with the wide prevalent but comparatively low virulent MBV, HPV, IHHNV and LSNV. These less virulent virus may not bring large scale mortality but able to reduce growth to significant extent and there by bringing huge production loss.
- *Risk associated with exotic pathogens*–Many of the exotic viruses are already shown to be highly virulent to several cultured shrimps. Some of these are YHV, TSV and IMNV. Introduction of these viruses may bring catastrophic situations if it escapes our quarantine systems to check the imported stocks.
- *Risk associated with the emerging diseases / pathogens*–Every now and then, new pathogens are reported which are found to bring emergency situations. This include the recently reported 'Early mortality syndrome (EMS)', 'Running mortality syndrome', 'Abdominal Segment Deformity Disease (ASDD)', 'Loose Shell Syndrome (LSS)' and 'Monodon Slow Growth Syndrome (MSGS)'. Many times the avirulent or less virulent bacteria/virus also becomes major pathogens after genetic modification.

Diseases of vannamei - Global scenario

The cultured shrimp species encounters diseases caused by various etiology of which the infectious disease caused by virus stands out to be more significant as the loss caused by them is more. The common diseases that have been reported to occur in vannamei, which are of economic importance are White spot Disease (WSD), Infectious Myonecrosis (IMN), Infectious Hypodermal and Hematopoietic Necrosis (IHHN), Taura Syndrome Disease (TS) and Yellow Head Disease (YHD). IMN disease has recently affected shrimp farms in the northeastern states of Brazil where it has caused serious mortality and production loss. An unpublished data from Brazilian Shrimp Farmers Association have estimated that the loss due to this disease from 2002 to 2006 in Brazil exceeds $100 million. Similarly in Indonesia this disease is very important and found that the expected loss may exceed $1 billion by 2010. A total of $100–200 million loss has occurred mainly because of this disease in America, but in Asia this disease has emerged in the year 2006 was and the estimated loss is about $1 billion. Significant loss from yellowhead virus (YHV) disease amounts to US $1 billion per year in Asia was reported. White Spot Syndrome Virus (WSSV) caused US $6 billion loss (emerged in the year 1992-

1993) in Asian cultured shrimp while in America WSSV has caused US $1–2 billion loss. *Pv*NV is a new pathogen first reported from Belize in 2004 as the cause of muscle necrosis leading to as much as 50% reduction in production. This is a relatively new disease reported from Thailand and Indonesia seen in *L. vannamei*. From Indonesia, high mortality (60% to 90%) of juvenile shrimps in *L. vannamei* hatcheries and shrimps which are less than one month old in grow-out ponds has occurred since 2007 in association with haplosporidian infections of the hepatopancreas. The loss due to this was estimated at approximately US$5 million since 2007. TSV in Asia is comparatively low because most of the domesticated and cultivated Specific Pathogen Free (SPF) stocks of *L. vannamei* have been selected for resistance to TSV. The impact of Taura syndrome on the shrimp-farming industry in the Americas was estimated to be $US1–2 billion up to 2001 while in Asia it is of $0.5–1 billion (Lightner, 2003). TSV had become endemic in most major shrimp-farming countries in East and South-East Asia, including China, Korea, Thailand, Myanmar, Indonesia and Vietnam. The IHHNV causes about $0.5–1 billion loss in America. An unique pattern of disease outbreak was recently noticed in *L. vannamei* farms in China the year 2009 followed by Vietnam in 2010, Malaysia in 2011, and in 2012 it has also been reported in Thailand where in 100% mortalities seen in affected during the first 20-30 days after stocking. It was named as early mortality syndrome (EMS) in shrimp (also termed acute hepatopancreatic necrosis syndrome or AHPNS). It has caused severe economic loss throughout these regions. The cause is not yet known.

Diseases of vannamei - Indian scenario

In the year 2008 the Indian government allowed the import of SPF *L. vannamei* broodstock by some selective breeders in order to introduce this species. With the introduction of this species into the Indian soil, people have started cultivating this species vigorously without any scientific intervention, which had lead to the development of many known and unknown diseases in this species. A study conducted by CIBA in collaboration with NFDB at different coastal states showed that *L. vannamei* also showed disease related mortality and crop failure in farms. The most commonly encountered diseases by these farms were WSSV and IHHNV. In few farms both these diseases has occurred together. Further, the samples which were screened were found to be negative for exotic pathogens like YSV, TSV and IMNV. Laem Singh Viral (LSNV) disease was reported in Juveniles and postlarvae of *L. vannamei* shrimps from farms in Andhra Pradesh (Kumar *et.al.,* 2011). Besides this, the most commonly noticed diseases in Indian farms are mortality often cristioned as 'running mortality syndrome' (unknown etiology), white discolouration of body (mineral deficiency?). Since the culture of *L. vannamei* was adopted by farmers who practiced tiger shrimps farming, the information generated on *L. vannamei* diseases is of limited value.

Conclusions

While some important strides have been made in developing new preventive methods to combat diseases in *L. vannamei* farms, additional research needs to be done, especially on many unexplained mortalities at different stages of culture and methods to control the disease. The risk of major disease incursions and newly emerging diseases will keep on threatening the sector, and unless appropriate health management measures are effectively implemented. The techniques like use of SPF broodstock and biosecure production systems have advanced shrimp farming and made the industry far more sustainable than it was before the emergence of the diseases. Maintaining strict biosecurity in aquaculture is difficult but is not an impossible task. In the coming years, there will be an increasing demand from the industry for improved aquatic animal biosecurity. This will be driven by various

factors like resource protection, food security, trade, consumer preference for high quality and safe products, production profitability, investment and development issues, and new threats of emerging health problems. Health management is a shared responsibility and each stakeholder's contribution is essential to the health management process. Further the association of people involved in this sector and institutions will maximize provision of diagnostic support and assist in building capacity for effective implementation of aquatic animal health management strategies.

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SHRIMP EARLY MORTALITY SYNDROME (EMS) / ACUTE HEPATOPANCREATIC NECROSIS SYNDROME (AHPNS)

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An emerging disease known as early mortality syndrome (EMS) or acute hepatopancreatic necrosis syndrome (AHPNS) has been recently causing significant losses in shrimp farms in China, Vietnam, Malaysia and Thailand since 2009. The disease affects both black tiger shrimp *Penaeus monodon* and Pacific white shrimp, *P. vannamei* and is characterised by mass mortalities during the first 20-30 days of stocking. Considering the severity of the disease, the Network of Aquaculture Centres in Asia-Pacific (NACA) jointly organized an emergency regional consultation on EMS/AHPNS with the Australian Department of Agriculture, Fisheries and Forestry (DAFF), Australia, in Bangkok, on 9-10 August 2012, to share information on this emerging disease, its occurrence, pathology and diagnosis, and to develop a coordinated regional response where international shrimp health experts, regional governments and industry personnel participated.

History of EMS / AHPNS and its impact

The EMS / AHPNS has been so far reported from Vietnam, China, Malaysia and Thailand since 2009. In Vietnam, the disease was observed since 2010, but the most widespread devastation due to EMS was reported since March 2011 in the Mekong Delta (South Vietnam). EMS affected the main shrimp production areas of Tien Gang, Ben Tre, Kien Giang, Soc Trang, Bac Lieu and Ca Mau provinces with a total shrimp pond area of around 98,000 hectares. In June 2011, unprecedented losses in *P. monodon* were reported in 11,000 ha of shrimp farms in Bac Lieu, 6,200 ha in Tra Vinh (causing a loss of over VND12 billion), and 20,000 ha in Soc Trang (causing VND1.5 trillion in losses) (Mooney, 2012). In China, the occurrence of EMS in 2009 was initially ignored by most farmers. But in 2011, outbreaks became more serious especially in farms with culture history of more than five years and those closer to the sea using high saline water (Panakorn, 2012). Shrimp farming in Hainan, Guangdong, Fujian and Guangxi suffered during the first half of 2011 with almost 80% losses. In Malaysia, EMS was first reported in mid-2010 in the east coast of peninsular states of Pahang and Johor. The outbreaks of EMS resulted in significant drop in *P. vannamei* production, from 70,000 mt in 2010 to 40,000 mt in 2011. Production for 2012 (up to May) was only 30,000 mt and worse was expected to come as unconfirmed reports on EMS outbreaks in the states of Sabah and Sarawak came in April 2012. In Thailand, so far 0.7% total shrimp ponds were reported to be affected by EMS, in Rayong, Chantaburi, Trat, Chacheongsao provinces located along the eastern Gulf.

Investigations on EMS/AHPNS and their findings

Pathology of EMS / AHPNS has been explicitly described by Dr. DV Lightner of University of Arizona, in both *P. monodon* and *P. vannamei* and he informed that the pathology appeared to be limited to the hepatopancreas (HP). He described EMS / AHPNS as idiopathic since no specific disease causing agent (infectious or toxic) was so far found to be associated. The clinical signs such as significant atrophy of the HP, which may be often pale to white due to pigment loss, sometimes with visible black spots or streaks, which does not squash easily between the thumb and forefinger could be used for presumptive diagnosis in cases of shrimp mortality starting as early as 10 days post-stocking. Progressive degeneration and dysfunction of the HP tubule epithelial cells progressing from proximal

to distal ends of HP tubules and its degenerative pathology suggested of a toxic etiology. However, he also mentioned that the anecdotal information suggested that disease spread patterns may be consistent with an infectious agent. According to Dr. Tim Flegel of CENTEX shrimp, in the EMS affected shrimp, in addition to various well studied pathogens such as WSSV, YHV and vibrios that are commonly linked to EMS, they also found certain groups of bacteria using metagenomics tools. While the role of certain crustaceacides such as cypermethrin was ruled out, the disease transmission trials are still inconclusive.

The potential pathogens are integral components of all ecosystems and disease emergence and subsequent spread often resulted from some disturbance in the ecology, which can upset the natural balance resulting in a normally innocuous organism emerging as a new disease agent. The current aquaculture practices are artificial and un-natural high density culture activities and promote emergence of pathogens (Dr. Peter Walker, CSIRO). Considering the need for identifying the etiology of EMS / AHPNS of shrimp, a number of new molecular methods such as sequence-assisted and sequence-independent virus discovery could be applied to discover viruses or other pathogens associated with AHPNS (Dr Jeff Cowley, CSIRO).

The epidemiology and risk factors involved in EMS / AHPNS require systematic studies. Until epidemiological approaches are applied systematically to include hatchery, transport, pond, farm and location specific data, it will be very difficult to pinpoint and prioritize risk factors for AHPNS (Dr Flavio Corsin and Dr Matthew Briggs). However, the potential risk factors included most of the generic factors such as high stocking densities, older farms closer to the sea using higher salinity water, farms not employing reservoirs, farms overusing chemicals, inadequate aeration, and presence of toxic levels of H_2S etc.

Way forward

While the role of cypermethrin was ruled out, involvement of biotic and abiotic factors and toxins was required to be investigated. An in depth analysis is required to identify the etiology that should unravel cryptic pathogens using molecular tools and pyro-sequencing. Robust challenge studies to prove Koch's and Rivers' postulates would have to be repeated using fresh tissues using appropriate challenge protocols by oral, reverse gavage, cohabitation, etc.

With regard to surveillance, reporting, disease prevention and control, quality data / information on suspect parameters such as pH, H_2S, use of probiotics etc to understand potential risk factors is essential. Information has to be provided to farmers on how the disease appears, what samples to be collected, mode of sample collection etc for lab investigations. It is necessary to provide training to the farmers on how to detect the disease and field officers on sampling.

Biosecurity, emergency response and disease management could be immediately addressed by imposing restrictions on movement of live affected animals to areas free from EMS and that buffer zones be created and monitored. Areas that are free from EMS should take precautions for stocking. It is also important to take utmost care in processing produce from affected EMS areas. Affected ponds must be treated before release of pond water into wild. Information on EMS in broodstock and PL, and wild organisms as carriers has to be generated.

Summary

For the purpose of detection and surveillance of EMS / AHPNS, the definition proposed by Prof Don Lightner, which relies mainly on histopathology of hepatopancreas may be employed along with

the clinical signs. It is important that histological examination be carried out to confirm that suspected occurrences that fit the AHPNS. Identifying the primary cause of EMS / AHPNS is necessary and can be addressed by robust scientific programme. Until this information becomes available, it is essential to increase disease awareness among the shrimp farmers. Considering the great economic loss that EMS is likely to cause in the region's shrimp aquaculture, ways of preventing the spread and/or occurrence of this disease should be formulated by concerned experts, officials and other regulatory bodies. Farmers, on the other hand, should also cooperate with the concerned agencies by promptly reporting any suspected mortalities among cultured shrimp that appear to be similar to the clinical description of EMS / AHPNS. Further, it is necessary to impose restrictions on movement of live affected animals to areas free from EMS either for culture or for processing purposes, and movement of live shrimp may be undertaken only after conducting robust import risk analyses.

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TRANSBOUNDARY DISEASES WITH RESPECT TO *Litopenaeus vannamei* CULTURE PRACTICE: AWARENESS AND PREPAREDNESS

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Pacific white shrimp, *Litopenaeus vannamei* native to Western Pacific coast, that introduced into most of South East Asian countries for commercial aquaculture gained momentum is visible by its a rapid culture area expansion. Consequently the production of *L. vannamei* has exceeded that of native cultured species in these countries *viz*., China, Thailand, Taiwan, Viet Nam and Indonesia. However, translocation of *alien* species poses potential risk of spread of pathogens that may lead to disease outbreaks that might associate with significant production and economic losses. Indian government has recently permitted *L. vannamei* for culture in India, with CAA approval by allowing importation of Specific Pathogen Free (SPF) broodstock from other countries after undergoing required quarantine of such consignments. SPF shrimp are expected to be free from the OIE listed pathogens that are of economic importance in shrimp aquaculture. However, SPF status cannot guarantee the disease free status after importation. In this context awareness on the transboundary diseases with respect to culture of *L. vannamei* and preparedness for health management are being discussed.

WHITE SPOT DISEASE (WSD)

This virus is the most serious threat facing the shrimp farming industry. WSSV was first reported in farmed *P. japonicus* from Japan in 1992/93, but was thought to have been imported with live infected PL from Mainland China. WSD has been identified from crustaceans in China, Japan, Korea, South-East Asia, South Asia, the Indian Continent, the Mediterranean, the Middle East, and the Americas. All penaeid shrimp species are highly susceptible to infection, often resulting in high mortality. Crabs, crayfish, freshwater prawns, spiny lobsters and clawed lobsters are susceptible to infection, but morbidity and mortality consequence of infection is highly variable. Prevalence of WSSV is reported highly variable, from <1% in infected wild populations to up to 100% in captive populations. WSD is caused by one of the largest animal viruses. WSSV is a large double-stranded DNA virus of 120-150 by 270-290 nm size, assigned to a new virus family, whispoviridae. WSSV had several names such as Chinese baculovirus (CBV), White spot syndrome baculovirus complex (WSBV), Shrimp Explosive Epidermic Disease (SEED), Penaeid Rod-shaped DNA Virus (PRDV), Japan's Rod-shaped Nuclear Virus (RV-PJ) of *P. japonicus*, Thailand's Systemic Ectodermal and Mesodermal Baculovirus (SEMBV) of *P. monodon*. WSSV can infect a wide range of aquatic crustaceans including marine, brackish and freshwater penaeids, crabs and crayfish. All decapod crustaceans from marine and brackish or freshwater sources are susceptible host species. WSSV affects most organs derived from ectodermal and mesodermal origin, including the cuticular epithelium, connective tissue, nervous tissues, muscle, and lymphoid organ and haematopoietic tissues. The virus also severely damages the stomach, gills, antennal gland, heart and eyes. During later stages of infection, these organs are destroyed and many cells are lysed. The shrimp then show reddish discoloration of the hepatopancreas and characteristic 1-2 mm diameter white spots on their carapace, appendages and inside surfaces of the body. Affected shrimp show lethargic behavior. Cumulative mortality typically reaches 100% within two to seven days of infection. It can be visually diagnosed through the presence of the characteristic white spots, which can be seen in advanced stage of infection. However, white spots may not be always present in infected shrimp. WSSV can be detected by using PCR, or with probes for dot-blot and *in situ*

hybridization tests. PCR detection efficiency can be increased by exposure to stressful conditions (e.g. eye-stalk ablation, spawning, moulting, changes in salinity, temperature or pH, and during plankton blooms). WSSV can be confirmed histologically (particularly in asymptomatic carriers) by the presence of large numbers of Cowdry A-type nuclear inclusions and hypertrophied nuclei in H&E-stained sectioned tissues, or simply by rapid fixation and staining of gill tissue. The mode of transmission of WSD around Asia was believed to be through exports of live PL and broodstock. The infection can be transmitted vertically, horizontally by cannibalism, predation, etc. and by water-borne routes. Some studies have shown that disinfection of water supplies and the washing and/or disinfection of the eggs and nauplius is reported to be successful in preventing its transmission from positive broodstock to their larvae.

