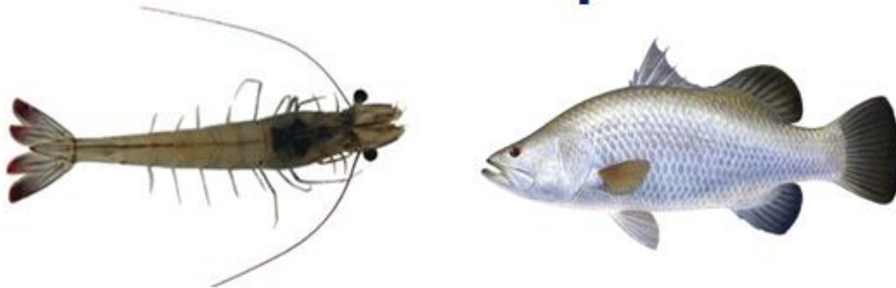
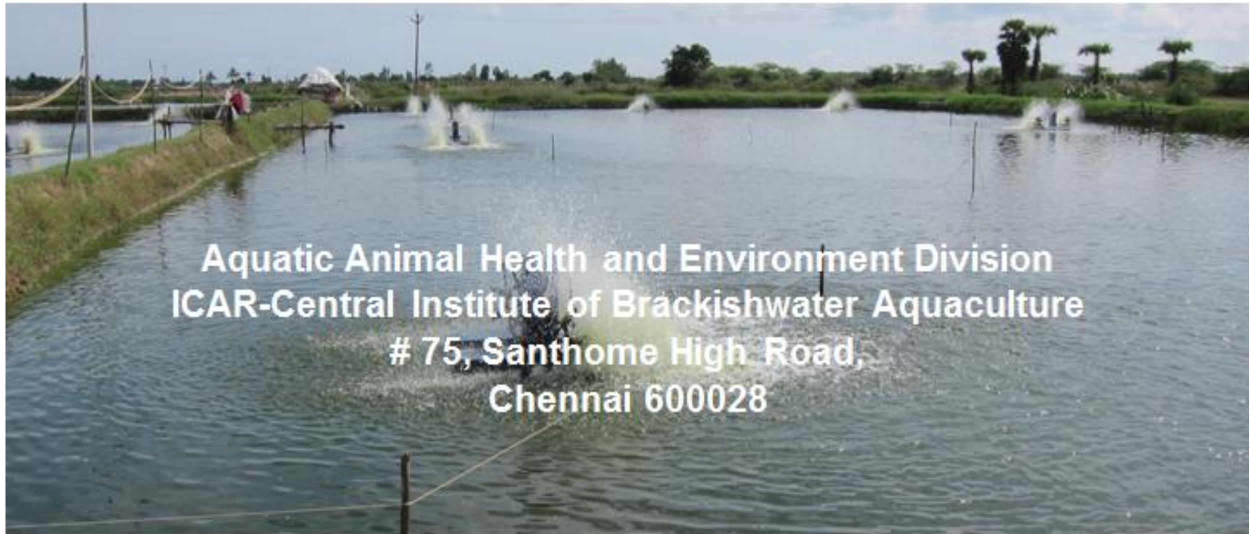
CIBA-TM Series- 2015- 2

Training Manual

On

Aquatic Animal Health Management in Brackishwater Aquaculture





Aquatic Animal Health and Environment Division
ICAR-Central Institute of Brackishwater Aquaculture
75, Santhome High Road,
Chennai 600028

CIBA-TM Series-2015-2

Training Manual

On

Aquatic Animal Health Management in Brackishwater Aquaculture

Contributed by:

K.K.Vijayan, S.V.Alavandi, K.V.Rajendran, K.P. Jithendran, C.P.Balasubramanian, M.Jayanthi, M. Muralidhar, A. Panigrahi, K.Ambasankar, J. SyamaDayal, T.K.Ghosal, Debasis De, M.Poornima, R. Saraswathy, P.K.Patil, S.K.Otta, Sanjoy Das, K.P.Kumaraguru Vasagam, P.Ezhil Praveena, T.Bhuvaneswari, GourangaBiswas ,
Prem Kumar, N. Lalitha, R. Kumara Raja, L. Christina, K.P. Sandeep, Satheesha Avunje, T. Sathish Kumar, and R. Vidya.

Aquatic Animal Health and Environment Division
Central Institute of Brackishwater Aquacutlrue,
75, Santhome High Road, R.A. Puram,
Chennai 600028

DISCLAIMER

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, recording or otherwise, without theprior written permission of Director, CIBA.
The author and publisher are providing this book and its contents on an “as is” basis and make no representations or warranties of any kind with respect to this book or its contents. In addition, the author and publisher do not represent or warrant that the information accessible via this book is accurate, complete or current.
Except as specifically stated in this book, neither the author or publisher, nor any authors, contributors, or other representatives will be liable for damages arising out of or inconnection with the use of this book.