YELLOW HEAD DISEASE (YHD)

Yellow head disease was the first major viral disease that caused extensive losses to shrimp farms in Thailand during 1990-91. YHD outbreaks have been reported in the black tiger prawn and the white Pacific shrimp. YHD has been reported in China, Taipei, India, Indonesia, Malaysia, the Philippines, Sri Lanka, Thailand and Vietnam. Outbreaks of YHD with heavy mortalities have been reported in farmed black tiger shrimp and pacific white shrimp. It is reported to be highly prevalent (>50%) sampled farmed and wild populations in Australia, Asia, East Africa and Mexico.YHD is caused by yellow head virus (YHV), and its close relatives such as gill-associated virus (GAV). YHV is rod-shaped enveloped viruses of 40-60 nm by 150-200 nm size, containing single stranded RNA. YHV affects tissues of ectodermal and mesodermal origin including lymphoid organ, haemocytes, haematopoietic tissue, gill lamellae and spongy connective tissue of the sub-cutis, gut, antennal gland, gonads, nerve tracts and ganglia. YHV principally affects pond reared juvenile stages of 5-15 g. Affected shrimp typically feed voraciously for two to three days and then stop feeding abruptly and are seen swimming near the periphery of the pond. YHV infections can cause swollen and light yellow colored hepatopancreas in infected shrimp, and a general pale appearance, before dying within a few hours. YHD can cause up to 100% mortality in infected *P. monodon* ponds within 3-5 days of the first appearance of clinical signs. GAV has been reported to be associated with mortalities of up to 80% in *P. monodon* ponds in Australia.Yellow head virus can be detected by RT-PCR or with a probe designed for dot-blot and *in situ* hybridization tests. It can also be diagnosed histologically in moribund shrimp by the presence of intensely basophilic inclusions, most easily in H&E stained sections of stomach or gill tissue, or simply by rapid fixation and staining of gill tissue and microscopic examination. The primary mechanism of spread of YHV in pond culture appears to be through water and mechanical means. Infected broodstock can pass on the virus to larvae in the maturation/hatchery facilities if thorough disinfection protocols are not strictly adhered to. Methods of YHV eradication in ponds are much the same as for other viruses and involve BMPs that include pond preparation by disinfection and elimination of carriers and production of virus free broodstock and PL for pond stocking.

TAURA SYNDROME (TS)

Taura Syndrome was first identified from farms around the Taura River in Ecuador in 1992 and the disease spread rapidly to the whole of Latin and North America within three years. Subsequently, TS was also reported from Asia including Mainland China and Taiwan (from 1999), and in late 2003 in Thailand, probably through the regional and international transfer of live PL and broodstock of *L. vannamei*. Initial work suggested that TS was caused by a toxic pesticide. However, it is now known that a single or perhaps several very closely related strains (mutants) of the Taura syndrome virus

(TSV) are responsible for the TS. TSV is a single stranded RNA virus of 32 nm size, non-enveloped icosahedrons and more prone to mutations causing more concern.

TSV infections occur in juvenile shrimp (0.1-1.5 g body weight) within two to four weeks of stocking ponds and occur largely within the period of a single moult cycle. In the acute phase of the disease, during pre-moult stage, the shrimp are weak, soft-shelled, have empty gut and diffuse expanded chromatophores that appear red, particularly in the tail (hence the common name - red tail disease). Such animals will usually die during moulting (5-95%). Adult shrimp are known to be more resistant than juveniles. Shrimp that survive infection show signs of recovery and enter the chronic phase of the disease. Such shrimp show multiple, randomly distributed, irregular, pitted, melanised lesions of the cuticle. These gross lesions will persist, but may be lost during moulting, and the shrimp thereafter appear normal. TS can be diagnosed using standard histological and molecular methods of detection. Specific DNA probes applied to *in situ* hybridization assays with paraffin sections provide the confirmatory diagnosis. Reverse transcriptase polymerase chain reaction (RT-PCR) assay is commonly used for larger sample sizes and non-lethal sampling for broodstock. Histological demonstration of enlarged lymphoid organs (LO) with multiple LO spheroids and multifocal areas of necrosis in the cuticular epithelium of the general body surface, appendages, gills, hindgut, and foregut (the oesophagus, anterior and posterior chambers of the stomach).The mechanism of transmission of TSV can be through contaminated PL and broodstock. Recently it has been shown that mechanical transfer through insect. The disease can be prevented by avoidance of reintroduction of the virus from wild shrimp and carriers and stocking with TSV-free PL produced from TSV-free broodstock.

INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS (IHHN)

IHHN was first discovered in *L. vannamei* and *P. stylirostris* in the Americas in the year 1981. However, it was thought to have been introduced along with live *P. monodon* from Asia. IHHNV has probably existed for some time in Asia without detection due to its insignificant effects on *P. monodon*, the major cultured species in Asia. Recent studies have revealed geographic variations in IHHNV isolates, and suggested that the Philippines was the source of the original infection in Hawaii, and subsequently in most shrimp farming areas of Latin America. Large-scale epizootics were responsible for multi-million dollar losses in *L. vannamei* culture in the Americas during the 1990s. IHHNV is caused by a small (20-22 nm) single-stranded DNA-containing parvovirus. Gross signs of disease are not specific to IHHN, but may include reduced feeding, elevated morbidity and mortality rates, fouling by epicommensals and bluish body coloration. Larvae, PL and broodstock rarely show symptoms. In *L. vannamei*, IHHNV can cause runt deformity syndrome (RDS), which typically results in cuticular deformities (particularly bent rostrums), slow growth, poor FCR and a greater size variation at harvest, contributing substantially to reduction in profits. IHHNV typically causes no problems for *P. monodon* since they have developed a tolerance to it over a long period of time, but they may suffer with RDS. *P. merguiensis* and *P. indicus* appear refractory to the IHHNV. However, these species may be life-long carriers of the virus and so could easily pass it onto *L. vannamei*, which typically suffer from RDS when exposed to IHHNV.IHHNV can be diagnosed using methods such as DNA probes in dot blot and *in situ* hybridization and PCR techniques (including real-time PCR) as well as histological analysis of H&E-stained sections looking for intracellular, Cowdry type A inclusion bodies in ectodermal and mesodermal tissues such as cuticular epithelium, gills, foregut, hind gut, lymphoid organ and connective tissues. Transmission of IHHN is known to occur rapidly by cannibalism shrimp. It can also be transmitted through waterborne route and cohabitation. Vertical transmission from broodstock to larvae is common. Strict hatchery biosecurity including checking of broodstock by PCR, or the use of SPF broodstock, washing and disinfecting of eggs and nauplii is essential in combating this disease.

INFECTIOUS MYONECROSIS (IMN)

Infectious myonecrosis is an emerging *L. vannamei* disease, first detected in Brazil during 2004, and then in Indonesia in 2006. To date, IMN has been detected in East Java, Bali, and West Nusa Tenggara provinces. The principal host species in which IMNV is known to cause significant disease outbreaks and mortalities is *L. vannamei*. IMN is caused by a virus, a putative totivirus. IMNV particles are icosahedral in shape and 40 nm in diameter. Juveniles and sub-adults of *L. vannamei*, farmed in marine or low salinity brackish water, appear to be the most severely affected by IMN disease. The principal target tissues for IMNV include the striated muscles (skeletal and less often cardiac), connective tissues, haemocytes, and the lymphoid organ parenchymal cells. IMN disease causes significant mortality in grow-out ponds and is characterized by acute onset of gross signs including focal to extensive whitish necrotic areas in the striated muscle, especially of the distal abdominal segments and the tail fan, which may become necrotic and reddened similar to the colour of cooked shrimp. Severely affected shrimp become moribund and mortalities can be instantaneously high and continue for several days. Mortalities from IMN range from 40 to 70% in cultivated *L. vannamei*, and food conversion ratios (FCR) of infected populations increase from normal values of ~ 1.5 to 4.0 or higher. IMN can be confirmed by histopathology, using routine haematoxylin-eosin (H&E) stained paraffin sections and demonstrating characteristic coagulative necrosis of striated skeletal muscle fibres, often with marked oedema among affected muscle fibres. IMN may be also rapidly diagnosed using a nested reverse-transcriptase polymerase chain reaction (RT-PCR) method which provides a rapid, sensitive and specific test to detect IMNV in penaeid shrimp. Published methods are available for the molecular detection of IMNV by *in-situ* hybridisation (ISH), nested RT-PCR and real-time RT-PCR. IMNV has been demonstrated to be transmitted through cannibalism. Transmission via water and vertical transmission from broodstock (transovarian or by contamination of the spawn eggs) to progeny is also likely to occur. IMNV may also be transmitted among farms by faeces of seabirds or shrimp carcasses. Outbreaks of IMN with sudden high mortalities may follow stressful events such as capture by cast-net, feeding, sudden changes in salinity or temperature, etc., in early juvenile, juvenile, or adult *L. vannamei* in regions where IMNV is enzootic. Stocking only pre-screened broodstock and/or their spawned eggs/ nauplii and discarding those that test positive for the IMN virus by reverse-transcription polymerase chain reaction (RT-PCR). The disease can be prevented by stocking with virus free PL produced from IMNV-free broodstock.

NECROTIZING HEPATOPANCREATITIS (NHP)

This disease is also known as Texas necrotizing hepatopancreatitis (TNHP), Texas pond mortality syndrome (TPMS), and Peru necrotizing hepatopancreatitis (PNHP). NHP has been reported as an important disease since its first diagnosis in 1985. It has been reported to cause mass mortalities to the tune of 20-90 percent of *L. vannamei* in highly saline commercial grow-out ponds nearly every year since then. By 1993, NHP spread to Ecuador, Guatemala, Honduras, Mexico and Peru and by 1995, coincided with warm waters with high salinity associated with El Nino, and caused severe mortalities (60-80 percent mortality) of *L. vannamei* and *L. stylirostris* throughout Ecuador. NHP has not yet been reported in Asia, but could cause significant damage were it to be transferred here with untested shrimp introduction. Necrotizing hepatopancreatitis is caused by obligate intracellular Rickettsia-like bacterium, a member of the order a-Proteobacteria (Gram-negative, pleomorphic, rod-shaped or helical-shaped bacterium). Affected shrimp are lethargic, anorexic with empty gut and show epibiotic fouling. Exoskeleton becomes soft and show abdominal muscle atrophy. Affected ponds have increased FCR and growth of affected shrimp is retarded. The hepatopancreas becomes watery with white or

black streaks. Mortality rates reach up to 90% within 30 days of the appearance of clinical signs. NHP can be diagnosed by demonstration of lipid droplets and melanisation of hepatopancreas by microscopic examination of wet mount of preparations. It may be confirmed by histopathological examination showing atrophy and the presence of granuloma in the hepatopancreas, and haemocyte aggregations around the hepatopancreatic tubules. Intra-cytoplasmic Rickettsia-like bacteria may be prominently seen in the cytoplasm. Molecular diagnostic tools such as *in situ* hybridization, dot blot hybridisation, and PCR for specific a-Proteobacterial DNA are also available. NHP could be transmitted horizontally with infected PLs. Maintaining optimal environmental parameters using BMPs will be useful in preventing NHP.

Aquatic animal health management

Risk of diseases is inherent with transboundary movement of live aquaculture species, health management practices should function to reduce the risk of pathogen incursion and associated diseases outbreaks for which diagnostic expertise and effective technologies to handle disease outbreaks need to be evolved. Minimisation of the risks of introduction of pathogens can be achieved through quarantine, health certification, disease zoning, disease surveillance and reporting, import risk analysis, contingency planning, capacity building especially for exotic disease diagnostics.

COMMON BACTERIAL AND VIRAL DISEASES OF PACIFIC WHITE SHRIMP, *Litopenaeus vannamei*

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Introduction

Recent past years advances in intensive aquaculture have brought parallel acute concern for problems associated with diseases both by sudden catastrophic epizootics and slow continuous attrition and leads to great economic loss. Generally infectious diseases of shrimp and other aquatic animals are caused by virus, bacteria, fungi and parasites, and other diseases and abnormalities due to environmental stresses, genetic factors and nutritional deficiencies. Diseases may be caused by a single or multiple pathogens. After introduction of Pacific white shrimp, *Litopenaeus vannamei* in to the country during 2008, the diseases of the species is of great concern to be known. Moreover, all other diseases not yet reported in *L. vannamei* in India but can affect native shrimp species need to be known for precautionary measures. This chapter is aimed at highlighting the major bacterial and viral diseases of *L. vannamei*.

Major bacterial diseases to be considered for *L. vannamei*

Bacterial diseases occur as a result of the complex interactions between pathogen, host and environmental stress. The shrimp pond is the natural habitat of several pathogenic and beneficial microbial populations. Environmental stresses can affect the homeostatic mechanism of shrimp thus reducing their resistance to disease-causing organisms. Hence, the bacterial infections of shrimp are primarily stress related with other predisposing factors like adverse environmental conditions or mechanical injuries. The most common gram negative bacteria pathogenic to shrimp belong to the genus *Vibrio*. Other Gram-negative bacteria such as *Leucothrix mucor*, *Thiothrix* spp., *Flavobacterium* spp., *Flexibacter* spp., *Cytophaga* spp., *Aeromonas* spp., and *Pseudomonas* spp. are also concerned in shrimp diseases.

i) Vibriosis

Vibriosis is one among the serious problems in shrimp culture operations with high epidemic in shrimp hatcheries, intensively raised farm and pond culture stocks especially at the juvenile stage. Stressors such as higher salinity, increased ammonia level in the culture environment, low dissolved oxygen, rise in temperature and higher stocking density are found to be responsible for Vibriosis. The identified and reported pathogenic *Vibrio* spp. for shrimp and fish are *Vibrio harveyi, V. alginolyticus, V. parahaemolyticus, V. anguillarum, V. mimicus, V. fluvialis, V. splendidus, V. penaeicida, V. campbellii, V. carchariae, V. cholerae, V. damsela, V. ordalii,* and *V. vulnificus*. Characteristic luminescence is seen in severe pathogenic *Vibrio* infection. Symptomatically the disease is associated with melanized cuticular lesions of the appendages and melanized nodules in the gills and other organs. Other major clinical signs such as disoriented shrimp swimming weakly, gathering along the edges of the pond, cloudiness of the musculature, red discoloration of the appendages and dorsal flexure at the third abdominal segment with slight rigidity. Fish eating birds gather and feed on the weakened shrimp. Haemolymph from moribund shrimp will fail to clot or will clot very slowly. Histopathological investigation of moribund shrimp reveals the presence of pathogens in different tissues. Vibriosis in nursery and grow out culture of shrimp can be managed with use of medicated feed, fertilization of pond with 20

kg/ha sucrose, partial harvest to reduce pond biomass, herbal treatment with application of neem leaves at the rate of 20 kg/ha, disinfection of pond bottom between grow out cycles by drying and removal of excessive organic sediments and application of quick lime. Immediate farm wide harvesting is advisable in extreme cases to minimize the economic losses.

ii) Fouling/black gill disease

Microbial colonization on the cuticle surface of crustaceans is a common occurrence in larvae, juveniles, and adult shrimp. Normally, the density of microorganisms attached to shrimp remains low without causing pathological changes. But, in poor management/culture conditions the increase in population of the fouling organisms can cause impairment of physiological functions of the host. A filamentous bacterium, *L. mucor* is the predominant bacterial fouling organism affecting shrimp culture. Sometimes *Vibrio* and other rod shaped organism also colonize cuticle surface. *Thiothrix* sp., *Flavobacterium* sp., *Flexibacter* sp., and *Cytophaga* sp. are the other related bacterial genera causing fouling. Usually, heterogenous mixture of filamentous and non-filamentous bacteria, blue green algae and protozoa (*Zoothamnium*) causes fouling and black gill. Disease occurs when there is abundant colonization on the gill lamellae, mouth parts and/or swimming appendages with respective physiological dysfunctions. Affected shrimps show slow growth rate associated with sporadic but persistent mortality.

iii) Necrotizing hepatopancreatitis (NHP) disease

Necrotizing hepatopancreatitis (NHP) disease is caused by infection with Gram-negative, pleomorphic intracellular á-proteobacterium. This is also called as Necrotizing hepatobacterium (NHPB), NHP bacterium (NHPB) or Rickettsial-like organism (RLO). The principal host species in which necrotizing hepatobacterium (NHPB) can cause significant disease outbreaks and mortalities are *Penaeus vannamei* and *P. stylirostris*. NHP has four distinct phases: initial, acute, transition and chronic. In acute- and transition-phase, pathognomonic lesions are typically present in histological sections of the hepatopancreas, while in the initial and chronic phases of the disease, there are no pathognomonic lesions, and molecular and antibody-based methods for NHPB detection are necessary for diagnosis. NHPB is a pleomorphic, Gram-negative, intracytoplasmic bacterium. It is a member of the á-subclass of proteobacteria and remains unclassified. Most penaeid species can be infected with NHPB, including *P. vannamei* (Pacific white shrimp) and *P. stylirostris* (Pacific blue shrimp). NHPB infections are most severe in *P. vannamei* where the intracellular bacterium can cause acute epizootics and mass mortality (>90%). In *P. vannamei,* the juvenile, sub adult and broodstock life stages are the most severely affected. NHPB causes chronic disease in *P. vannamei,* the main effects of which are slow growth, a soft cuticle and a flaccid body. The target tissue is the hepatopancreas, with NHPB infection reported in all hepatopancreatic cell types. Some members of *P. vannamei* populations that survive NHPB infections and/or epizootics may carry the intracellular bacteria for life and pass it on to other populations by horizontal transmission. Natural transmission of NHPB occurs by cannibalism, cohabitation and dissemination of NHPB via the water column. NHPB in faeces shed into pond water has also been suggested as a possible means of transmission. Outbreaks of disease are often preceded by prolonged periods of high water temperature (approximately 30°C) and salinity (up to 40ppt). In *P. vannamei*, infection by NHPB results in an acute, usually catastrophic disease with mortalities approaching 100%.