CONTENT

Part I

Sl. No	Title	Page No
1.	Overview of BrackishwaterAquaculture	1
2.	Better management practices (BMPs)	9
3.	Biosecurity and Quarantine Measures for Aquaculture Health Management	25
4.	Diseases surveillance in brackishwater aquaculture: recent developments	31
5.	Nutrition and feed management in brackishwater aquaculture with special reference to aquatic animal health	36
6.	Live feeds and health management in the brackishwater larviculture	40
7.	Application of probiotics and immunostimulants in aquaculture for pond health and disease management	44
8.	Pond preparation and management practices for prevention of diseases in brackish water aquaculture	48
9.	Water and soil quality requirements and their management in brackish water aquaculture	55
10.	Introduction to Nucleic Acids	62
11.	Viruses, structure, biology – viral replication, infection, variations / methods	67
12.	Viral diseases and their management with special reference to white spot disease and Indian shrimp farming	74
13.	Update on Acute hepatopancreatic necrosis disease (AHPND)	78
14.	Diseases in shrimp aquaculture-Current scenario in India: white faeces/gut syndrome (WFS), growth variation / retardation, white muscle syndrome, running mortality syndrome (RMS) and and Hepatopancreatic microsporidiosis	81
15.	Shrimp Diseases : An overview	86

Part II

Sl.No	Title	Page No
1.	Requirements for Aquatic Animal Disease Diagnostic Laboratory	93
2.	Investigating disease in brackishwater aquaculture: Overview	95
3.	Shrimp anatomy, dissection, tissue preservation; sampling, preservation / fixation and transport of samples for disease investigation	100
4.	Bacteriological methods for disease investigation: Sampling, isolation and identification of bacteria	108
5.	Application of molecular diagnostics in disease management	114
6.	Extraction and quantification of nucleic acids	118
7.	Principles and practice of polymerase chain reaction	123
8.	PCR diagnosis of some important OIE listed viruses and bacterial pathogens of shrimp	127
9.	Histopathological techniques in shrimp disease diagnosis	134
10.	Collection, preservation and despatch of aquatic animal sample to a diagnostic laboratory / referral laboratory for disease diagnosis	136

Part I

Concepts in Aquatic Animal Health Management

Overview of Brackishwater Aquaculture in India

Ever increasing human population coupled with decreasing availability of space for land-based food production system (such as agriculture, animal husbandry) has resultedshortageandinconsistentsupplyofqualityfoodforlargenumberofpeople across the globe. A realistic solution of the problem could be sustainable utilization of water resources as most of the global water resources are lying unutilized or underutilized. Amongst a variety of food items present in the aquatic system, fishes are considered as the most important group of the organisms suitable for human consumption. While capture fisheries is showing the signs of almost stagnation for more than a decade, aquaculture offers a vast scope of expansion.

India is the second most populous country in the world with a total population of 1.21 billion as in 2011 representing 17.5% of the world's population and occupying only 2.4% of the world's landmass. In line with the global trend, greatest challenge the country faces is to ensure food security of the largely undernourished protein starved population in rural as well as urban areas, especially in the context of declining land resources available for agriculture and animal husbandry. Hence fisheries, mainly aquaculture sector would have to emerge as the saviour to meet increased food demand. With its limited resources of man and materials various central and state govt. agencies are making all out efforts at augmenting production by way of exploiting sustainably all potential sources of aquatic organisms in various kinds of waterresources.

Indian aquaculture has demonstrated a six and half fold growth over the last two decades. Carp in freshwater and shrimps in brackishwater form the major areas of activity. Aquaculture in India is generally practiced with the utilization of lowtomoderatelevelsofinputs,especiallyorganic-basedfertilizersandfeed.About 40% of the available 2.36 million hectares of freshwater resources and 13% of a total potential brackishwater resource of 1.24 million hectares is under use at present. This offers vast scope for both horizontal and vertical expansion of these sectors. As aquaculture plays vital role in socio-economic development in terms of income and employment, environment friendly aquaculture has been accepted as a tool for rural development. It also has huge potential for foreign exchangeearnings.

Initiatives have also been taken up to use the unutilized and underutilized resources in several regions of the country. Issues like investment in fish and shrimp hatcheries, establishment of aquaculture estates, feed mills, R&D support and ancillary industries have been given special emphasis to strengthen the pace of growth of thesector.

Origin and development

Brackishwater farming in India is an age-old traditional system confined mainly to the'*bheries*'(manmadeimpoundmentsincoastalwetlands) of WestBengal, "*gheris*" in Odisha,"*pokkali*' (salt resistantdeepwaterpaddy)fieldsinKerala,"*kharlands*" in Karnatakaand"*khazans*" in Goa coasts.Thesesystemshave been sustaining production of 500–750 kg/ha/year with shrimp contributing 20–25% with no

additional input, except that of trapping the naturally bred juvenile fish andshrimp seed during tidal influx. In this traditional method, low lying areas near the banks of saline water rivers and creeks are encircled by peripheral dyke and tidal water is allowed to enter the impoundment along with natural seeds of various species of shrimps, crabs and fish. Water is retained with periodical exchanges during lunar cycles and the animals are allowed togrow.

Realizing the importance of shrimp farming in Indian economy, Central Inland Fisheries Research Institute (CIFRI) under the Indian Council of Agricultural Research (ICAR) established first Experimental Brackish water Fish Farm at Kakdwip, West Bengal in 1973. This was followed by inception of All-India Coordinated Research Project on Brackish water Fish Farming in 1975 by the ICAR with centres in West Bengal, Odisha, Andhra Pradesh, Tamilnadu, Kerala and Goa. At the same time, shrimp seed production studies were initiated by the Central Marine Fisheries Research Institute (CMFRI) of ICAR. Commercial scale shrimp farming started gaining roots only after 1988–1989 and the semi-intensive farming technology demonstrated production levels reaching 4–6 tons/ha. Area under shrimp farming increased substantially during 1990–1994 with remarkable growth rate till 1995 as the boom period of commercial-scale shrimp culture and the bust came in 1995-96, with the outbreak of viral disease. The fact that most of the farmers were new to commercial scale and high intensive shrimp farming, the general ignorance of good aquaculture practices and the lack of suitable extension services, led to a host of problems. Later with the advent of bio-secured closed culture technology using better management practices, semi-intensiveand intensive shrimp farming again started to regain its lost glory during early years of the present century. Farmed shrimp production increased from 102940 tons in 2001-02 to record production of 144346 tons in 2006-2007 and operating at around 100000 tons over the years.

Brackishwater aquaculture development in India was mostly oriented till 2009 to tiger shrimp, *Penaeus monodon* culture only. Other shrimp species like *P. indicus*, *P. merguiensis*, *P. penicillatus*, *P. japonicus* and *P. semisulcatus* are not yet cultured on large commercial level. As tiger shrimp farming became regressed by viral diseases since 1995 and profitability was decreasing due to abnormal hike in input cost and decreasing unit sale value, Indian farmers were looking an alternative. In 2009, the Coastal Aquaculture Authority of India (CAA) permittedthe entrepreneurs to introduce a new species, *P. vannamei* (Pacific white leg shrimp) in India with prescribed guidelines. Before introduction, risk analysis was carried out by Central Institute of Brackishwater Aquaculture (CIBA) and National Bureau for Fish Genetic Resources (NBFGR) after pilot scale initiation 2003.At the same time CAA is very keen in the bio security and approval for culture of *P. vannamei*. Since its introduction, vannamei farming showed rapid growth.

The state wise area and production of shrimpis asfollows:

Among Indian states, Andhra Pradesh is leading in *P. vannamei* farming contributingnearly80%ofIndianproduction .Table3depictsstatewiseareaunder culture and production of *P. vannamei* (Pacific white leg shrimp) inIndia.

Table 1: Areas (ha) under shrimp cultivation by state

State	1990	1994	1999	2014
WestBengal	33815	34400	42525	48410
Orissa	7075	8500	11332	6302
AndhraPradesh	6000	34500	84269	36123
TamilNadu	250	2000	2670	7804
Kerala	13000	14100	14595	12917
Karnataka	2500	3500	3540	394
Goa	525	600	650	31
Maharashtra	1800	2400	970	1486
Gujarat	125	700	997	2359
Total	**65090**	**100700**	**161548**	**115826**

Table 2: Area (ha) under farming, production and percent of total production during2013-2014

State	Area under farming	Production(mt)	Percent of total production
WestBengal	48410	52581	19.4
Orissa	6302	14532	5.4
AndhraPradesh	36123	159083	58.7
TamilNadu	7804	25815	9.5
Kerala	12917	5175	1.9
Karnataka	394	664	0.2
Goa	31	63	0.02
Maharashtra	1486	3513	1.3
Gujarat	2359	9393	3.5
Total	**115826**	**270189**	**-**

Source: MPEDA,2014

There is huge potential for mud crab farming in the country. Still there is no organized aquaculture of mud crab for supporting the export trade. Major reason is the non-availability or inconsistent availability of crab seeds for farming. Technology for seed production, culture and fattening of green mud crab *Scyllla serreta* has been developed by CIBA. Some farmers are practicing crab fattening in the coastal regions with considerable success. After the inception of crab hatchery by the Rajiv Gandhi Centre for Aquaculture (RGCA) in Nagapattinam district in Tamil Nadu, hatchery produced crab seeds are now available. Some progressive farmers have started crab grow-out farming with better performance of hatchery producedseedscomparedtowildones.

Table 3: State wise area under culture (ha) and production (tons) of *P. vannamei* inIndia

States		2009-10	2010-11	2011-12	1012-13	1013-14	2014-15
WestBengal	Area	0	0	0	0	130	326
	Production	0	0	0	0	479	395
Orissa	Area	0	0	25	46	485	2340
	Production	0		100	436	2907	11866
Andhra	Area	264	2739	7128	20198	49764	37560
Pradesh	Production	1655	16913	75385	133135	210639	276077
Tamil Nadu &	Area	0	34	397	1511	5087	5037.1
Pondicherry	Production	0	109	2863	8595	26281	32687.8
Kerala	Area	0	0	0	0	0	5.8
	Production	0	0	0	0	0	11.75
Karnataka	Area	0	0	72	154	157	124.76
	Production	0	0	232	484	517	623.2
Goa	Area	0	0	0	1	29	27.2
	Production	0	0	0	15	67	88.2
Maharashtra	Area	10	94	127	439	908	1274.51
	Production	30	508	941	1503	3291	4901.04
Gujarat	Area	9	64	88	366.71	707	3545.4
	Production	46	717	1195	3348.19	6326	26763
Total	**Area**	**283**	**2931**	**7837**	**22715.71**	**57267**	**50240.77**
	Production	**1731**	**18247**	**80717**	**147516.2**	**250507**	**353413.1**

Source: MPEDA(Kochi)

High value carnivorous fishes like Asian seabass (*Lates calcarifer*), snappers (*Lutjanus sp.*) and herbivorous/omnivorous fishes like Striped grey mullet (*Mugil cephalus*), Tade mullet (*Liza tade*), Parsia (*Liza parsia*), Milk fish (*Chanos chanos*) and Pearl spot (*Etroplus suratensis*) are available for farming in the Indian coastal ecosystem. In addition to this, fishes like *Mystus gulio*, are also being cultured. Successful technology has been developed for the seed production of Asian seabass under controlled conditions and farming by CIBA since 1997. Some enterprising farmers in Tamilnadu and West Bengal have taken up seabass culture. Monoculture of Asian seabass is in practice in those areas where cheap trash fish is available in plenty. Polyculture of Asian seabass following "predator-prey culture" system using tilapia as prey material has also been tried with considerable success. In addition to this an avenue has come by successful breeding and seed production of cobia (*Rachycentron canadam*) using land based pond reared brooders by CIBA. Cobia farming in India is gaining momentum. Controlled breeding of grouper (*Epinephelus tauvina*), striped grey mullet and pearl spot has also been successful. Mullets, milkfish and pearl spot have shown promises for commercial aquaculture in inland saline soil / water areas. Production potential ranging from 0.5 to 3 tons/ hectare/ year has been demonstrated from such waters.