Major viral diseases to be considered for *L. vannamei*

The shrimp industry experienced parallel increase in quantum of diseases with million dollar questions unanswered despite increased scientific contribution to the field of profit. In last two decades

many new emerging diseases have been identified which led to search for innovative means of BMPs and therapeutic applications for control. Even though no published data is available for serious viral disease out break in *L. vannamei* in India as on date, it is pertinent to know the importance of the diseases experienced by western countries.

i) Spherical Baculovirosis (*Penaeus monodon*-type baculovirus, Monodon baculovirus)

It is associated with high mortalities in hatchery-reared larval, post-larval and early juvenile stages of *Penaeus monodon*, caused by dsDNA virus, PmSNPV (for singly enveloped nuclear polyhedrosis virus from *P. monodon*) belonging to the genus *Nucleopolyherdovirus* which was first reported in Taiwan during 1983 and in India during 1995. The existence of different strains of this virus is likely based on its wide geographical and host species range. It infects other penaeid species also but in *L. vannamei* it causes no pathogenesis.

ii) Tetrahedral Baculovirosis (Baculovirus penaei, PvSNPV-singly enveloped nucleopolyhedrovirus from *Penaeus vannamei*)

The International Committee on Virus Taxonomy lists the related virus MBV (spherical baculovirosis) as a tentative species in the genus *Nucleopolyherdovirus*. Therefore, this should also be considered as a tentative species in this genus. At least three geographical strains have been demonstrated so far. Baculovirus penaei (BP) infections have been reported in one or more species of penaeid genera or subgenera. All penaeid species may be potential hosts in all life stages, except eggs and nauplii. BP is strictly enteric infecting mucosal epithelial cells of the hepatopancreas tubules and the anterior midgut. Persistent infection occurs commonly in penaeid hosts of BP. Wild adult *P. vannamei* females that are heavily infected with BP have been shown to excrete BP-contaminated faeces when spawning, thereby contaminating the eggs and passing the virus to the next generation. None are known in natural infections, but the rotifer *Brachionus plicatilis* and *Artemia salina* nauplii were used as passive carriers of BP to deliver the virus to larval stages of *P. vannamei* in experimental infections. Transmission of BP is horizontal by ingestion of infected tissue (cannibalism), faeces, occlusion bodies, or contaminated detritus or water. Highest mortality occurs in protozoa, mysis and post larval stages. High mortality rates are unusual as a consequence of BP infection in the juvenile or adult stages, but infection may cause poor growth and reduced survival in nursery or grow-out ponds.

iii) White spot disease (WSD)

It is an acute, highly contagious disease of shrimp, caused by a large, ovoid, bacilliform, non-occluded enveloped dsDNA virus belonging to the family *Nimaviridae* (Nima: In Latin, means thread) and genus *Whispovirus* consisting of thread-like polar extension. The virus is inactivated in <120 minutes at 50°C and <1 minute at 60°C and is viable for at least 30 days at 30°C in seawater under laboratory conditions and is viable in ponds for at least 3-4 days. It infects all life stages of decapod crustaceans of marine and brackishwater sources. The virus infects ectodermal and mesodermal tissues, especially the cuticular epithelium and subcuticular connective tissues. Both vertical and horizontal transmission is reported. Clinically, the disease is characterized by lethargy, inappetence, crowded at pond margin, red to pink discoloration of the body, loose cuticle, swelling of branchiostegites, broken antennae, damaged appendages, and the most conspicuous feature of small to large white spots on the inner side of the carapace which spread all over the body in advanced infection. Cumulative mortalities in infected populations may reach 100% within 3 to 7 days of the onset of clinical signs. Histologically, degenerated cells are characterized by hypertrophied nuclei with marginated chromatin and eosinophilic to basophilic intranuclear inclusions.

iv) Infectious hypodermal and hematopoietic necrosis (IHHN) disease

The disease is caused by ssDNA virus of family *Parvoviridae*. IHHNV is the smallest of the known penaeid shrimp viruses but believed to be the most stable virus of the known penaeid shrimp viruses. Transmission of IHHNV can be by horizontal and/or vertical routes. At least three distinct genotypes of IHHNV such as Type 1 from the Americas and East Asia (principally the Philippines); Type 2 from South-East Asia; Type 3A from East Africa, India and Australia; and Type 3B from the western Indo-Pacific region including Madagascar, Mauritius and Tanzania are identified so far. The first two genotypes are infectious to the representative penaeids, *P. vannamei* and *P. monodon*, while the latter two genetic variants are not infectious to these species. In *P. vannamei* it causes the chronic disease called runt deformity syndrome (RDS) characterized by lower overall crop production, shrimp with increased size variability, and cuticular deformity. Infected shrimps have been observed to rise to the water surface, remain motionless for a few moments then roll over and sink to the bottom. This behavior may be repeated until mortality occurs. In juvenile *P. stylirostris*, more than 90% mortality reported within several weeks of onset of infection. Gross signs of infection include white to buff mottling of the cuticle, opacity of striated muscle and melanized foci within the hypodermis. In the later stages of infection *P. stylirostris* and *P. monodon* may appear bluish in color. Infected *P. vannamei* display deformed rostrums, cuticle and antennal flagella. IHHNV forms Cowdry Type A intranuclear inclusion bodies (IB's) associated with widespread cytopathological changes including hypertrophy of the nucleus and margination of the chromatin in cells of ectodermal (epidermis, gills, fore and hind gut, antennal gland and neurons) and mesodermal origin (hematopoietic tissue, haemocytes, striated muscle, heart, lymphoid organ and connective tissues).

v) Infectious myonecrosis (IMN)

Infectious myonecrosis (IMN) is a recently identified viral disease caused by dsRNA infectious myonecrosis virus (IMNV), a putative totivirus. IMNV particles are icosahedral in shape and 40 nm in diameter. It causes mortalities in juvenile and sub adult pond-reared stocks of *L. vannamei* and the mortality range from 40 to 70%. Outbreaks of the disease seems to be associated with certain types of environment and physical stresses (i.e. extremes in salinity and temperature, collection by cast net, etc.), and possibly with the use of low quality feeds. Experimental infection is observed in tiger shrimp, *P. monodon* and blue shrimp, *P. stylirostris*. IMN affected shrimp presents focal to extensive white necrotic areas in the striated (skeletal) muscle, especially of the distal abdominal segments and tail fan, which can become necrotic and reddened in some affected shrimp. By histopathology, shrimp with acute phase disease presents lesions with coagulative necrosis of skeletal muscle. In shrimp recovering from acute disease or those in the more chronic phase of the disease, the myonecrosis appears to progress from coagulative to liquefactive necrosis accompanied with hemocytic infiltration and fibrosis. Significant lymphoid organ spheroid formation is typically present, and ectopic lymphoid organ spheroids are often found in the hemocoel and loose connective tissues, especially in the heart lumen and adjacent to antennal gland tubules. In some histological preparations, perinuclear pale basophilic to dark basophilic inclusion bodies are evident in muscle cells, connective tissue cells, hemocytes, and in cells that comprise lymphoid organ spheroids.

vi) Yellowhead disease (YHD)

Yellow-head disease is first noted by Limsuwan in cultured *P. monodon* adults in central Thailand during 1991. It appears to be widespread in cultured stocks of *P. monodon* and *L. vannamei*. It is caused by ssRNA virus of genus *Okavirus*, family *Roniviridae* of the order *Nidovirales*. Yellow head virus

(genotype 1) is one of six known genotypes in the yellow head complex of viruses and is the only known agent of YHD. Gill-associated virus (GAV) is designated as genotype 2. GAV and four other known genotypes in the complex (genotypes 3–6) occur commonly in healthy *P. monodon* in East Africa, Asia and Australia and are rarely or never associated with disease. Contaminated water, cannibalism of weak or moribund shrimp, animate vector, net and other equipments transmit the disease. YHV remains viable in aerated seawater for up to 72 hours. YHV can be inactivated by heating at 60°C for 15 minutes and treating with chlorine at 30ppm. Vertical transmission has not been reported. Clinical signs of YHD occur in *P. monodon* within 7–10 days of exposure. Shrimps with YHD display yellow colouration of the dorsal cephalothorax caused by the underlying yellow hepatopancreas showing through the translucent carapace. Within the ponds, infected animals, usually between 5 and 15 g, begin consuming feed at an abnormally high rate for several days then cease feeding entirely. One day after cessation of feeding, moribund shrimps may be seen swimming slowly near the edges of the pond. By the third day, mass mortality occurs and the entire crop is typically lost. Histologically, moribund shrimps suffering YHD usually have extensive abnormalities in the lymphoid organ. These include foci of necrotic cells which resemble degenerate tubules with occluded lumens and contain cells with hypertrophied nuclei, pyknotic nuclei, large vacuoles and cytoplasmic, basophilic, Feulgen-positive inclusions. Similar inclusions may also be found in the interstitial tissues of the hepatopancreas, connective tissues underlying the midgut, cardiac tissues, haematopoietic tissues, haemocytes and gill tissues.

vii) Taura syndrome (TS) / Red tailed disease

It is caused by a nonenveloped, linear, ssRNA virus called Taura syndrome virus belonging to the family *Dicistroviridae*. TS is widely distributed in the shrimp-farming regions of the Americas and South-East Asia. Both horizontal and vertical transmission is reported. During the preacute/acute phase of infection, shrimps appear pale red while their tail fans become bright red. They are soft shelled, lethargic and anorexic. Those with severe infections die during moult and cumulative mortalities may reach 80-95%. Recovering, chronically infected shrimps generally display multifocal, melanized cuticular lesions and may also have soft cuticles and red body coloration with normal feeding. Microscopically, Feulgen-negative inclusion bodies, which may first appear eosinophilic then change to basophilic observed in the cytoplasm of cells in areas of necrosis.

Diagnosis

The correct diagnosis is obviously a critical step in any control program. The available diagnostic methods that may be selected for diagnosis of the shrimp diseases or detection of their etiological agents are based on:

1) Anamnesis.
2) Gross and clinical signs.
3) Direct bright-field, phase-contrast or dark-field microscopy with whole stained or unstained tissue wet-mounts, tissue squashes, and impression smears; and wet-mounts of faecal strands.
4) Post mortem findings.
5) Histology of fixed specimens.
6) Bioassays of suspect or subclinical carriers using a highly susceptible host (life stage or species) as the indicator for the presence of the pathogen.

7) Transmission or Scanning Electron Microscopy (TEM or SEM).

8) Antibody-based tests for pathogen detection using immune sera polyclonal antibodies (PAbs) or monoclonal antibodies (MAbs).

9) Molecular methods.

Disease prevention and control strategy

The disease prevention and control strategy is the best practice for successful hatchery and grow-out culture practices in shrimp industry.

1. Ponds should be dried before starting the culture.
2. Strict biosecurity measures to be adopted.
3. Sieve should be used at water inlet and the water should be bleached before stocking to weed out wild shrimp, fishes and intermediate hosts.
4. Good water quality should be maintained through out the culture.
5. Zero water exchange or minimal water exchange from reservoir ponds.
6. Disease-free stock should be used from good genetic strain of broodstock.
7. Development and use of disease resistant stocks will help in prevention of catastrophic disease out break and loss.
8. Coastal Aquaculture Authority (CAA) guidelines should be followed for optimum shrimp stocking density in grow out culture system.
9. Quarantine measures should strictly be adopted to import broodstock to avoid entry of existing or emerging pathogen.
10. Adequate balanced good nutrition to be made available to avoid problems associated with cannibalism and horizontal spread of diseases.
11. Proper destruction and disposal of infected as well as dead animals to be regularly monitored.
12. Animals should be handled with good care to avoid unwanted stress.
13. Proper chemical prophylaxis and vaccine development is needed for immunological protection.
14. Regulations are required to prevent transfer of pathogens from one host population to another, nationally or internationally.
15. Sanitation and disinfection of hatchery and equipments are to be strictly followed.

Conclusion

Even though sporadic disease conditions of the Pacific white shrimp are reported in India at present, there is every possibility for serious outbreaks both due to exotic and existing pathogens. Therefore, knowledge on various shrimp pathogens is very much essential to tackle any future problems.

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SHRIMP IMMUNITY AND DISEASE RESISTANCE

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Diseases are one of the major constraints for the economic sustainability of modern intensive shrimp culture systems. High-density stocking, deteriorating environments, accumulating pollutants and improper nutrition impose the stress on cultured animals. Immuno-supression caused by these abiotic and biotic stress favour opportunistic pathogens to infect causing retarded growth, mortality and economic loss. Because of the environmental concerns and legal sanctions use of antibiotics and other chemicals in culture ponds is not feasible. Alternatively, the shrimp health can be maintained through approaches like enhancing the immune status by enhancing the disease resistance competence of the host. These economic concerns drive the need for understanding the mechanisms behind the antimicrobial immune system for developing the effective disease prevention and control systems.

Invertebrate animals do not have adaptive immune system like higher animals and depend mostly on innate immune mechanism. Humoral and cellular immune systems are the two main branches of host immunity which work together upon activation by the invading pathogen. The cellular component contains circulating hemocytes while cell free molecules in the haemolymph form the humoral component.

Circulating haemocytes are classified in to three main cell populations based on the cytoplasmic granules, the hyaline or agranular, the semigranular (with small granules) and the granular (with large granules) cells. These haemocytes in addition to the defence system also participate in wound and shell repair, nutrient transport, digestion and excretion processes. Hyaline haemocytes are known to involve mainly in coagulation processes while granular haemocytes perform phagocytosis, encapsulation and regulation of prophenoloxidase (proPO) system. The haemocytes originate from hematopoietic tissue, located in the dorsal and dorsolateral sides of the stomach, surrounding the antennal artery, and at the base of the maxillipedes beside the epigastric region as densely packed lobules. Lymphoid organ located at the anterior part of the hepatopancreas is responsible for filtration and elimination of bacteria.

The humoral immune response includes the diverse array components like, melanization and clotting cascades, anti-oxidant defense enzymes like superoxide dismutase, peroxidase, catalase and nitric oxide synthase, defensive enzymes like lysozyme, acid phosphatase and alkaline phosphatase, reactive oxygen and nitrogen intermediates and antimicrobial peptides.

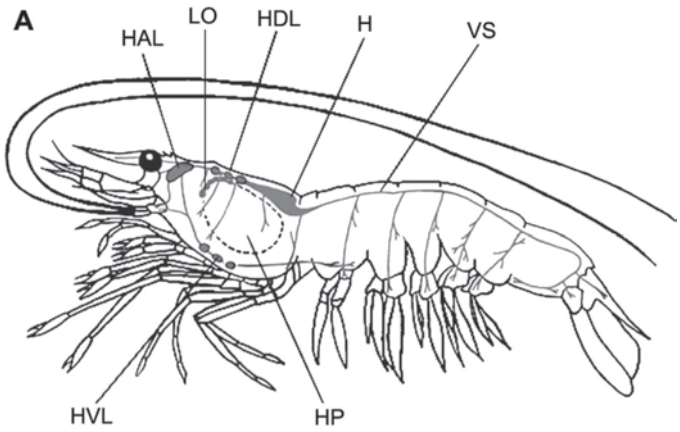

Fig. 1. Shrimp circulatory systems of shrimp. H: Heart, HP: hepatopancreas, LO: lymphoid organ HDL: hematopoietic dorsal obules, HVL: hematopoietic ventral lobules, HAL: hematopoietic antennal lobules, VS: vascular system (Bachere *et al.* 2004).

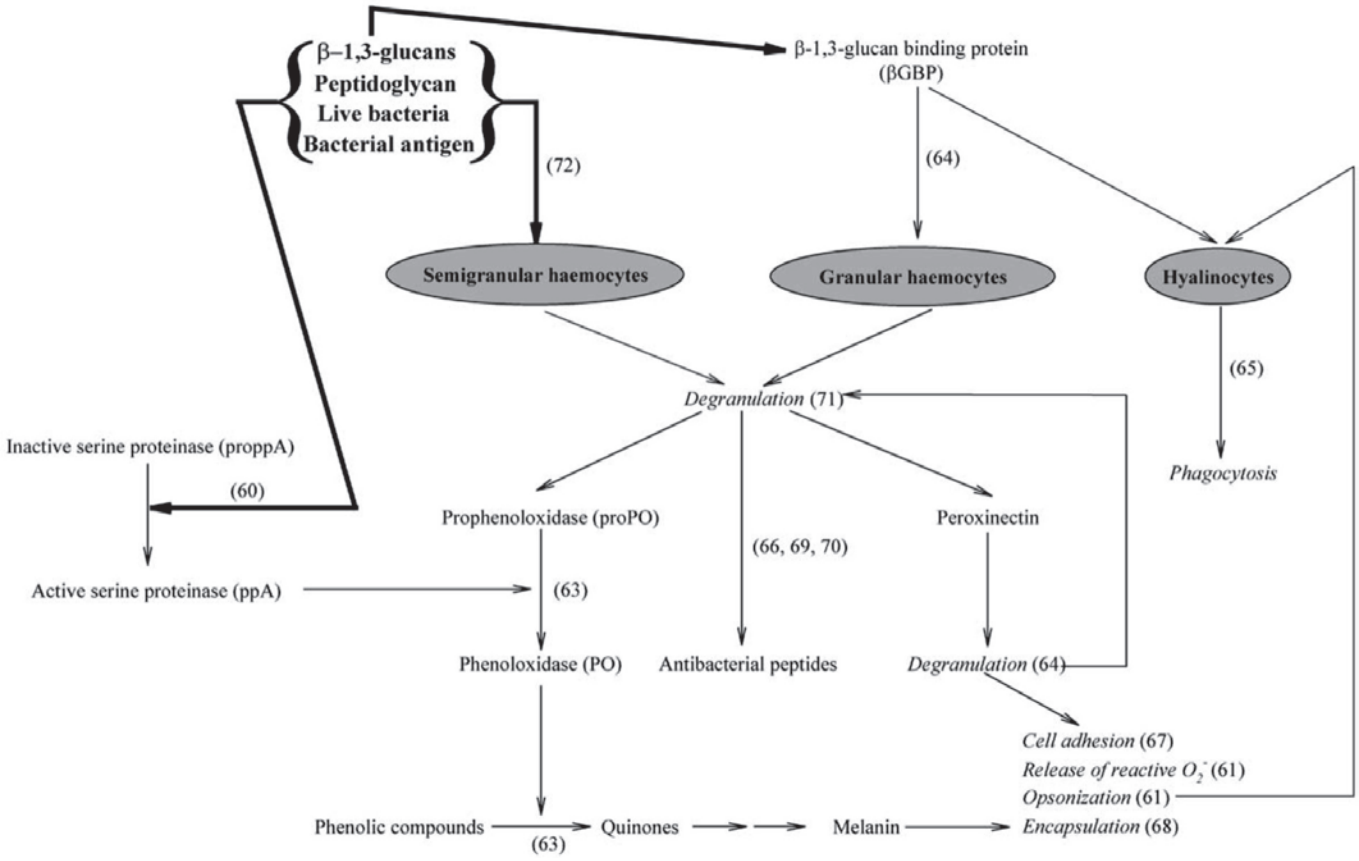

Fig. 2. Simplified flow diagram of the crustacean host defence system (Smith *et al.* 2003)

Invertebrates have evolved innate immune mechanisms to resist infection and maintain tissue integrity through three broad phases:

1. recognition of a threat (detection of non-self; allorecognition)
2. signalling pathways to activate appropriate defence mechanisms
3. effector responses which are responsible for eliminating the pathogen

Currently the research is focused on the key effector responses like, antimicrobial peptides (AMPs), melanin synthesis, coagulation and immune cell activation.

(a) Recognition

The invading pathogen is recognized through array of pattern recognition receptors (PRRs), which are germ-line encoded proteins, different from vertebrate antibodies. The process of recognition leads to rapid humoral and cellular immune responses. These PRRs bind to conserved pathogen-associated molecular patterns (PAMPs), such as the lipopolysaccharides (LPS) and induce appropriate signalling pathways. Though numerous PRRs have been identified in higher animals, TLRs, lectins and integrin have been investigated extensively in invetebrates. Groups of PRRs in invertebrates studied extensively are peptidoglycan recognition proteins (PGRPs), Gram-negative binding proteins (GNBP) or lipopolysaccharide and β-1,3-glucan binding proteins (LGBPs), C-type lectins, galectins, thioestercontaining proteins (TEPs), fibrinogen-related proteins (FREPs), scavenger receptors (SRs), Down syndrome cell adhesion molecules (DSCAMs) and Toll like receptors (TLRs). The invading pathogen is recognized by PRRs through the molecular structure/pattern unique to the pathogen. Pathogen counterparts recognized by the host immune system are called pathogen associated molecular

patterns (PAMPs) which are distributed in wide variety of pathogens interestingly these patterns are highly essential for the survival of the pathogen itself. Most commonly encountered PAMPs are polysaccharides and glycoproteins on the surface of microbes, such as lipopolysaccharide (LPS) from Gram-negative bacteria, peptidoglycan (PGN) and lipotechoic acid (LTA) from Gram-positive bacteria, and glucans from fungal cells. Further, these patterns could be polynucleotides, such as bacterial and viral unmethylated CpG DNA, single-strand and double-strand RNA from viruses. Distribution of the PAMPs effectively means that shrimp immune system can recognize almost every invading pathogen.

(b) Signalling pathways

Immune system kicks off the varieties of signalling pathways following the recognition of invading pathogen by the appropriate receptors. The most recognised pathways in invertebrate immune systems are, Antimicrobial peptide synthesis pathways, Proteolytic cascades (Melanin-synthesis pathway and Coagulation cascades) and Immune cell activation.

Anti-microbial peptides (AMPs)

Anti-microbial peptides (AMPs) are a diverse group of key effector molecules in the innate immunity. These are small in size, generally less than 150-200 amino acid residues, and have an amphipathic structure produced and stored in haemocytes. They exhibit wide range of anti-microbial activity against bacteria, virus, yeast, parasite and fungi including anti-tumor activity. Families of shrimp AMPs studied extensively are penaeidins, lysozymes, crustins, ALFs and stylicins. These AMPs are distributed in variety of organs;

Hepatopancreas : Hemocyanin C-type Lectin ALF Lysozyme

Lymphoid organ : ALF Lysozyme

Gill : Lysozyme ALF Penaeidin

Epidermis : Lysozyme Penaeidin

Intestine : Lysozyme Penaeidins ALF

Hemocytes : Penaeidins Crustins SWD, ALF Lysozyme Histones

ProPO cascade and Melanization

This is the rapid and most effective immune functions of invertebrates, the cascade requires the combination of circulating hemocytes and several associated proteins of the prophenoloxidase (proPO)-activating system. Main functions of this process are wound healing, pathogen encapsulation and killing. ProPO-activating system recognizes nonself and play important role in hemocyte attraction, induction of phagocytosis and melanization, production of cytotoxic reactant, particle encapsulation, and finally formation of nodules and capsules. This system is strictly regulated as slight reductions in the activity might lead to the failure of resistance and excess activation might lead to cytotoxicity and subsequent host tissue damage and cell death. The most common PAMPs activate by the proPO cascade are lipopolysaccharide (LPS) from Gram-negative bacteria, peptidoglycan (PGN) from Gram-positive bacteria and b-1,3-glucans from fungi.

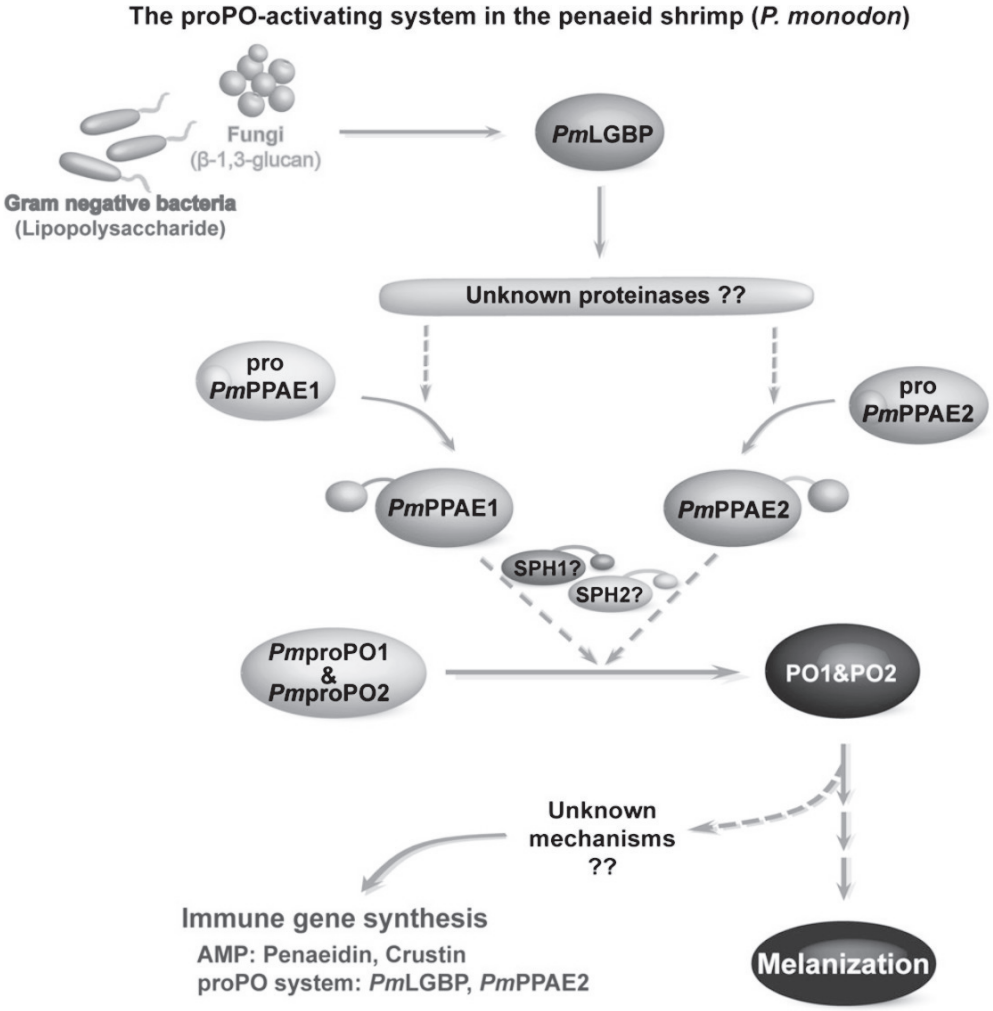

Fig. 3. A proposed mechanism for the activation of the proPO cascade in the penaeid shrimp *P. monodon*. PmLGBP is a pattern-recognition protein (PRP); PmPPAE1 and PmPPAE2 are prophenoloxidase activating enzymes (PPAEs); PmMasSPH1 and PmMasSPH2 are clip-domain serine proteinase homologs (Clip-SPHs) and PmproPO1 and PmproPO2 are prophenoloxidases (proPOs). (Amparyup *et al*. 2012)

Coagulation cascades and clotting

Among the humoral immune responses clotting system is the first line of defense especially in invertebrate immune system. Coagulation cascade in invertebrates preventing the haemolymph loss help in immobilizing and inactivating microorganisms by entrapping them together with immune effectors through a complex clotting mechanism has been identified in higher animals, crustaceans seem to have adapted the simplest coagulation process. Upon stimulation by foreign particle or tissue damage haemocytes release calcium-dependent transglutaminase (TGase) which catalyzes the formation of g-glutamyl- ε-lysine cross links between glutamine and lysine residues of the clotting protein leading to cross-linking of the aggregates.

(c) Effector responses

After the PAMPs are recognised and signalling pathways are started the effector responses are initiated. Clearly understood effector responses are antimicrobial peptide activity (AMPs) are located within granular immune cells and on epithelial layers and act by disrupting microbial cell membranes. Melanin synthesis pathways produce melanin which creates a physical barrier, encapsulating invading organism and cytotoxic by-products, like reactive oxygen species (ROS) which assist in antimicrobial

defence by immobilizing and killing pathogens. Clot formation is the effector response associated with the activation of the coagulation pathway help in formation of cross-linking among immune pathways. The immune cells help in activation of phagocytosis and encapsulation of pathogens and re-establish the tissue integrity.

Apoptosis is one of the important cellular mechanisms to inhibit viral multiplication and eliminates infected cells thereby limiting the spread of the virus. Apoptotic cell could be identified by several characteristic signs like, nuclear disassembly, fragmentation of DNA into a ladder and increased caspase-3 activity. To counter this WSSV has developed the strategies to inhibit the process of apoptosis in the early phase of infection so that target cells are available for viral multiplication and induce the apoptosis at the latter stages so that infective virions are released in large numbers.

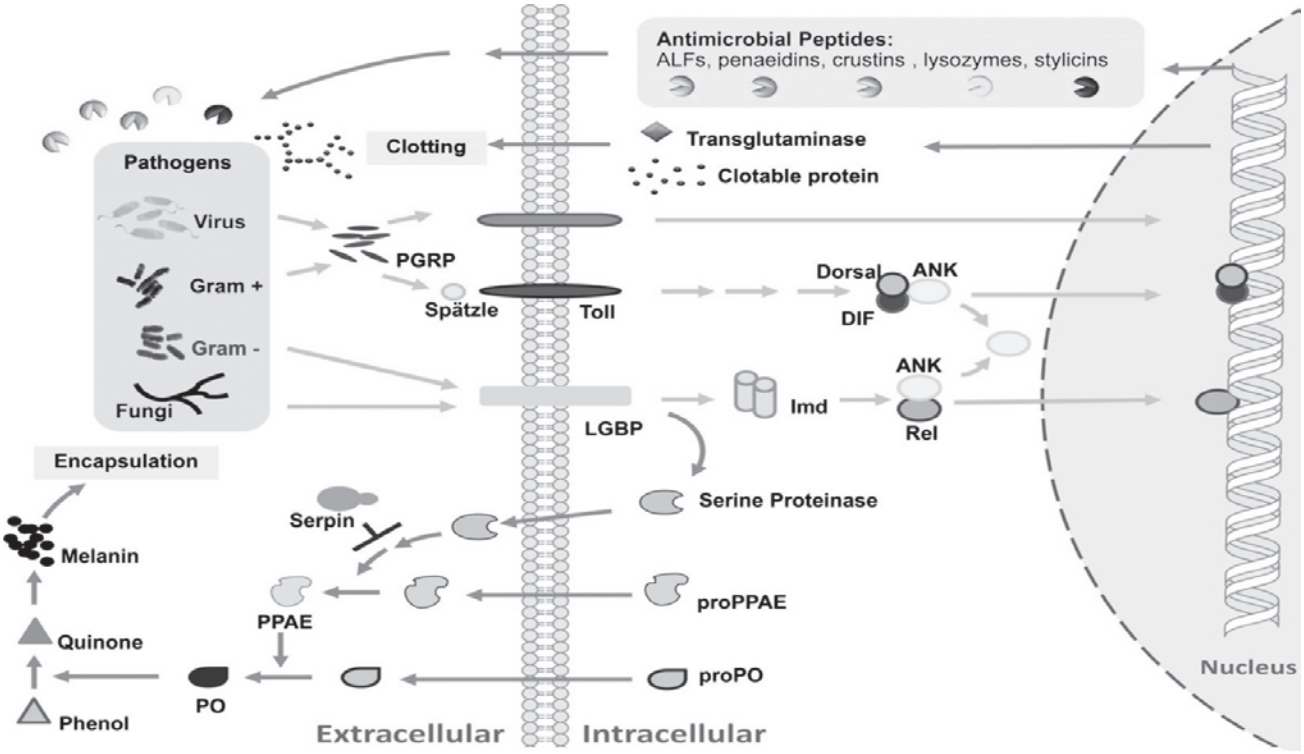

Fig. 4. Schematic representation of the molecular mechanisms involved in shrimp immune system (Tassanakajon *et al*. 2012)

Implication of RNAi machinery in antiviral activity

RNAi is a highly conserved nucleic acid-based mechanism, involved in the regulation of endogenous gene expression and antiviral defence mechanisms in plants, animals and fungi. Investigations are revealing the role of RNAi in host innate immune cell functioning. Since, invertebrates depends almost entirely on innate immunity, it is interesting to understand the role of this machinery in regulation of shrimp immune system. RNAi system senses the dsRNA, in viral replication complex and initiates the antiviral function through miRNA and siRNA pathways. Information is emerging on the role of miRNAs in regulation of most important innate immune responses, phagocytosis, apoptosis and pro-phenoloxidase system in marine invertebrates. Recently, it has been identified that 24 miRNAs are involved in regulating the above mentioned functions in kuruma shrimp *M. japonicas* and these functions are conserved among different vertebrate animals. Based on these observations it is expected that techniques to enhance the functioning of these miRNAs will help in improving the immune responses shrimp against invading pathogens in culture systems.

Immune molecules and defence reactions involved in immune functions

Class of molecule	Molecules	Species	Function
Antimicrobial peptides (AMPs)	Penaeidins	*L. vannamei* *P. monodon* *L. setiferus* *M. japonicus* *F. chinensis* *F. paulensis* *L. schmitti* *L. stylirostris* *F. indicus*	antifungal and anti-Gram-positive bacterial immunomodulation pro-inflammatory cytokine
	Crustin	*L. vannamei* *L. setiferus* *M. japonicus* *F. chinensis* *P. monodon*	antimicrobial activity against Gram-positive
	ALFs	*M. japonicus*	broad antimicrobial activity against bacteria and fungi, *V. harveyi* and WSSV Immune related gene regulation
	Lysozyme	*M. japonicus* *L. vannamei* *P. monodon* *F.merguiensis* *L. stylirostris* *F. chinensis*	Gram-positive and Gram-negative bacteria causes cell lysis.
	Stylicins	*L. vannamei* *P. monodon* *L. stylirostris*	Bacteriostatic activity, agglutination activity, strong antifungal activity
Clotting	clottable protein (CP)	*P. monodon* *F. chinensis* *M. japonicus* *L. vannamei* *F. paulensis*	Prevent loss of hemolymph upon injury and invasion of microorganisms

Pattern recognition receptors (PRRs), or pattern recognition proteins (PRPs)	LGBP BG-binding protein (GBP) lectin.	*P. monodon* *L. vannamei* *F. chinensis* *L. stylirostris* *F. paulensis* *L. schmitti*	Bind to pathogen associated molecular patterns (PAMPs), such as LPS, PG and BGs, which are present on the surface of microorganisms Trigger signaling pathways of the immune responses, including phagocytosis, nodule formation, encapsulation and synthesis of AMPs
Proteinases and proteinase inhibitors	Clip domain serine proteinases (clip-SPs) Kazal-type serine proteinase inhibitors (KPIs), serpins, and alpha-2-macroglobulins (A2Ms)	*P. monodon,* *L. vannamei* *F. chinensis* *F. paulensis*	Proteinases - apoptosis and melanization Proteinase inhibitors - regulate the pathway to prevent excessive activation of cascades and consequent damage to host tissue.
Cascade/pathways			
Melanization	Phenol oxidase	Most of important shrimp species	Activate proPO system
Apoptosis pathway	Caspases	*P.monodon* *L.vannamei* *M. japonicus*	antiviral defense
Signal transduction pathway	Toll, Immunodeficiency (IMD) and the Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathways	*L. vannamei* *P. monodon* *F. chinensis* *M. japonicus*	Antiviral defense
Antioxidant enzymes	Reactive Oxygen species, superoxide anion (O_2) hydroxyl radical (OH) and hydrogen peroxide (H_2O_2)	*P. monodon* *F. chinensis* *F. indicus*	Immune defense system

This knowledge of immune system has helped immensely in generating the interest in the area of developing immune stimulating agents as alternative to the drugs, chemicals and antibiotics for health management in aquaculture. These immunostimulating agents are known to enhance the innate (non-specific) immune response. The major advantage of immunostimulants is that they can be administered by bathing or orally as feed top-dressing to shrimp live and killed bacteria, bacterial cell wall lipopolysaccharides, peptidoglycans, glucans and chitin/chitosane are the some of the extensively studied immune stimulating agents in aquatic species. However, synthetic compounds, polysaccharides, vitamins and animal and plant extracts are also reported to enhance the non-specific immune response in finfish and shellfish. In the last few years voluminous information on the molecular mechanisms involved in invertebrate immunology is emerging. With mounting losses due to diseases and no feasibility of therapeutic approach it is pertinent to focus on development of methodologies for improving the shrimp health through immune enhancement. It is expected that knowledge regarding the underline principles of immune responses will help in devising strategies for developing novel immune response modifiers for effective control of diseases in shrimp culture operations.