Farmingsystems

There are five different shrimp aquaculture practices mentioned in the literature, ranging from traditional to ultra-intensive techniques, but the most common techniques followed in India are traditional, extensive, semi-intensive, and intensive. These three categories are divided, according to their stocking densities (shrimp/ m^2), and the extent of

management over grow-out parameters, i.e. level of inputs (Table4).

Traditional culture practices dependent completely on the natural tidal entry for seed, food and water exchange. Furthermore, traditional systems are often characterized by polyculture with fish or by rotation with rice, e.g. in the *bheris* of West Bengal and *pokkalis* of Kerala in India. In this method of aquaculture low lying areas near the banks of saline water rivers and creeks are encircled by peripheral dyke and tidal water is allowed to enter in the impoundment along with natural seeds of various species of shrimps, crabs and fishes. Water is retained with periodical exchanges during lunar cycles and the animals are allowed to grow. After 3–4 months harvesting is done partially during lunar cycles. Productivity in this system ranged between 500–750 kg/ha, of which about 30 percent is contributed by prawns/ shrimps and 70 per cent bymullets

Extensive shrimp aquaculture is primarily used in areas with limited infrastructure. Producers rely on the tides to provide most of the food for the shrimp and as a means of water exchange. Feed for shrimp is naturally occurring, in some cases fertilizers or manure is added to promote algal growth. Low stocking densities result in modest yields. Land and labour are the principal inputs, which keeps operational cost at a minimum. The extensive farming is commonly known as improved traditional farming. This system involves construction of peripheral canals/ ponds of size ranging from 1–5 ha. Shrimp seed at the rate of 15000 – 20000/ha are stocked. Water management is done by tidal effect. The average yield is 1500 – 1700 kg/ha, including fin fishes. In most of the cases, the stock is left atthe mercy of nature and the predators. Supplementary feeding is not generally practiced as the entire production system is dependent on utilization of natural productivities. However some farmers use oil cakes and rice bran as supplementary feed.

Semi-intensive cultivation involves stocking densities beyond those that the natural environment can sustain without additional inputs. Consequently these systems depend on a reliable shrimp post larvae (PL) supply, and a greater management intervention in the pond‟s operation compared to extensive ponds. Semi-intensive shrimp aquaculture relies on water pumps to exchange up to 25% of pond volume daily; however, mostly closed culture is practiced at present. With stocking rates of 6-20 shrimp PL per m^2, fertilizers are applied to augment natural foodintheponds.Supplementaryfeedingis doneusingformulatedfeedspreferably in pelletized form. Maximum annual yields range from 2 to 6 tons per hectare. The risk of crop failure increases with increasing farming intensity, which is mainly due to the impact on water quality exerted by the high stocking densities and supplementary feeding. All of the costs associated with semi-intensive production are much higher relative to those for extensive production, including a more complex system of ponds, installation of a pump system to regulate waterexchange, skilled management, labour, purchased feed and seed stock,and increased energy usage for aeration and other purposes. The higher the culture intensity, the higher the capital required and the higher the risks involved. Thus,theincreasedcapitalinputsrequiredforsemi-intensivecultureoftenprecludeits adoption by small-scale producers. Tiger

shrimp farming and low density pacific white leg shrimp farming in India falls under thiscategory.

Intensive grow-out systems utilize ample supplies of clean sea / estuarine water, adequate infrastructure, and well-developed hatchery and feed industries. Intensive shrimp farming introduces small enclosures (down to 0.1 ha), high stocking densities (20-50 hatchery-produced shrimps/m^2), around-the-clock management, very high inputs of formulated feeds, and aeration. Aeration, the addition of oxygen to the water permits much higher stocking and feeding levels. Yields range from 7 to 15 tons per hectare per year. The risk of disease can be serious in intensive culture, especially if water discharge from one pond or farm is taken into another to be reused. Most of the *P. vannamei* farming in India is conducted in this method using specific pathogen free (SPF) seeds under strict biosecurityprotocol.

Table 4: Farming practices based on level of management, stocking density and production followed in India:

	Traditional	Extensive	Semi-intensive	Intensive
Pond size(ha)	0.1-50	1-10	0.2-2	0.1-1
Stocking	Natural	Natural +artificial	Artificial	Artificial
Stocking density (seed/m^2)	Unregulated	2-6	6-20	20-50
Seedsource	Wild	Wild + Hatchery	Hatchery + wild	Hatchery
Annual Production (ton/ha/yr)	<0.6	0.6-1.5	2-6	7-15
Feedsource	Natural	Natural	Natural + Formulated	Formulated
Fertilisers	No	Yes	Yes	Yes
Waterexchange	Tidal	Tidal +pumping	Pumping	Pumping
Aeration	No	No	Yes	Yes
Diversity of crops	Polyculture	Monoculture, polyculturerarely	Monoculture	Monoculture
Disease problems	Rare	Rare	Moderate to frequent	Frequent
Employment (persons/ha)	1-2	2-3	3-4	4-5

Human resource development through brackishwater aquaculture

Employment opportunities in coastal areas have increased greatly with the development of shrimp farming. According to an estimate, the average labour requirement in shrimp farming has been estimated to be about 600 labour days/crop/ha. In contrast, labour days/crop/ha in paddy cultivation is 180, which is much lower than shrimp farming. Case studies carried out at a sea-

based farm in the Nellore District of Andhra Pradesh showed an increase of 2–15 percent employment and 6–22 percent income for farm labourers following the establishment of shrimp farms. Hatcheries and feed mills in the brackish water sector are also providing substantial employment opportunities. Jobs generated in the main and supporting sectors of the shrimp aquaculture sector was estimated to be over three lakhs inIndia.