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IMPORTANCE OF DISEASE SURVEILLANCE IN *Litopenaeus vannamei* FARMS AND HATCHERIES

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Shrimp aquaculture is a well established and high profitable aqua industry in many parts of the world. Till the late part of 90's, the native shrimp, *Penaeus monodon* dominated the culture practice in India. However, regular occurrence of viral disease, particularly because of white spot disease, created panic and forced many farmers to avoid this practice. At the same time, the biology of this shrimp was not known to the full extent and therefore captive maturation was not possible. As a result of this, development of Specific Pathogen Free (SPF) stocks or genetically improved stocks to have better disease resistance was not possible for this species. Subsequently, it was thought to exploit the possibility of culturing genetically improved and SPF non-native species. In this regards, the preference for Pacific white shrimp, *Litopenaeus vannamei* was at higher side because of several positive traits associated with it. As an effort from the Govt. of India, Coastal Aquaculture Authority was established and several guide lines were formulated to import vannamei for culture practice in India. Prior to this vannamei had been introduced successfully in China, Thailand, Indonesia and Vietnam. Considering the commercial availability of SPF broodstock and high production and productivity, Government of India finally permitted the introduction of *L. vannamei* with strict guidelines for import, seed production and culture. However, there is a need to initiate monitoring of disease prevalence in *L. vannamei* culture to develop an understanding of the disease development and transmission and to locate the point of entry of pathogens.

Surveillance is a method to collect, analyze and interpret data on the health status of specific cultured organism with respect to specific diseases. This should be carried out at regular intervals and also at the time of emergency as and when required. Therefore, a surveillance programme generally involves both surveillance and monitoring the health status. Surveillance is very very essential due to globalization, increased aquaculture production and microbial adaptation.

Disease surveillance has several benefits;

1. Informs the health status of cultured organisms
2. Provides information regarding the presence, distribution and spread of various pathogens
3. Helps to detect the entry point of pathogens
4. Helps to create zoning and there by prevents spread of pathogen to other areas
5. Prevents entry of exotic pathogens
6. Establishment of proper coordination between farmers and researchers and thereby it helps to educate the farmers

Different points of surveillance

1. Starting point at import

A clear health certificate regarding the absence of specific pathogens should be obtained from the importer. The animals imported should be from a well established SPF facility with associated

laboratory for health check up and pathogen detection. A detailed history regarding the genetically improved stock should also be provided.

2. At quarantine

Animals imported for culture practice in a different country should not directly be used for culture practice. Quarantine facilities should be established at different locations based on the demand for the culture and number of animals established. After the acclimatization period, the animals should once again be tested for the presence of pathogens that are mentioned to declare it as SPF. In this case, non-lethal samplings from the pleopods of shrimps should be carried out. Sufficient number of random samples from different broods should be taken to show that these are really free from these pathogens. This will help to cross check the report provided by the importer and also to rule out the presence of cryptic pathogens that able to multiply and cause health problem due to transport stress.

3. At hatchery

It is necessary to do the sampling at different points of a hatchery operation. Some of the shrimp virus stay in dormant stage and then multiply due to spawning stress. Therefore, a sampling should be tested after the initial spawning. Subsequently, samplings should be carried out from different stages of larval rearing. In order to detect the point of entry, different live feeds such as polycheat worm, artemia and algae should also be tested for the presence of different pathogens. The monitoring programme should be carried out during the entire hatchery cycle till it is ready to go for culture. After successful numbers of spawning, the broods should again be sampled for pathogen detection before these are discarded.

4. During culture

Farmers who take seeds from different hatcheries should subject it for testing before putting them in the culture ponds. Prior to the culture, care should be taken to test the other crustaceans that are present in the pond to check their carrier status. The ponds then should regularly be monitored at intervals. Sufficient number of samples from different parts of a farm should be carried out to ensure that these are disease free and negative for important pathogens. Surveillance can be a general surveillance or a targeted surveillance.

General surveillance is carried out for the determination of disease conditions in the pond by the native or existing pathogens. This is a routine process to check the general health status of animals and to ensure that all the biosecurity measures are properly followed. This will help to detect the time point and conditions for disease outbreak. Accordingly, precautions can be carried out to control the disease and prevent spreading to other areas.

Targeted surveillance aims at detecting specific pathogens of interest. It is generally associated with the detection of exotic pathogens. This type of surveillance is strictly carried out at different entry points and also during the entire practice.

Both general and targeted surveillance are very much necessary for a successful vannamei culture practice.

Important viral pathogens for general surveillance

The native viral pathogens such as white spot viral disease (WSSV) and infectious hypodermal necrotic disease (IHHNV) are most important for vannamei culture. Vannamei has been found to be

susceptible to both these viruses. While WSSV has been reported to be highly pathogenic in bringing mass mortality, IHHNV has not yet been reported to be lethal for vannamei. However, it can cause significant economical loss due to partial mortality and due to reduced commercial value because of body deformity.

These are the general information about both the viruses

White spot syndrome virus (WSSV)

WSSV is known for its high virulent nature and thereby frequently brings mass mortalities in shrimp culture ponds. The virus can be transmitted by vertical and horiziontal means. This has extensive host range and has a wide range of vectors. It infects all stages of shrimp from eggs to broodstock. About 80-100 % mortality is seen within 5-10 days of the first appearance of clinical signs. Environmental factors and presence of secondary pathogens trigger this disease. Tissues of ectodermal and mesodermal embryonic origin, especially the cuticular epithelium and subcuticular connective tissues are the major target organs for this virus. WSSV infected shrimp exhibit wide range of clinical signs. Gross lesions include reddish discoloration, reduced feeding and congregation at the pond edges with white spots on carapace and body cuticle. The most characteristic histological lesion is the presence of eosinophilic Cowdry types A inclusions in hypertrophied nuclei with marginated chromatin that become lightly basophilic in late infection.

Infectious hypodermal and hematopoietic necrosis virus (IHHNV)

The disease caused by this virus called infectious hypodermal and hematopoietic necrosis (IHHN). The virus is also called as PstDNV (for *Penaeus stylirostris* densovirus). It is the smallest known shrimp virus. First to be reported in blue shrimp, this virus was subsequently found to have three distinct genotypes. They are Type1, Type 2 and Type 3, of which Type 1 and 2 are found to be infectious for shrimp. Pacific blue shrimp, *Penaeus stylirostris* is affected severely by this disease. In other shrimps it causes chronic disease known as "runt deformity syndrome" (RDS) which is characterized by irregular growth, cuticular deformities and short bent rostra. The principal target organs are gills, cuticular epithelium, all connective tissues, the haematopoeitic tissues, the lymphoid organ, antennal gland and the ventral nerve cord, its branches and its ganglia. Transmission is by horizontal and vertical routes. The prominent histological lesion is the presence of intranuclear, Cowdry type A inclusion bodies, which are eosinophilic and often haloed within chromatin-marginated, hypertrophied nuclei of cells in tissues of ectodermal and mesodermal origin.

Important viral pathogens for targeted surveillance

Several exotic viruses have been reported to be highly virulent to vannamei. Outbreaks of diseases and mass mortality have been reported in farms of several countries. Among all the exotic viruses, Yellow Head Virus (YHV), Taura Syndrome Virus (TSV) and Infectious Myonecrosis Virus (IMNV) are considered as most virulent ones. Other viruses have been reported to cause either growth retardation or decrease production due to other reasons.

Yellow Head Virus (YHV)

The virus was first reported in 1991 and was responsible for an epizootic in Thai shrimp farms. YHV has also been reported to have high prevalent in many other South East Asian countries. Gross signs of disease and mortality occur within 2 to 4 days following an interval of exceptionally high feeding activity that ends in abrupt cessation of feeding. Mortalities can reach 100% within 3-5 days.

The hepatopancreas becomes discoloured which gives the cephalothorax a yellowish appearance, hence the name of the disease. The overall appearance of the shrimp is abnormally pale. Post-larvae (PL) at 20-25 days and older shrimp appear particularly susceptible, while PL<15 appear resistant. Histological diagnosis of YHV disease should not be based solely on the presence of lymphoid organ necrosis and systemic necrosis of the connective tissue.

Taura Syndrome Virus (TSV)

The disease and the virus associated with this was first detected in shrimp farms near the Taura River of Ecuador in 1992. It has then rapidly spread to cultured penaeid shrimp-farming regions of many other countries. Taura Syndrome (TS) is particularly devastating to post-larval *L. vannamei* within approximately 14 to 40 days of stocking into grow-out ponds or tanks. However, larger stages may also be severely affected. Three distinct phases characterize TS disease progression: i) the acute stage, during which most mortality occurs; ii) a brief transition phase, and iii) a chronic 'carrier' stage. In the acute phase, the cuticular epithelium is the most severely affected tissue. In the chronic phase, the lymphoid organ becomes the predominant site of infection. In *L. vannamei*, the acute phase of infection may result in high mortalities (40-90 %). Histopathological examination at chronic stages of infection are characterised by the presence of spherical accumulations of cells in the lymphoid organ, referred to as 'lymphoid organ spheroids' (LOS). Another feature of acute TS is the absence of haemocyte infiltration, or other signs of a host defense response. These features combine to give acute phase TS lesions a "peppered" appearance that is considered to be diagnostic for the disease, and can be considered confirmatory.

Infectious Myonecrosis Virus (IMNV)

The disease is also called as white tail disease because of the discoloration of shrimp body. *L. vannamei* cultures in Brazil were found to be affected with focal to diffuse necrosis of skeletal muscle tissues, particularly in the distal abdominal segments and the tail region during 2002. The persistent mortality seems to exist throughout the culture period with significant harvest loss to the farmers. The etiological agent was identified as Infectious myonecrosis virus. The gross changes in chronic infection were lethargy, opacity of abdominal muscles and red coloration of tail fan. Histological examination revealed numerous spheroids in their lymphoid organs and haemocytic infiltrations in muscle tissues.

Laem Singh Virus (LSNV)

This virus has been recently identified to be the cause for growth retardation. The disease was first identified in Thailand in the year 2008. Because of this, the farmers encountered shrimp with retarded growth rate. This virus is also associated with retinopathy in stunted shrimp from Monodon slow growth syndrome (MSGS) ponds and not with normal size. It is believed to be one of the contributing factors for MSGS which is seen in *P.monodon*. Gill, pleopod and lymphoid organ can be used for screening this virus in shrimps. Studies on tissue tropism of this virus have shown that infection occurs throughout neural tissues such as the thoracic ganglion, abdominal ganglion, brain and optical lobe.

Penaeus vannamei nodavirus (PvNV)

The virus is also reported to cause muscle necrosis. This was first reported in 2004 from *L. vannamei* cultured in Belize. Infection with this virus resulted up to 50% reduction in production in some ponds of the affected farms. Because of molecular & ultrastructural characteristics, *Pv*NV is placed in the

Nodaviridae family. *Pv*NV is most similar to *Mr*NV, agent of White Tail Disease in *Macrobrachium rosenbergii*. It is not highly virulent in lab challenges. Not associated with major on-farm mortality. The gross and histological signs were whitened abdominal muscles, coagulative muscle necrosis with haemocytic aggregation and basophilic inclusions similar to the signs of IMNV. Effect (s) on farmed shrimp has not been fully evaluated and is not clear at present. RT-PCR and In-situ Hybridization (ISH) methods are needed to detect *Pv*NV & differentiate it from IMNV.

Conclusion

Disease surveillance appears to have high importance for successful culture practice of any shrimp farm. This is particularly important for vannamei farming as the species is non-native and has a high risk for the introduction of transboundary diseases. A proper disease surveillance programme will help to increase the shrimp production and will also help for a sustainable culture practice in India.

PATHOLOGY OF MICROBIAL INFECTION IN *Litopenaeus vannamei*

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Introduction

Farmed raise shrimp is one of the fastest growing sectors in aquaculture in tropics, but the growth of this sector is markedly affected by various factors due to the intensification of aquaculture activities. As farm-raised shrimp production of *L. vannamei* has increased in market share and industry size, the impact of infectious diseases caused by microbial pathogens has taken an advantage on production and resulting in devastating monetary losses to *L. vannamei* farmers. 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 anaerobe bacteria of the family *Vibrionaceae*. It is ubiquitous throughout the world and all marine crustaceans, including shrimp, are susceptible. They become opportunistic pathogens when the natural defence mechanisms are suppressed. Vibriosis is caused by a number of Vibrio species of bacteria, like *V. harveyi*, *V. vulnificus*, *V. parahaemolyticus*, *V. alginolyticus* and *V. penaeicida*. Vibrio infections are commonly known as black shell disease, tail rot, septic hepatopancreatic necrosis, 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 with the presence of clumping (aggregation of digestive cells), red discolouration of the body, yellowing of the gill tissue and white patches in the abdominal muscle, melanisation, granulomatous encapsulation, necrosis and inflammation of organs (lymphoid organ, gills, heart etc.) and luminescence. Adult shrimps suffering from vibriosis may appear hypoxic, show reddening of the body with red to brown gills, reduce feed intake and seen swimming lethargically 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 characterised 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 melanization are the changes observed in the hepatopancreas or midgut region.

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 an atrophied hepatopancreas. Infected shrimp display empty midguts with increased superficial epicommensal cuticular fouling and/or opportunistic infections (i.e. black spots) also present. There is degeneration of digestive gland (hepatopancreas) which appears pale to white. The hepatopancreas is the target tissue for this disease. Microscopic exam of unstained tissue squashes from suspect hepatopancreata may show reduced lipid and dark melanized necrotic tubules.

Histopathology

Histologically, infected tubular epithelial cells will appear initially hypertrophied with a generalized basophilic intracytoplasmic granularity due to the presence of numerous pleomorphic intracytoplasmic rickettsial-like organisms. Three stages of infection have been described in histologic studies. In early NHP infection (stage I), intracytoplasmic 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 hemocytic 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 *Penaeus japonicus* in Japan in 1993. White Spot Syndrome Virus (WSSV) belongs to a family of *Nimaviridae* under 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 abdominal muscle 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 infected and are not appropriate tissues for detection. It has wide range of vectors which includes rotifers, marine molluscs, polychaete worms and non-decapodal crustaceans including artemia and copepods, as well as non-crustacean aquatic arthropods such as sea slaters and *Ephydridae* insect larvae. All these species can accumulate high concentrations of viable 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 manifestation WSD 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 lethargy and 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 into larger plates. The white spots represent abnormal deposits of calcium salts by 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 completely absent) 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 haemotopoietic 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 exhibit severe 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*. YHV virions are enveloped, rod-shaped, positive, single stranded RNA genome. This disease 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 and is the only known agent of YHD. Gill-associated virus (GAV) is designated as genotype 2. GAV and four other known genotypes in the complex (genotypes 3–6) occur commonly in healthy *P. monodon* and are rarely or not at all associated with disease. Vectors include 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 of the 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 hepatopancreas which 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 feed 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, total crop 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 the pond edges, cessation of feeding, a reddening of body and appendages, and pink to yellow colouration of the gills. However, these signs occur commonly seen in diseased shrimp and are not considered a reliable method for diagnosis of GAV disease. Shrimp chronically infected with YHV or GAV display normal appearance and behaviour.

Histopathology

Presence of moderate to large numbers of deeply basophilic, evenly stained, spherical, cytoplasmic inclusions approximately 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 target tissues and organs for YHV infection. Systemic necrosis of ectodermal and mesodermal tissues with prominent nuclear pyknosis and karyorrhexis is also another feature of this disease. Cellular changes in early infections may include nuclear hypertrophy, chromatin diminution and margination, and lateral displacement of the nucleolus. Loss of tissue structure within lymphoid organ, stromal matrix cells that comprise tubules become infected leading to loss of tubular structure, tubules appear degenerate. Lymphoid organ spheroids (LOS) develop during infection, ectopic spheroids may lodge in constricted areas of the haemocoel (heart, gills, subcuticular connective tissues etc). Necrosis of the lymphoid organ in YHD infections can be used to distinguish YHD from acute Taura Syndrome (TS) in penaeid 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 to 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). Hence, enteric tissues are inappropriate samples for detection of infection by TSV. TSV infects tissues of ectodermal and mesodermal origin. The principal target tissue in the acute phase of TS is the cuticular epithelium. In chronic infections the 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 are 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 can produce 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 cuticular epithelium 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 melanized cuticular 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 the infected shrimp show no obvious signs of disease.