Laws andregulations

There are many laws and regulations, which are relevant to aquaculture adopted at state level, several key laws and regulations relevant to aquaculture at the central level. Those include the century-old Indian Fisheries Act (1897), which penalizes the killing of fish by poisoning water and by using explosives; the Environment Protection Act (1986) with provisions for all environment related issues; Water (Prevention and Control of Pollution) Act (1974) and the Wild Life Protection Act (1972). The Supreme Court prohibited the construction / set up of shrimp culture ponds except traditional and improved traditional types of ponds within the Coastal Regulation Zone (CRZ) on 11th December, 1996. It also ruled that an authority should be constituted to protect the ecologically fragile coastal areas, sea shore, water front and other coastal areas and specially to deal with the situation created by the shrimp culture industry in the coastal states / union territories. To perform the functions indicated by the Supreme Court, Coastal Aquaculture Authority was formed in accordance with the Environment (Protection) Act. The Authority, to which specific responsibilities for aquaculture have been allocated, falls under the administrative control of the Ministry of Agriculture.

Institutionalsetup

Division of Fisheries under the Department of Animal Husbandry, Dairying, and Fisheries of the Ministry of Agriculture, Govt. of India is the nodal agency for planning, monitoring and the funding of several centrally sponsored developmental schemes related to fisheries and aquaculture in all of the Indian States. Most of the states possess a separate Ministry for Fisheries and also have well-organized fisheries departments, with fisheries executive officers at district level and fisheries extension officers at block level, who are involved in the overall development of the sector. Centrally sponsored schemes are implemented through 422 (Fish Farmer‟s Development Agency) FFDAs in freshwater sector and 39 (Brackishwater Fish Farmer‟s Development Agency) BFDAs in the maritime districts substantially contributes to brackishwater aquaculture development. The ICAR under the Department of Agricultural Research and Education, which in turn is within the Indian Ministry of Agriculture, has a Division of Fisheries, which undertakes the R&D on aquaculture and fisheries through a number of research institutes. There are about 400 Krishi Vigyan Kendras (KVKs) operated through State Agricultural Universities, ICAR Research Institutes and NGOs, most of which also undertake aquaculture development activities. The MPEDA functioning under the Ministry of Commerce, besides its role in the export of aquatic products also contributes towards the promotion of coastal aquaculture. Many other organizations like Department of Science and Technology, Departments of Biotechnology, University Grants

Commission also support or conduct R&D in the subject. Various NGOs and private organizations contribute substantially in thiscontext.

Research andDevelopment

Eight fisheries research institutes are there under ICAR, the nodal agency for aquaculture research in India, of which CIBA, in Chennai is responsible for research on brackishwater aquaculture. These institutes have their regional centres located in different agro-ecological regions to undertake research on problems of regional importance. Research programmes are set depending on national priority and regional necessity, farmers' feedback is also given due emphasis. Fisheries colleges under different State Agriculture Universities, as well as other universities and organizations also undertake aquaculture research. The institutes transfer the developed techniques and technology through research publications and on-farm demonstrations. To disseminate the emerging technologies,electronicmediaalsoplayvital role.

Wayforward

Exports will remain the mainstay of the sector for years to come. Institutional agencies focused towards this end must seek to examine the scope for diversification of markets, communicate to exporters and processors the niche markets that exist for exclusive markets for value added fish and shrimp. Non-tariff barriers to trade will continue to assume different forms and dimensions. It is the versatility and capability of the seafood industry to adapt that will enable them to survive such onslaughts on their territory. Domestic markets for fish and fish products not only provide an entirely new opportunity for growth but also can act as a buffer in case of gluts in the international markets. Institutional agencies such as National Fisheries Development Board (NFDB) have an onerous task on hand for enabling the domestic markets for fish to establish itself. Mud crab farming is one of the avocations started in the brackishwater sector recently to enhance the production of mud crabs as well as to uplift the socio-economic condition of coastal rural population. In the brackish water sector there were issues of waste generation, conversion of agricultural land, salinization, and degradation of soil and the environment due to the extensive use of drugs and chemicals and destruction of mangroves. Efforts towards adoption of improved farming technologies like recirculatory aquaculture system (RAS), improved polyculture, integrated multitrophic aquaculture (IMTA) may make brackishwater aquaculture more environmentallyacceptable.

Better management practices (BMPs)

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 preparation
- Pond water preparation
- Seed quality and screening
- Water management
- Pond bottom management
- Feed 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) Drying

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 hatcheryshould 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 quality 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 *Litopenaeusvannamei* Clifford (1999) proposed a scale. Length uniformity was evaluated using the coefficient ofvariation (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 favourable 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.

CIBA

Biosecurity and Quarantine Measures for Aquaculture Health Management

Aquaculture has rapidly grown during the last couple of decades, contributing significantly to the National economic development through export of fish and fishery products and providing food security to the country. Indian aquaculture sector has achieved remarkable growth during the past five years especially, with respect to the shrimp production through aquaculture after the introduction of Pacific white shrimp. During 2014-15, seafood exports crossed 10,51,243 tons valued at ` 33,441.61 crore (USD 5511.12 million). However, disease related losses in aquaculture have been a challenge to achieve higher productivity.Suchsetbackshavebeenexperiencedcountriesworldover.

Aquatic animals are widely translocated across countries for enhancing aquaculture productions and species diversification. Such trans-boundary movement of live aquatic animals has the risk of introduction of new diseases. Responsible fisheries emphasizes the need to minimize the risk of disease transfer and adverse effects on wild and cultured stocks associated with the introduction of non-native species and transport of eggs, larvae, brood stock and other live organisms. Presently two amphibian, ten fish, seven molluscan and eightcrustacean diseases have been listed by the World Organization for Animal Health (OIE). In molluscs, parasitic diseases are important, while in fish and crustaceans viral diseases are cause of concern. Whether a listed disease is due to a virus, fungus, bacterium or a parasite, the occurrence of the disease may adversely affect international trade among trading partners that have, or do not have, the listed disease. The purpose of this article is to emphasize the importance of quarantine measures and biosecurity of aquaculture in minimizing risks of disease and maximizing aquacultureproductions.

Introduction of exotic species, import risk analysis and quarantine

Introduction of exotic species has both benefits and adverse impact. The exotics may be introduced with a view to enhancing growth, genetic up-gradation of local stocks, as biological control agents, disease resistance and for ornamental purpose.

The introduced species may escape into natural waters and establish in the wild. Theadverseeffectassociatedwithexoticscanbedividedintothreemaincategories

i.e. ecological, genetic and health. Ecological impacts may be due to habitat alteration, competition and predation. The genetic impact may result in the loss of native germplasm and genetic diversity, which has evolved through centuries to adapt to our conditions. The exotic may introduce pathogens of concern in the open water and destroy the native species. For the economic, social and trade development, movement of live aquatic organisms across national boundaries is necessary even though such activities may lead to the introduction of new pathogens and pose risks to the importing country. However, movement of live aquatic animals into our country should be considered only

after critically examining the options for utilizing the natural biodiversity of India. The import of live aquatic organisms has to be considered taking into consideration the ConventiononBiodiversity(CBD)aswellas theBiodiversityActofIndia.

Asthemovementofliveaquaticanimalsi nvolvescertaindegreeofrisktothe importing country, an import risk analysis (IRA) to assess the possible risk associated with the import is imperative. The main components of IRA are hazard identification, risk assessment, risk management and risk communication. Though various countries use different methods, they should be science-based and transparent with detailed documentation. The importing country has an obligation to ensure that IRA is scientifically sound, adequately documented and critically evaluated as per international obligations. The claims about its own aquatic animal health status should be accurate and based on scientific data and rigorous surveillance as demanded by the exporting country. The exporting country should also ensure that the information provided on its health status is accurate and based on international standards. It also has an obligation to report any significant change in the health status to all trading partners and international conventions. In India, *Penaeus vannamei*, a non-native species of shrimp, has been introduced to brackishwater aquaculture system as an alternative to *P. monodon*, culture, recently. The introduction was mainly driven by the negative impact of white spot disease(WSD)causedbywhitespotvirus(WSV)onthesustainabilityof*P.monodon* culture. The introduction of this exotic species was facilitated by the Coastal Aquaculture Authority and ICAR-CIBA and NBFGR carried out import risk analysis prior to the introduction of the species in the country and recommended for establishment and maintenance of quarantine facility byGovernment.

The strategy of the aquatic quarantine and health management system, primarily involves the protection of a country's aquatic biodiversity from exotic organisms, pathogens and containment of the diseases. The guiding principles in establishing a quarantine policy for responsible movement of aquatic organism and products have been formulated by consensus among various countries. According to FAO (2006), *"Quarantine means maintaining a group of aquatic animals inisolation with no direct or indirect contact with other aquatic animals, in order to undergo observation for a specified length of time and, if appropriate, testing and treatment, including proper treatment of the effluent waters*." Detailed aquatic animal quarantine guidelines are provided in Asia Regional Technical Guidelines on Health Management for the Responsible Movement of Live Aquatic Animals and the Beijing Consensus and Implementation Strategy (FAO/NACA, 2000). For the success in formulating and implementing aquatic quarantine, health management and certification system, it is necessary to identify pathogens of concern. Viruses such as white spot virus and bacteria such as vibrios are of great concern. International Aquatic Animal Health code requires that all the member countries make available through OIE whatever information is necessary to minimize the spread of important pathogens and their etiological agents to assist in achieving better worldwide control of these diseases. The OIE has two lists of Diseases of Aquatic animals i.e. diseases notifiable to OIE‟ and „other significant diseases‟. These diseases,

especially the notifiable ones, are of significance in international trade. At present, the OIE notifiable and other significant diseases are only considered for quarantine purposes. But there are other diseases, which are infectious and capable of causing economic, and biodiversity loss. To comply with the OIE guidelines to deal with the diseases, it is essential that a country has to develop disease information and reporting system built on a disease surveillance programme. In this direction, Aquatic Animal Disease Surveillance programme has been implemented in India since 2013, and diseases of concern are being listed. India also established its Aquatic Quarantine Facility (AQF) in the year 2009 at Chennai at the behest of Ministry of Agriculture for quarantining the *P. vannamei* broodstock imported to India. Establishment of this facility has boosted vannamei farming inIndia.