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 cuticular epithelium 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 and adjacent striated muscle fibres are occasionally affected. In some severe cases the antennal gland tubule epithelium is also destroyed. Cytoplasmic remnants of necrotic cells are often extremely abundant and these are seen as spherical bodies (1–20ìm in diameter) which are eosinophilic to pale basophilic. These structures, along with pyknotic and karyorrhectic nuclei, give a characteristic 'peppered' or 'buckshot-riddled' appearance, which is considered to be pathognomonic for TS disease when there is no concurrent necrosis of the parenchymal cells of the lymphoid organ (LO) tubules. The absence of necrosis of the LO in acute-phase TSV infections distinguishes TS disease from acute phase yellow head disease in which similar lesion is seen. In this phase there is absence of haemocytic infiltration or other host-inflammatory response which distinguishes it from the transitional phase of the disease.

In the transitional phase typical acute-phase cuticular lesions decline in abundance and severity are 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 that characterize 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 of an enlarged LO with numerous Lymphoid Organ spheroids (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 *Penaeus stylirostris* 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. The virion is 20–22 nm in size, nonenveloped, 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 3A and 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 epizootics and mass mortality in *P. stylirostris*. By contrast, it does not cause 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 for life and pass it on by vertical and horizontal transmission. The infected adult carriers show no signs 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 haematopoietic tissues, 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 tests using 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, or its 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 or right) or otherwise deformed rostrum, a deformed sixth abdominal segment, wrinkled antennal flagella, cuticular roughness, 'bubble-heads', and other cuticular deformities. Populations of juvenile shrimp with RDS 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 a provisional diagnosis of IHHNV infection. The characteristic IHHN inclusion bodies are eosinophilic and often haloed, intranuclear inclusion bodies within chromatin-marginated, hypertrophied nuclei of cells in tissues of ectodermal (epidermis, hypodermal epithelium of fore- and hindgut, 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 be easily confused with inclusion bodies seen in WSSV infection.

Infectious Myonecrosis (IMN)

Infectious myonecrosis (IMN) is a recently identified disease in cultured *L. vannamei*. IMN virus found 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 cannibalism and 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 lymphoid organ may 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 of muscle, often with edema. In chronic phase liquefactive necrosis of the muscle is seen which is accompanied by hemocytic infiltration and fibrosis. Significant lymphoid organ spheroid (LOS) formation is seen and ectopic lymphoid organ spheroids are often found in the hemocoel 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, hemocytes, and in cells that comprise lymphoid organ spheroids.

***Penaeus vannamei* Nodavirus (*Pv*NV)**

*Pv*NV is a new pathogen first reported from Belize in 2004 and caused by a nodavirus called *Penaeus vannamei* nodavirus (*Pv*NV). This disease causes 50% reduction in production. The gross and histological sign mimics IMNV, i.e., whitened abdominal muscles, coagulative muscle necrosis with hemocytic aggregation, and basophilic inclusions.

Conclusions

In India shrimp farming has changed considerably because of the extensive adoption of domesticated, SPF stocks of exotic *L. vannamei* as the source of broodstock that operate under obligatory biosecurity conditions. WSSV is still remains the most important serious threats which still haunt this industry, for all cultivated species in all countries. The relative threat from other pathogens can be eliminated by adopting scientific farming, use of SPF stocks and by proper biosecurity measures.

MICROBIAL MONITORING OF *Litopenaeus vannamei* HATCHERIES

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Introduction

Commercial shrimp farming in India had reached a magnificent height in yielding income to the farmer and the country as a whole. To sustain production and to improve the quality of production the shrimp hatcheries should develop strategic planning in obtaining and managing a disease free broodstock to provide healthy *L. vannamei* seeds. Shrimp diseases have had a devastating effect on world shrimp farming to deter investment and economic development. Most of the diseases affecting shrimps are of microbial origin. Microbes are abundant in environment, some are beneficial and some are pathogenic. Hence it is more important for hatcheries to follow standard operating procedures and microbial monitoring protocols in every cycle to control the transmission of pathogenic microbes and to maintain disease free status in their hatchery environment. The aim of microbial monitoring is to prevent the disease from occurring, to reduce the incidence of infectious diseases and to reduce the severity of the disease when it occurs.

Microbial Evaluation in Hatchery

i. Broodstock Health Screening

Shrimp broodstock assessment is more significant as it plays a major role in the production of high health seed. Healthy seeds can with stand the risks associated with disease attacks even at adverse condition. Hence, SPF broodstocks are preferred at present, yet it has to be certified as free from diseases such as WSSV, HPV, IHHNV and other transboundary diseases like TSV, YHV, IMNV through quarantine from competent authority. In the quarantine area the broodstock are passed through a dip of iodine-PVP solution (20 ppm) or formalin (50–100 ppm). The sampling has to be done on the third day of quarantine period with a removal of pleopod from each shrimp (if held individually) or a sample from the population (if held as a group) for analysis. Random samples should be taken from each container to evaluate the general condition of the population held in that container. Groups of ten pleopods can be analysed as one sample. Groups which are found positive can be discarded or, in the case of a pooled sample from animals held individually, the shrimp can then be tested on an individual basis to identify and discard only the positive individuals.

Shrimp broodstocks may have pathogens in carrier state which can be transmitted vertically or horizontally to progeny. Viruses are intracellular pathogens that may be transmitted vertically through the egg or ovarian fluid during spawning, or they can be transmitted horizontally from host to host. Although PCR testing should be conducted on broodstock upon arrival during their quarantine, it is worthwhile to conduct additional PCR testing after spawning. This is because there are evidences that broodstock tested as PCR-negative for WSSV during quarantine may test positive if analysed following exposure to a stress such as spawning. Broodstock found infected with severe untreatable diseases should be eliminated immediately and only animals negative for pathogens are introduced to the maturation unit to produce healthy seed. Routine monthly screening for pathogenic vibrio and viruses like HPV, WSSV and IHHNV is required to maintain their high health status. Infected animals should be disposed of by incineration or some other method (e.g. autoclaving and deep burial) that will prevent the potential spread of virus.

ii. Evaluation of broodstock rearing water

Spawning systems should have the best water quality through which the risk of horizontal transfer of diseases between females will be reduced. It has been shown that the tissues exuded during spawning and faeces can contain high levels of some viruses (IHHNV, HPV, BP, MBV etc.) and exposure to this can result in infection of uninfected females during the collective spawning. If collective spawning must be carried out, the number of females per tank should be as low as possible to limit the number of females exposed to potential infection (i.e. one female to 200–300 litres of water). The tanks may be flat bottomed, but if they are slightly conical, or at least angled to the outlet, it allows easier and less damaging in harvesting of all the eggs. Tanks should allow the harvest of the eggs in such a way that they can be subjected to washing or a disinfection bath after collection using formalin (100 ppm for 30 sec), or iodine PVP (50–100 ppm for 1–3 min). Treflan may also be added at 0.05–0.1 ppm to combat fungal infections. This disinfection will help to reduce the risk of disease transmission.

iii. Evaluation of larval and Postlarval quality

Shrimp larval quality plays a key role in success of commercial shrimp culture. Good management in hatchery and nursery rearing tanks would ensure better survival and growth rate of shrimp post-larvae before stocking. The major problem faced by shrimp hatcheries are the mortality caused by diseases. Diseases in larvae are caused by numerous microbes like vius, bacteria, fungi and protozoa. Bacterial diseases particularly vibriosis is considered to be a major cause of mortality in shrimp hatcheries. Alavandi *et al* (2006) carried out phenotypic and molecular typing of *V. harveyi* isolates and their pathogenicity to tiger shrimp larvae and reported sucrose-fermenting biotypes of *V. harveyi* appeared to be associated with pathogenicity to larval shrimp. It is also named Luminescent shrimp disease, caused by luminescent vibrio strains is a major problem in hatchery and infected shrimp often appear luminescent at night. The larvae became host to a range of fouling organisms ranging from bacteria and fungi to protozoans of many species such as *Zoothamnium*, *Vorticella*, *Epistylis* or *Acineta*. Postlarval stages must be examined regularly for necrosis, luminous bacteria, WSSV, IHHNV, HPV, ecto and endoparasites etc.

iv. Evaluation of larval and postlarval rearing water

The bacterial flora of *P. monodon* shrimp hatcheries in India was investigated by Otta et al (2001) which revealed that the total plate counts of raw sea water on tryptic soya agar ranged from 10^2 to 10^4 /ml, whereas it ranged from $l0^4$ to 10^6 /ml in the larval tanks. The proportion of vibrio species ranged from 50% to 73%, as compared to 31% in raw sea water. A mixed bacterial flora was observed in hatchery water but in the larval tanks, the flora in the larvae was predominantly made up of vibrio species. Mourino *et al.* (2008) has noted that more than 60% of the mortalities in mysis 3 and post-larva 1 in the hatcheries of *Litopenaeus vannamei* in Santa Catarina state are related to the presence of a great number of filamentous bacteria. *Flexibacter maritimus*, Gram-negative bacilli of 0.4-0.5 μm width and 15 μm in length is pathogenic and can cause massive mortality of *L. vannamei* post-larvae. It is presumed that the presence of highly virulent strains of *Flexibacter* sp. is associated with the presence of organic matter dissolved in water, which leads to the development of the disease. This is also correlated to *Flavobacterium columnare* (Bernardet and Grimont, 1989), according to Hanson and Grizzle (1985), since water quality is directly related to their appearance. Maintenance of good water quality, use of adequate and balanced diets, and prophylactic treatments (e.g. formalin) can result in low filamentous bacteria levels in the system (Skjermo and Vadstein, 1999).

General Assesment of Larval Condition in Hatchery System

The assessment of larval condition is usually done in the morning, and decisions on water exchange, feeding and other management activities made to carry out such activities in the afternoon. The larvae in each tank should be inspected two to four times each day. Initially, a visual inspection of the larvae, the condition of the water in the rearing tank and the feed is made. Samples of larvae and postlarvae for routine checking should be taken in disposable paper/plastic containers (300 ml plastic beakers) that are disposed of once used. After the daily check is complete, the larvae or postlarvae should be discarded into a plastic container with sodium hypochlorite (20 ppm active ingredient) or another suitable disinfectant. Larvae and postlarvae used in the daily checks must never be returned to the larval rearing rooms or larval tanks. Records may also be taken for water quality parameters and the amount of food in the tank. The sample of larvae should also be taken to the laboratory for a more detailed microscopic examination. This will provide information on the stage, condition, feeding and digestion and presence of any disease or physical deformity. Three levels of observations are made based on the health assessment.

Level 1 observation by simple visual features

Swimming activity - This activity of the larvae changes dramatically but characteristically through the larval cycle. Zoea l stages will swim rapidly and consistently forwards, usually in circles, filter feeding on phytoplankton. Mysis, by comparison, swim backwards with intermittent flicks of their tails, maintaining themselves in the water column and feeding visually on phyto- and zooplankton. PL, again turn to swimming rapidly and consistently forward, initially planktonically, but at least from PL4–5 onwards, benthically, searching for food, unless maintained in the water column by strong aeration.

Phototaxi*s* - Zoea stage larvae should retain a strong positive phototaxis and move towards light. To test this, a sample of larvae is placed in a translucent container next to a light source and the displacement of the animals is observed. The weak ones do not move towards light.

Faecal string (cord)- During the zoea l stages, when the zoea are feeding almost exclusively on algae, long faecal strings can be seen projecting from the anus and loose in the water column. If the strings are short and discontinuous it indicates that the larvae are avoid of feed and not healthy.

Luminescence **-** This factor is observed directly in the larval rearing tank in absolute darkness. Larval luminescence is generally due to the presence of luminescent bacteria such as *Vibrio harveyi*.

Stage homogeneity **-** This indicates the uniformity of larval stages in a tank. If 80% or more of the population is in the same stage is considered good. It should be noted that when larval shrimp moult, it is normal to see a decrease in the stage homogeneity, so the time at which the stage homogeneity is determined has to be taken into consideration. This is also true for postlarvae when they are moulting.

Intestinal contents **-** It can be observed in older larval stages. The intestine is visible as a dark line from the hepatopancreas in the larva's head region that is easily observed in larvae held in a clear container, such as a glass beaker. This is useful as a guide to larval feeding and feed availability.

Level 2 observation using microscopic examination and squash mounts

Condition of the hepatopancreas and gut contents **-** It is an indication of larval feeding and digestion. It is observed using a wet mount of a sample of larvae on a microscope slide at a magnification of 40X.

In healthy larvae showing active feeding and digestion, the hepatopancreas and midgut will be full of small, easily observed bubbles (digestive or "lipid" vacuoles) and strong peristalsis will be seen in the intestine. Unhealthy ones will have empty intestine.

Necrosis - larval body and limbs with necrosis indicates cannibalism or possible bacterial infection, can be observed by light microscope under low power.

Deformities - indicate poor quality nauplii, if in the early stages, and bacterial infections or mishandling and stress later on. Typically, the fine setae on the limbs of the larvae and/or their rostrums may appear bent, broken or missing; the tail may appear bent; or the gut may terminate before the anus. Typically, no remedies exist for these problems (unless due to rough handling), and such deformed larvae will die. In severe cases, it may be preferable to discard the whole tank as soon as possible to prevent infection of other tanks.

Epibiont fouling - The larvae may become host to a range of fouling organisms ranging from bacteria and fungi through to protozoans of many species. These will typically attach to the exoskeleton on the head and body, and particularly around the gills of the larvae. Where the infections are slight, the next moult may remove the fouling without further problems, but in severe cases, the fouling will persist or reoccur in the next stage, indicating poor water quality and necessitating action.

Baculovirus - usually detected in whole or squashed (stained with malachite green for *Monodon baculovirus*) preparations of hepatopancreas or faecal strands from larger-sized larvae, using a high powered light microscope to spot the characteristic viral occlusion bodies (which, in the case of MBV, are dark coloured and tetrahedral) . The expression of bacculoviruses is often mediated by stress, and if seen, reductions in levels of stress can often reduce prevalence and the associated problems of growth depression.

"Bolitas"- 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. It is believed to be caused by bacteria and can be fatal. Rapid stocking of the hatchery (within three to four days), use of probiotics, good health and feeding management practices are done to overcome this condition.

Level 3 observation using molecular techniques and immunodiagnostics

These techniques are not normally required until the postlarvae are ready to be transferred to on-growing facilities. PCR and/or dot-blot techniques are commonly used to test for major viral pathogens. However, PCR is recommended as it is relatively more sensitive than dot-blot.

Conclusion

Production of healthy shrimp relies on good biosecurity and better managemental practices. The management includes nutrition aspects, phytoplankton density maintenance, water quality and other environmental conditions. Microbial monitoring at various levels is more significant for success of hatchery production, Level 1 observations are also frequently sufficient to make a decision about the fate of a hatchery tank or batch of larvae. Selection of nauplii, for example, generally includes a decision based on phototactic response without the need for a more detailed microscopic examination. If a batch of nauplii shows poor phototaxis and weak swimming behaviour, it will be rejected without further examination. But, Level 2 basic bacteriology is done to gain an understanding of the bacterial flora of the tanks and to identify possible pathogens when the larvae become weak or sick. This information may then be used to make a decision on whether the tank should be discarded or treated.

Level 3 techniques are becoming more commonly employed in shrimp hatcheries for the screening of post-larvae and broodstock for viral diseases. Routine monitoring, disinfection, dry-out of hatchery facilities and adequate aeration in larval tanks will control the growth of pathogenic microbes.

Reference

FAO (2003). FAO Fisheries Technical Paper on Health management and biosecurity maintenance in white shrimp (*Penaeus vannamei*) hatcheries in Latin America.

PROBIOTICS FOR HEALTHY SHRIMP AND POND ENVIRONMENT IN INTENSIVE AQUACULTURE

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Diseases are considered the limiting factor in development of shrimp culture operations. Control of diseases using conventional disinfectants and antimicrobial drugs resulted in limited success. With growing concern over the use of antibiotics in aquaculture, focus is shifting to alternatives like, development of vaccines, probiotics, bioaugmenters, nonspecific immune enhancers and immuno-stimulants. As an alternate to use of chemicals and antibiotics it will be wiser to use beneficial bacteria (probiotics) to competitively inhibit the harmful bacteria. Development of probiotics is one of the most significant technologies evolved in response to disease control in medicine. Administration of probiotics is widely practised both in human and veterinary medicine as an alternative to antibiotics. In recent years application of probiotics has gained importance in aquaculture, especially in intensive shrimp culture systems which are mainly export-oriented. Though only gut acting probiotics are used in human and veterinary medicine, soil and water probiotics are commonly used for management of pond environment in aquaculture.

Definition

The general definition being used for probiotics is 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance'. However, an extended definition covering the usage of probiotics in aquaculture would be 'a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment. Hence, based on this definition, probiotics include microbial adjuncts that prevent pathogens from proliferating in the intestinal tract, on the superficial structures, and in the culture environment, that secure optimal use of the feed by aiding in its digestion, that improve water quality or that stimulate the immune system of the host.

Under intensive aquaculture operations, it is difficult to expect the establishment of stable microbial community due to drastic changes in the pond environments. Major factors that influence the microbial development in aquaculture systems are changes in salinity, temperature, oxygen concentration, and quantity and quality of the feed administered in addition to the availability of particular microbe and its ability to utilize the environmental conditions for its proliferation. Since, the development of microbial community in aquaculture operations is based mainly on the availability of the microbe and favorable conditions for them to grow; there is possibility of intervention in the system. The availability of the microbe in the given culture environment can be manipulated instead of allowing microbe accidentally present in the system to grow and colonize. Provided the prevailing environmental conditions are optimum for the selected microbe, addition of such microbe should be able proliferate and dominate system. This artificial dominance could be achieved more efficiently in the presently

followed lined pond as in *L. vannamei* cultures which limit the environmental variations. In general, for single probiotic microbe to establish the dominance in the culture system it is essential that, the selected species of bacteria should be native or closely related to the native microbiota and supplied in sufficiently large numbers on regular basis.