Aquaculturebiosecurity

Biosecurity is a broad concept and the application of biosecurity concepts to shrimp aquaculture will contribute significantly to reduce losses due to diseases and make this sector more sustainable and environmentally responsible. Biosecurity means „life protecting", but its use appears to be restricted to issues related to preventing the introduction, establishment or spread of unwanted biological organisms or agents. The principles of biosecurity should be considered to keep the pathogen not only out of the culture environment but also out of the country and the region. Implementation of biosecurity practices is an increasingly pressing issue for fisheries and aquaculture managers, considering the importance of this sector in terms of economic development of the people. Resource protection, food security, trade, production and development issues are driving this change (Beers,etal,2005).Developingbiosecurityprogrammeincludesidentificationofthe

species at-risk that is required to be protected by the programme, threats,pathways of hazards; assessment of the level of harm that would result; measures that could be used to mitigate the risk; documentation of the programme, its performance and auditing of the programme, preparation of contingency plans, and finally provision of adequate resources to implement the programme. The sanitary and phyto-sanitary measures (SPS Agreement) provide an internationally enforceable set of rights and obligations on the use of biosecurity measures by governments.

A biosecurity programme is developed after scientific analysis of information with the aim of adopting procedures to manage risks to an acceptably low level. Theuse of sound epidemiological principles and a logical, structured approach will help in achieving the biosecurity. At farm level, implementing biosecurity plan requires modifying existing farms and management routines. In this instance, white spot disease (WSD) is taken as a case considering its devastating nature and biosecurity measures required for minimizing risks associated with this disease in aquaculture are outlined. Basic husbandry practices have to be expanded to include elements of disease prevention and control. Biosecurity measures implemented appropriately can be a cost-effective way of managing disease risks.

Among the viruses causing diseases in shrimp, the white spot syndrome virus (WSSV) is an extremely virulent pathogen. It is one of the most prevalent

CIBA

and widespread viruses in shrimp aquaculture systems around the globe. Transmission of viral diseases in aquaculture occurs through two pathways, viz., horizontally (transmitted by direct contact, or indirectly, by ingestion of infected organisms, and water), and vertically (virus is passed from an infected female parent to her progeny). A number of vectors such as rotifers, marine molluscs, polychaete worms and non-decapod crustaceans including *Artemia salina* and the copepods, as wellas non-crustacean aquatic arthropods such as sea Slaters (Isopoda) and Euphydradae insect larvae can serve as host to this virus and transmit the disease in cultured stock. To date, more than 93 species of arthropods have been reported as hosts or carriers of WSSV either from culture facilities, the wild or experimental infection. Birds also can serve as potential sources of disease transmission. At present there is no treatment available to interfere with the unrestrained occurrence and spread of the disease, while biosecurity and better management practices involving rigorous sanitation practices on shrimp farms have been helpful in prevention and control of thedisease.

Farm level of biosecurity measures have to be implemented by the farmers. Achieving biosecurity in hatcheries and farms requires preventing the entry of WSV into hatcheries and farms, monitoring the health status of the shrimp population, adoption of better management practices including recommended protocols from using pathogen-free stock, pond preparation and management measures.

Preventing the entry of WSSV in hatcheries and grow-out phase of aquaculture can be achieved primarily using specific pathogen-free (SPF) or specific pathogen- resistant (SPR) and genetically improved (selective breeding method) stock. Specific pathogen-free history comes only from a long-term captive breeding and disease surveillance programme at a facility that has a fully functional and effective biosecurity plan. Pathogen carriers such as vectors, intermediate hosts, reservoir hosts, non-host biological carriers such as birds, insects, other predators, human beings and, fomites such as water, vehicles, buckets, shoes, nets, clothing also pose serious threat and management measures need to be incorporated to prevent entry of pathogens from these sources.

Adopting quarantine measures for broodstock prior to their use, adopting better management practices (BMPs) and standard operating protocols (SOPs) by implementing good sanitary practices, treating water before use, optimizing stocking density of larvae and maintaining good water quality, treating hatchery effluent during seed production in hatchery will help in achieving biosecurity in hatcheries.

Main preventive measures at pond / farm level include proper pond preparation to eliminate pathogens and their carriers, treatment of water in reservoirstoinactivatefreevirusesandkillvi ruscarriers,waterfiltration usingfine filters to keep carriers out, closed systems to avoid contamination from source water, reduced water exchange to minimize the entry from source water and even changing the water source. Transmission of virus can be prevented by providing crab fencing, fencing, foot baths, wheel baths, and disinfection protocols. Improved husbandry practices have been successfully employed for the control of diseases. Shrimp ponds with a history of disease outbreaks have a

greater likelihood of future disease outbreaks, and hence, special attention is required during pond preparation in suchfarms.