Mode of action

Though the mechanisms of probiotics action have been well established in human and veterinary medicine, the same is not the case in aquaculture may be due to variety of species and the culture environments. The suggested modes of action for probiotics are;

- Production of inhibitory compounds
- Competition for chemicals or available energy
- Competition for adhesion sites
- Enhancement of the immune response
- Improvement of water quality
- Interaction with phytoplankton
- Source of macro-and micronutrients
- Enzymatic contribution to digestion

Methods of probiotic selection

- Collection of background information
- Acquisition of potential probiotics
- Evaluation of the ability of potential probiotics to out-compete pathogenic strains
- Assessment of the pathogenicity of the potential probiotics
- Evaluation of the effect of the potential probiotics in the host
- Economic cost/benefit analysis

Methods of probiotic application

- Addition via live food
- Bathing
- Addition to culture water
- Addition to artificial diet to larvae

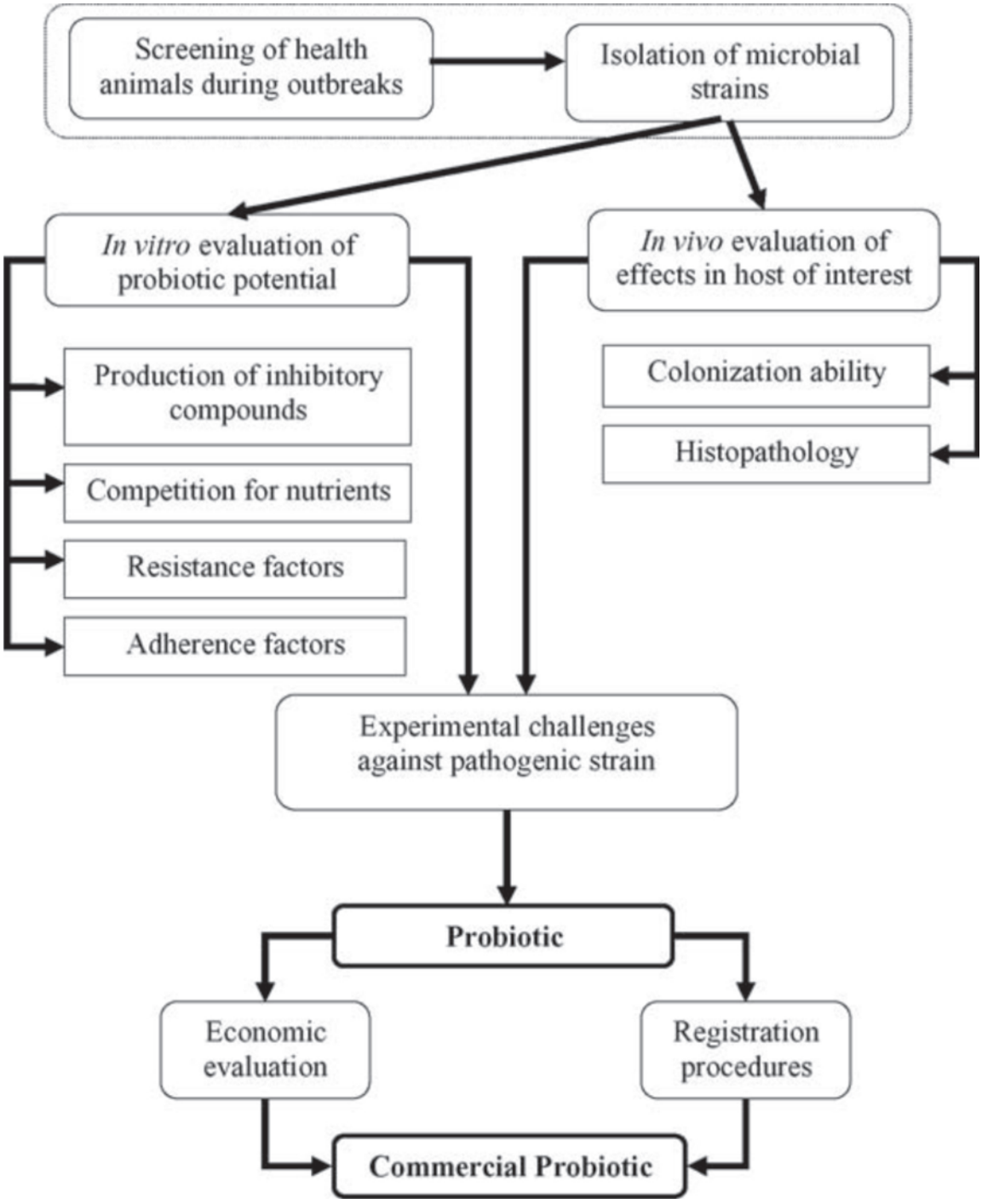

Fig.1. Diagram for selection of probiotics as biocontrol agents in aquaculture (Balca´zar *et al*., 2006).

Generally probiotic bacteria are selected based on the ability of the bacteria to inhibit shrimp specific pathogen (vibrios) and to survive pH and enzymes of host gut environment. More importantly, these bacteria should be able to colonize the gut epithelium, induce beneficial effect through enhanced nutrition and immune response and lastly probiotic bacteria should be amenable for industrial production and storage. Hence, the criteria used for selection of probiotics for application in different aquaculture systems are very crucial.

Since, economically important aquatic organisms are grown in variety of environments; arrays of probiotic bacterial species are used depending upon the salinity, pH, and temperature of the pond. Recent report on the availability of spurious probiotic products in Indian market has raised many doubts on their efficacy in the minds of technicians and farmers. Common problems of such products

are inclusion of inappropriate species of bacteria and at too low densities to be effective in target pond environment. There is a need to strictly regulate the manufacture and use of these probiotics to obtain their intended prophylactic and therapeutic effect. Such regulations are strictly monitored by European Food Safety Authority (EFSA) in European Union, Food and Drug Administration (FDA) in the US and by Health Food and Nutrition Food Association in Japan. Unfortunately, there is no such regulatory body in India to specifically monitor the sale and use of substandard probiotic products. It is important for farmers to understand that probiotics are prophylactic but not therapeutic agents hence they need continuous application throughout the culture period and the intended effect will be slow to realize.

Common microbial compositions in probiotic products

Gut probiotics: *Bacillus pumilus, Bacillus megaterium, Lactobacillus thermophilus, L.halveticus, L.plantarium, L.bulgaricus, Streptococcus lactis, Vibrio alginolyticus.*

Soil and water probiotic: *Rhodococcus* sp., *Rhodobacter* sp., *Nitrobacter* sp., *Nitrosomonas* sp., *Nitrocystis* sp. *Aerobacter* sp., *Paracoccus* sp., *Thiobacillus* sp., *Cellolomonas* spp. *Bacillus subtilis*

Application of probiotics in hatcheries

Soon after hatching, larvae get exposed to immediate environment and different types of bacteria present in the water colonise the gut. Hence it is essential to maintain the composition of beneficial bacteria in larval rearing system so that the potentially pathogenic microbes do not get colonise. It is also possible to manipulate the gut microbiota in the early stages of larval rearing by enriching the live feed and feed the larvae. Such practises in hatchery have proved to enhance the disease resistance, suppress the growth of opportunistic vibrios and help to improve the larval quality.

Activity of the probiotics in the aquaculture environment depends on microbial composition, dosage applied, route, frequency and duration of application. Though the mechanism behind the beneficial effects of probiotic application in aquaculture is not unequivocally established, it is definitely an important activity in intensive aquaculture practices especially the recently being practiced in *L.vannamei* both in hatchery and grow-out cultures. It is essential to understand the composition of bacteria in the probiotic product and mode of application, suitable for the use in different types of culture systems.

Prebiotics

Major limitations encountered during application of probiotics were high cost, adverse effect on natural microbial diversity and the susceptibility for inactivation during incorporation in feed. Potential constraints to probiotic application as well as advantages of live microbes resulted in introduction of the new concept of prebiotics. A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon. The concept of prebiotics has resulted in alternate method for manipulating microbial population in the culture, in the place of adding beneficial bacteria as probiotics prebiotics selectively stimulate the growth of indigenous favourable microbes. Prebiotics are defined as non-digestible components that are metabolised by specific health promoting bacteria such as *Lactobacillus* and *Bifidobacterium.*

Along with the application of probiotics, the use of prebiotics is a relatively new concept in aquaculture which is gaining attention worldwide. Prebiotics are not live organisms but a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the

gastrointestinal microflora that confers benefits by improving the health and productivity. Commonly used substances as prebiotic are inulin, lactose, oligofructose, mannanoligosaccharides, galacto-oligosaccharides etc. These prebiotics are selectively fermented by Bifidobacteria, Lactobacillus and Bacteroides to release short chain fatty acids and lactates which are easily absorbed in the gut.

Despite the potential benefits to health and performance, as noted in various terrestrial animals, the use of prebiotics in the farming of fish and shellfish has been less investigated. The studies have shown the beneficial effect of prebiotics in fish and shellfish on growth, feed conversion, gut microbiota, resistance against pathogenic bacteria and innate immune parameters. Additionally mechanism attributed to the beneficial effect of prebiotics are, increase in intestinal short chain fatty acids production and enhanced binding of these fatty acids to G-coupled protein receptors on leycocytes, interaction with carbohydrate receptors on intestinal epithelial and immune cells, partial absorption resulting in local and systemic contact with the immune system.

Commonly used prebiotic in aquaculture contain mixture of partially autolyzed brewer's yeast, dairy ingredient components and dried fermentation products (35.2 percent crude protein, 1.7 percent crude lipid and 53 percent simple and complex carbohydrates including oligosaccharides). Since, prebiotic substances are natural feed ingredients and hence regulatory controls are expected to be limited. Substances need to fulfil the certain criteria to be selected as prebiotics like, resistance to gastric acidity, hydrolysis by digestive enzymes and gastrointestinal absorption, fermentation by intestinal microflora and selective stimulation of the growth and or the activity of intestinal bacteria associated with health.

Recent publications reveal that application of prebiotics improve (i) growth, (ii) immune response, (iii) gut microbial community, (iv) feed efficiency and (v) survival in several species of fishes. Further, prebiotic substances are known to increase the nutrient uptake and enhance the bioavailability of trace elements in fish and reportedly improve the growth, survival and non-specific immunity in *Litopenaeus vannamei.*

Though presently the application of prebiotics is limited, in future it holds considerable potential in aquaculture. The outlook is that these preliminary observations would be confirmed further. Once this happens, prebiotics might have the potential to increase the efficiency and sustainability of aquaculture production. More of research is needed to fully conclude the effects of prebiotic application in shrimp aquaculture. In order to exploit the maximum utility of prebiotics application, factors like chemical nature of prebiotic substance, type of aquaculture system (fish/shrimp, freshwater/saline, hatchery/grow-out) and mode of application needs to be standardised. However, it is interesting to note here that application of fermented juice, jaggery and molasses is practised traditionally in Indian aquaculture. In this context, it seems that principles behind the prebiotic functions may work in this case.

Substances having prebiotic effect

- Inulin, lactose, oligofructose, mannanoligosaccharides, galactooligosaccharides
- Mixture of partially autolysed brewer's yeast, dairy ingredient components and dried fermentation products
- Mixture of molasses, rice bran and yeast

Synbiotics

As the name itself suggests is a combination of probiotics and prebiotics which is considered as having ability to enhance the effect of each component and to obtain the synergistic benefit. Synbiotics is a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastro-intestinal tract by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health promoting bacteria. Synbiotics help in implantation of beneficial live microbes in the gastrointestinal tract by selectively stimulating the growth and activating the metabolism of health promoting bacteria, and thus improving growth and productivity. The application of synbiotics, is based on the principle of providing a probiont with a competitive advantage over endogenous populations, improving the survival and implantation of the live microbial dietary supplement in the gastrointestinal tract of the host.

Reports on utility of synbiotics application in aquaculture are limited. Recent studies in fish culture have demonstrated the beneficial effect of synbiotics, a combination of *Enterococcus* spp. and *Bacillus* spp. with fructose oligosaccharides and mannan oligosaccharides. Positive effects of prebiotics observed in fish culture are improvement in (i) survival and growth, (ii) feed utilisation, (iii) body composition, (iv) immunological and biochemical functions, (v) digestive enzyme activity and (vi) disease resistance. This is a new concept for aquaculture and further studies are required to standardise the suitable combinations of prebiotics and probiotics for the intended aquaculture system.

Probiotics for maintaining the healthy pond environment

In intensive aquaculture when feeding rates are high organic matter get accumulated in the pond bottom leading to accumulation of toxicant like ammonia, nitrate, nitre, sulphate and rapid depletion of oxygen. Additionally, fermentative bacteria like vibrios increase in number and release organic acids which are toxic to shrimp. Accumulation of these toxicants cause stress to animals threatening the health and production of the cultured shrimp. Hence it is essential to ensure that feed, faeces and dead algae settle in the bottom get decomposed rapidly and clean environment is maintained. Though regular aeration and water exchange can help to some extent it is not economically viable and practically feasible in the current aquaculture scenario. Fortunately, diverse group of microbes play important role in degrading the complex organic molecules in nature. Such microbial populations present in the culture pond environment can be exploited to maintain the quality of water for healthy shrimp production. Hence the success of modern shrimp farming depends on the art of maintaining the water quality by balancing beneficial and pathogenic bacteria. As the mechanism behind the effect some of the bacteria in the probiotic preparation produce a wide range of enzymes that are very efficient in breaking down the large molecules like proteins and lipids.

It is well known fact that substancial quantity of feed gets accumulated in the pond bottom posing the threat of nitrogen waste. In a natural situation, ammonia generated in the systems is eliminated through the process of nitrification and denitrification. This essential step in nitrogen recycling in aquaculture ponds is the oxidation of ammonia to nitrate via nitrite. This is microbial process involving two physiological types of autotrophic bacteria, ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), belong to group of â- and ã-proteobacteria and cyanobaceria. â-proteobacteria are the major group of bacteria involved in degradation, nitrogen fixation and ammonia oxidation. Mainly AOB are classified under genera: *Nitrosomonas, Nitrosovibrio, Nitrosococcus, Nitrsolobus* and *Nitrospira*, while nitrite oxidizing bacteria are classified under genera: *Nitrobacter, Nitrococcus* and

Nitrospira. Commercially combination of *Nitrosomonas* and *Nitrobacter* are commonly used in aquaculture as water probiotics to maintain the commercially in aquaculture as bioremediators to control the problem of nitrogenous waste.

Generally, the single bacterial populations are studied for their probiotic effects; however the mixed cultures are expected to perform better. Since, the pond microbial environment is very much dynamic, dominance of single bacterial populations throughout the culture period is highly unexpected. When a mixture of probiotic bacteria are administered on regular basis in sufficient numbers, probabilities are high that one of the beneficial bacterium dominates the system.

It is essential to understand the interrelationships between various functional groups within these microbial communities. Further, concentrations, growth and function of these microbes also depends on intensity of culture systems, temperature, salinity, nutrient availability, water quality and other biotic and abiotic factors. In addition to the external application it is important to create conditions favoring the growth of these bacterial populations from the natural environment.

Conclusion

Application of probitotics should be considered as insurance policy and the effect is not appreciated when the culture is operational in optimal conditions, but when the circumstances deteriorate and risk of disease occurrence increase benefits are visible. The positive effects of prebiotic, probiotic and synbiotic application is proven in human and veterinary medicine. Further studies are required to understand the mechanism in different aquaculture systems especially related to suitable combinations, level of inclusion, duration of application for individual aquatic species of animals cultured to obtain the maximum intended benefit. It is expected that research to understand the basics of the microbial communities will help in effective usage of these beneficial bacteria for maintaining the healthy shrimp culture environments. It has to be kept in mind that prebiotics, probiotics and synbiotics will be effective only when applied along with proper biosecurity and standard farm management practises.

PRACTICALS

GENERAL MICROSCOPY AND BACTERIOLOGICAL ANALYSIS

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Introduction

Microbes are tiny living organisms, especially viruses, bacteria and fungi cannot be seen in naked eye. Bacteria can only be seen through microscope, fungi associated with shrimp are microscopic plants that develop interconnecting tubular structures. Viruses are many times smaller than bacteria but may be made clearly visible at high magnification provided by an electron microscope. The microscope is a basic tool in any disease diagnostic laboratory; most specimens are treated with stains that colour pathogens, causing them to stand out from the background, although wet mounts of unstained samples can be used to detect fungi, parasites (including helminth eggs and larvae) and motile organisms. Visibility of fungi can be increased by applying 10% potassium hydroxide (KOH) to dissolve surrounding tissues and nonfungal organisms.

Sampling methods for bacterial diseases

- 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 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 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 of bacteria

Principle

Gram staining is widely used for classification of bacteria into gram-positive and gram-negative bacteria. The cell wall of bacteria contains peptidoglycans, which is a thick layer in the Gram-positive bacteria. The pararozaniline dye such as crystal violet treated with iodine mordant remains trapped in the cell wall and hence, the cells are not destained when treated with alcohol. Gram negative bacteria are the primary concern in shrimp culture, mostly belonging to *Vibrio* spp.

Material required

1. Primary stain – Crystal violet
2. Mordant – Gram's iodine
3. Decolorizer – 95% ethanol

4. Counter stain – Safranin
5. Glass slide with smear
6. Immersion oil
7. Blotting paper
8. Microscope

Protocol

Prepare smear of bacteria on clean glass slide using sterile nichrome loop by mixing with a drop of sterile normal saline.

1. Fix the smear after air-drying by gently passing the slide over flame.
2. Stain the smear with crystal violet solution for 1 minute
3. Wash in tap water for few seconds.
4. Flood the smear with iodine solution for 30 seconds.
5. Wash in tap water for 15 seconds.
6. De-colorize with 95% ethanol for 10 seconds.
7. Counters stain with safranin for 1 min.
8. Wash in tap water, blot dry and observe upon oil immersion.
9. Violet coloured bacteria – gram positive. Red/Pink coloured bacteria – gram negative.
10. Record size, shape and arrangement and other morphological characteristics.

B. Observation of bacterial motility by hanging drop technique

Principle

This test is performed to find out the motility of the bacteria by examining the living bacteria microscopically by the hanging drop method.

Material required

1. Cavity glass slide
2. Cover slip
3. Vaseline
4. Microscope

Protocol

1. Sterilize the inoculating loop, take a drop of culture and place it on the centre of the clean cover slip.
2. Add a small drop of Vaseline at the four corners of the clover glass.
3. Invert the cleaned cavity slide and keep it on the cover glass carefully in such a way that when the slide is inverted, the hanging drop in suspended in the depression on the slide.
4. Keep the hanging drop preparation on the microscope stage and allow the path of light through the object (hanging drop) by raising the condenser. Focus and observe the edge of the hanging drop using low power objective.

5. Turn to high power and using fine adjustment focus the bacteria and observe motility.
6. A darting zig–zag movement indicate polar flagellation and less vigorous and more vibratory movement indicates peritrichate flagellation.

C. Total Plate Count

Principle

In this method, serial dilution of the inoculum is made before spreading on the surface of an agar plate. At higher dilutions the number of bacteria will be less and small number of well isolated colonies will be formed in the agar plates inoculated with them. This is to determine the number of bacteria/ml in a specific solution. The hatchery and aquaculture farm samples were analysed for total bacterial loads.

Materials required

1. Inoculum
2. Sterile saline
3. Sterile test tubes
4. Zobell marine Agar medium in petridish
5. Bunsen burner
6. Sterile L-shaped bent glass rods

Protocol

1. Prepare serial ten fold dilutions of the inoculum (10^{-1} to 10^{-7})
2. Place 0.1 ml of the inoculum on the agar surface and the evenly spread the entire surface using a sterile bent glass rod.
3. For each dilution one plate is inoculated as described above.
4. All the inoculated plates are incubated at 36°C for 24-48 hours.
5. After incubation the plates are observed for bacterial growth and colonies are counted. In the higher dilutions discrete colonies can be observed.
6. TPC (cfu/g or cfu/ml) = Average Count X dilution factor

D. Total Vibrio Counts (TVC)

Protocol

0.5 ml each of the appropriate sample dilution was pipetted into sterile pre-dried petri plates containing TCBS agar, in duplicate and distributed over the surface with sterile bent glass rods. The plates were incubated in inverted position at 36°C for 18-24 hours. The total of yellow and green colonies for each dilution was recorded as presumptive Vibrio count.

Total Vibrio count (cfu/g or cfu/ml) = Average Count X dilution factor X 2

E. 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, Thiosulfate Citrate Bile Salts sucrose (TCBS) agar selective for *Vibrio* spp. can be used to make tentative diagnosis to involvement of potentially pathogenic *Vibrio* spp. to bacterial infection.

F. 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 by streak dilution technique to obtain pure colonies. One well separated pure colony from each plate was streaked on TSA slants for further confirmation, identification and storage purpose, 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 – Isolated bacteria were subjected to a series of phenotypic and biochemical tests such as Grams stain, motility, oxidase, salt tolerance (0%, 3%, 8% and 11%), amino acid decarboxylation (arginine, lysine and ornithine), production of indole, methyl red and acetyl methyl carbinol, sugar fermentation test as described in Bergey's manual of Systematic Bacteriology (2005). For identification of Vibrios, the criteria proposed by Alsina and Blanch (1994, 1994a).
   c. Antibiotic sensitivity of the bacteria.

POLYMERASE CHAIN REACTION (PCR) FOR THE DETECTION OF SHRIMP VIRAL DISEASES

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Disease diagnosis is an important step in shrimp aquaculture system and it helps to take the right decision at a particular time. Rapid, accurate and sensitive disease detection methods have always been popular and need of the hour where it helps to take the possible preventive measures at the earliest possible time. In this respect, several molecular methods, particularly PCR has been very helpful and therefore being widely used for shrimp disease diagnosis. PCR targets a particular part of pathogen nucleic acid (DNA or RNA) and amplifies the targets to several folds with the help of deoxynucleotides (dATP, dGTP, dCTP and dTTP) and DNA polymerase. Therefore, it becomes easier to detect it in agarose gel. Every now and then, the methods are being modified to make it more sensitive and more rapid. While direct PCR can detect comparatively higher load of pathogens (moderate to advance stage of infection), nested PCR can even detect the presence of very low number of pathogens in the host (initial stage of infection or asymptomatic carriers).

Comparatively it is easy to deal with pathogens having DNA as nucleic acid component than RNA. This is because DNA is more stable and less prone to degradation. Extra precautions and cares have to be taken into consideration while doing with RNA pathogens. Similarly, while doing PCR, it is first necessary to convert the RNA to cDNA before proceeding into the final reaction.

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

Sampling can be either lethal or non-lethal. Non-lethal sampling is usually carried out with broodstocks or cultured adult shrimps where a piece of pleopod is cut and used for PCR without sacrificing the animal. For lethal sampling, entire animal (in case of larvae) or any tissue material can be collected based on the type of viral pathogen needed for detection. Different parts those are usually collected 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 (*Pv*NV)	RNA	Larvae, **Muscle**, Pleopod, LO, Gill

Letters in bold indicates preferred tissues for PCR

1. Extraction of nucleic acid

a. DNA

While using any kit, the instruction given by the manufacturer should be followed step wise to extract DNA. There are several other methods by which DNA can be extracted. One of the examples is given below:

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) - Homogenise - 30 minutes incubation at room temperature (RT) - Centrifuge 5000 rpm 5 mins at 4^0C - Take 300 µl supernatant - Add 300 µl of ice cold ethanol - Vertex well - Centrifuge at 14000 rpm for 10 mins at 4^0C - 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

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^{0}C 30 mins, 85^{0}C 5 mins

2. 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 $MgCl_2$ (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.

3. 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

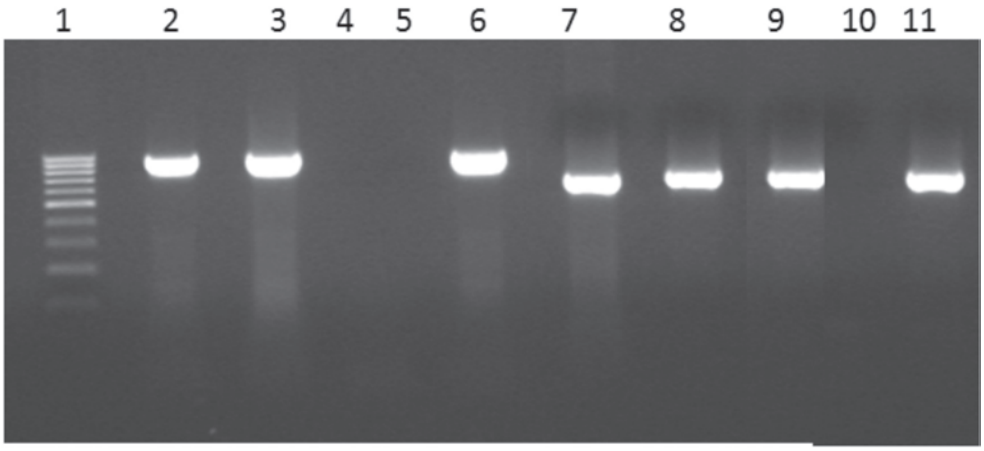
4. Gel separation of PCR products

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.

5. 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

1. 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.

Conclusion

PCR is a useful tool for rapid identification of viral and other shrimp pathogens. Early detection in larvae will help to discard the batch before taking it into culture practice. Detection in culture ponds will provide clue to take biosecurity measures to prevent the spreading

PROCESSING AND ANALYSIS OF SHRIMP SAMPLES FOR HISTOPATHOLOGY

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Microscopical examination of tissues for the presence of any pathological alterations in it is called histopathology. This involves collection of morbid tissues from necropsy, fixation, preparation of sections, staining and finally microscopic interpretation. The various steps involved in histopathology are

A. Collection of Materials

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

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 3-5 μ size sections are 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.

HEMATOLOGY IN PACIFIC WHITE SHRIMP, *Litopenaeus vannamei*

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Introduction

Hematology diagnostic tests are those that use blood to diagnose illnesses or disorders. Blood is the transport medium in the body so any toxins or deviations from normal range will be found in it. The normal range for all chemicals found in blood is known and an abnormal level signifies something wrong. Hematology and clinical chemistry is one of the principal diagnostic tools in human and veterinary medicine which can also be used as a diagnostic tool in shrimp and fish pathology as well. However, changes in haemolymph parameters such as haemocyte count, clotting time, glucose, non-protein nitrogen, ammonia, aspartate transaminase (AST/SGOT), alkaline phosphatase (ALT/SGPT), total serum protein, phenoloxidase activity, reactive oxygen intermediates (ROIs), etc., are found to show changes due to infectious diseases in shrimp and fish, almost none of these test have been adapted to routine diagnostic use. The reason might be due to lack of extensive study and popularity in shrimp and fish hematology. Therefore, this practical is aimed at knowing the methodology in basic hematology i.e., total (THC), Granular (GH) and nongranular (NGH) haemocyte count.

Equipments required

1. Tuberculin syringe.
2. Microcentrifuge tube.
3. Micropipette (100µL).
4. Microtips.
5. Scissors.
6. Binocular microscope.
7. Hemocytometer.
8. Cover glass.
9. Digital timer count down/up.

Reagents required

1. Formalin.
2. Sodium chloride.
3. Rose Bengal.
4. Haematoxylin solution.
5. Ethanol.
6. Xylene.
7. DPX mountant.

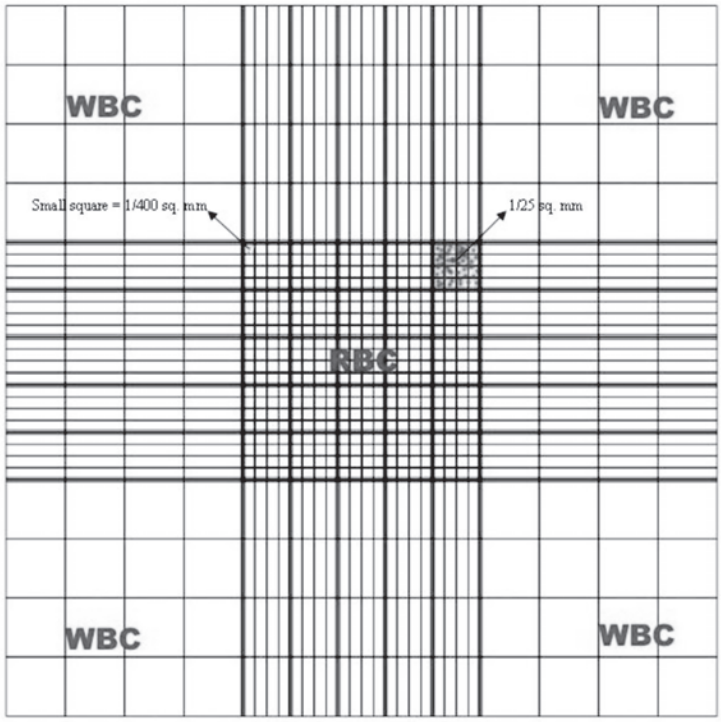
The shrimp used should be live and freshly collected from the pond.

Procedure for THC

1. Withdraw haemolymph (0.1 mL) from the ventral sinus of the first abdominal segment into a syringe containing equal volume of fixative (10% formalin in 0.45 M NaCl).
2. Transfer to a microcentrifuge tube gently.
3. After 10 minutes at room temperature, mix 20 µl of the fixed haemocyte suspension with same volume of Rose Bengal solution (1.2% Rose Bengal in 50% ethanol).
4. Incubate at ambient temperature (27-35^{o}C) for 20 minutes.
5. Load the stained mixture in to the space between cover slip and Haemocytometer.
6. Count the cells found in five RBC squares (volume of one square = 0.2x0.2x0.1 mm^3).
7. Calculate the THC as: THC ml^{-1} of haemolymph = 5 × count × 10^4 × dilution factor.

Procedure for GH and NGH

1. Prepare tongue shaped smears from the fixed and Rose Bengal stained haemocyte suspension.
2. Dry the smears in air before counterstaining with haematoxylin solution for 7 to 10 minutes.
3. Rinse the slides with tap water for 10 minutes followed by dehydration with ascending grades of ethanol (10 dips each).
4. After dehydration, clear the slides in xylene I and II (3 times for 3 minutes each) before being mounted with DPX mountant and covered with a cover glass.
5. Care must be taken to avoid air bubbles.
6. Record the proportions of GH that included both large-granular and small-granular/ semigranular haemocytes in 200 total haemocytes.
7. Calculate the total number of GH (i.e. GH count/200 × THC).
8. Calculate the total number of NGH (i.e. NGH count/200 × THC).



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Training Programme on

MANAGEMENT OF EMERGING DISEASES OF SHRIMP WITH SPECIAL REFERENCE TO PACIFIC WHITE SHRIMP, *LITOPENAEUS VANNAMEI*

Sponsored by

National Fisheries Development Board, Hyderabad

10th – 14th December, 2012

Convenors

Dr. Subhendu Kumar Otta

Dr. Prasanna Kumar Patil

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DR.A.G.PONNIAH
DIRECTOR

Forward

Pacific white shrimp, *Litopenaeus vannamei* culture has started gaining popularity in India as seen from the increased shrimp production in 2012. Based on the risk assessment carried out by CIBA, Govt. of India permitted its culture based on Coastal Aquaculture Authority guidelines for seed production and farming. This was well accepted by the farmers as an alternate species to tiger shrimp, *Penaeus monodon*. Till the late of 90's, tiger shrimp culture had gained immense popularity amongst the farmers of India. However, appearance of white spot syndrome virus (WSSV) resulted in repeated loss of crop. Due to difficulties in domestication of tiger shrimp, developing Specific Pathogen Free (SPF) stocks has been limited to few players. On the other hand, there are number of sources for Pacific white shrimp, *Litopenaeus vannamei*. In addition, Pacific white shrimp has higher growth rate and suitable for high density culture system. All these favourable attributes has led to increased number of farmers opting for vannamei culture.

Success of any cultured species to a large extent depends on the disease prevalence. The devastating role of many shrimp viruses such as WSSV, Yellow head virus (YHV), Taura syndrome virus (TSV) and Infectious Myonecrosis virus (IMNV) is well documented. Therefore, it is highly essential to create a better environment and take all necessary precautions to avoid the disease in the culture system. At the same time, it is also absolutely essential to take extra precautionary measures for the disease detection in vannamei considering the fact that seed production is based on imported stock. Introduction of any exotic pathogens will have high negative impact on the native species that are generally cultured in India. Therefore, along with developing culture system, awareness on the disease risk is very much necessary on the part of farmers and technical persons involved in vannamei culture.

I am extremely happy to know that the Aquatic Animal Health and Environmental Division is conducting a 5 day training programme on "Management of emerging diseases of shrimp with special reference to Pacific white shrimp, *Litopenaeus vannamei*" from 10th to 14th December, 2012. A manual is also being released on this occasion. I am grateful to the National Fisheries Development Board (NFDB), Hyderabad for sponsoring this programme. This training is the need of the hour and I am quite hopeful that it will provide immense benefit to the participants. My best wishes for all the participants and compliments to both the convenors, Dr. S.K. Otta and Dr. P.K. Patil for conducting this training programme.

(A.G.Ponniah)

Preface

Culture of Pacific white shrimp, *Litopenaeus vannamei*, has been taken up by the farmers of India on an extensive manner. Though, Specific Pathogen Free brooders are used to produce the larvae, the culture environment remains the same. Therefore, there is every possibility of disease outbreak by the existing pathogens like WSSV. There is also the likelihood of introducing transboundary pathogens considering the non-native status of vannamei. A number of infectious viral pathogens, have been documented in vannamei. In this regards, special attention by all the personnel involved with vannamei culture practice with respect to disease is highly essential. The necessity for rapid, sensitive and accurate diagnosis as well as biosecurity measures to prevent the disease outbreak should be well understood. The present training programme has therefore been organised for the benefit of participants. Fortunately, this training programme has been proposed in a time when vannamei culture is gaining popularity.

We are very much grateful to NFDB for their support and encouragement to conduct this training for the benefit of people who are interested in vannamei culture. We have received overwhelming response from several parts of the country regarding the participation in this programme.

The programme has been planned for a period of 5 days from 10th to 14th of December and consists of both theory and practical classes by the scientists from CIBA as well as eminent scientists from outside. Materials pertaining to all these classes have been compiled and put in the form of this manual. All the lecture notes and the protocols for practical have been designed to be very specific and relevant to the disease management in vannamei culture system. We are very much hopeful that this manual will provide sufficient information and guidance to all the participants of this programme.

We are very much grateful to Dr. A. G. Ponniah, Director for his encouragement and support to conduct the training programme. Our sincere gratitude to Dr. K. P. Jithendran, Scientist In-Charge, Aquatic Animal Health and Environment Division, who has been with us from the very beginning and provided all possible support for the improvement of this manual. We thank all the scientists for their prompt contribution to bring out this manual in time. A special thanks to all the scientists of Aquatic Animal Health section for their unconditional support.

We are sure that the manual to be a helpful guide to all the participants for a successful and sustainable vannamei culture.

Convenors

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