Pond preparation is essential to reduce the risks of shrimpdisease outbreaks. Removal of bottom sludge, especially in ponds with high stocking densities, ploughing of soil when wet, use of lime during pond preparation will help in minimizing disease risks. Farms with poor bottom soil quality such as presenceof a black soil layer, will suffer crop failures. Hence, the sludge must be removed and disposed away from the pond site. Extra precaution should be taken while disposing sludge from farms affected by disease outbreak during the last crop. Sludge removal should pay attention to areas of the pond where there is a high accumulation of organic matter from pervious crop, such as feeding areas. Ponds must be ploughed to expose the black soil layer underneath bottom soil to sunlight and atmospheric oxygen. By this process, the organic waste (sludge) will be oxidized. Ploughing on wet soil is particularly recommended for ponds if the sludge cannotberemovedproperlybymanualorm echanicalmethods.Afterploughing,

Ponds must be dried for 2-3 weeks and even more when pond had a history of WSD outbreak since WSSV can be viable for three weeks despite sun-drying. In case a heavy tractor is used for ploughing, then plough the dry soil and then fill the pond with water to wet the soil and then again dry. Ploughed pond bottom leads toturbid water conditions during culture period. Therefore, compaction of the bottom using heavy rollers after the whole process of pond preparation, i.e., before water intake, helps avoid the turbid water condition. Liming during pond preparation optimizes pH and alkalinity conditions of soil and water. The type and amount of lime to be added depends mainly on the soil pH and also on pond waterpH.

Some important biosecurity measures to be adopted in the shrimpfarm:

- Stocking should be done with disease-free SPFpost-larvae.
- The post-larvae should only be procured from registeredhatchery.
- The stocking density should not be very high.
- The crab fencing and bird scare lines should be inplace.
- Movement of people should be restricted inside thefarm.
- There should be proper education and awareness among the farmers on biosecurity.
- Environmental cleanliness should bemaintained.
- Proper pond preparation measures should be followed.
- Foot bath and hand disinfection should be there at the entrance.
- If possible, the separate feeding trays, boats, refractometer and

Potassium permanganate footbath

sampling materials should be used for eachpond.

Summary

Aquaculture has been contributing significantly to the National economic development in India. However, diseases have become the major limiting factor determining aquaculture production. Further, as translocation of aquatic animals for diversification has emerged as a potential threat and source of new diseases, quarantine and health management have become integral components of aquaculture. The strategy of the aquatic quarantine and health management system, primarily involves the protection of our country‟s aquatic biodiversity from exotic organisms, pathogens and containment of the diseases. In the process of introducing *P. vannamei* in India, ICAR-CIBA and NBFGR carried out import risk analysis and recommended introduction of the species and this introduction has resulted in substantial increase in shrimp production. This is also facilitated by the establishment of Aquatic Quarantine Facility (AQF) in the year 2009 at Chennai for *P. vannamei* broodstock imported to India. In this situation, application of biosecurity concepts will contribute significantly to reduce losses due to diseases and make shrimp aquaculture sustainable and environmentallyresponsible.

Diseases surveillance in brackishwater aquaculture: recent developments

Introduction

Global wild fisheries have been enduring under pressure of continuous exploitation, and during the last many years the catches are showing a flattened curve, the trend in Indian capture fisheries also shows similar stagnation. The promotion and progress of aquaculture sector is emerging as the only viable alternative, in meeting the ever increasing valuable fish protein. The Food and Agriculture Organization (FAO, 2012) estimates show that the catch production will remain relatively the same at about 90 million tonnes until the year 2030, while the production of aquaculture is expected to increase approximately to about 93 million tonnes by 2030 from the 63.6 million tonnes in 2011. In India barcksihwater aquaculture, especially the shrimp farming is the engine of industrial phase of aquaculture, contributing a major share in the export earnings, which crossed 30,000 crores during 2013-14. The Indian shrimp farming sector follows a bubble and bust cycle, where early nineties showed a remarkable growth with tiger shrimp farming, but later collapsed with the outbreak of white spot disease (WSSV) pandemic, even forcing a crop holiday for shrimp farming during 1995. However from 2009, with the introduction of specific pathogen free (SPF) Pacific white shrimp, India's brackishwater aquaculture saw a new dawn with productions crossing three lakh metric tonnes in 2013. Fisheries sector in India contributes about 0.83% of the total GDP and about 4.75% of agricultural GDP (Anon, 2014) valued at about `30213.26 crores during 2013-14.This scenario could be sustained provided aquaculture intensification progresses on a responsible aquaculture mode. Long-term sustainability of brackishwater aquaculture sector can be achieved with meticulous planning and effective management of aquaculture inputs and rearing systems, with proper use of novel biotechnologies in seed, feed and health aspects of the host and farming systems. As in the case of other rearing systems such as agriculture and animal husbandry, disease issues and related crop failures is the most significant challenge in the growth, sustainability and profitability of brackishwater aquaculture sector.

Diseases problems and related crop loss are the major constraints in aquaculture production

Across the world, the aquaculture production is showing an impressive growth, and the expansion is also significant, where the introduction of new species, including exotic species is playing a major role. In the process of introduction and movements of live aquatic animals and animal products and their life stages (broodstock, seed, and feed), has become a normal activity in India and other aquaculture countries. This intensified aquaculture, along with the introduction process, is opening up new opportunities for opportunistic and exotic pathogens, ultimately resulting in the spread and incursion of diseases in shellfish and finfish rearing systems. Disease outbreaks in aquatic environment are complex and the etiology is often difficult to determine since a series of events which are involved (environmental factors, stock health condition, infectious

agent, poor husbandry and management practices), in the process of disease development and outbreaks. Aquatic animal disease caused by viruses, bacteria, fungi, parasites and other undiagnosed and emerging pathogens continue to have significant impacts, as the industry expands to meet the challenges of increased production, and the unavoidable intensification.

Over the last couple of decades, diseases such as white spot syndrome caused by white spot virus (WSSV), yellowhead disease caused by yellow head virus (YHV) and Taura syndrome caused by taura virus (TSV), heavily impacted shrimp aquaculture in the Asiatic region and in Americas and caused the collapse of the *Penaeus monodon* industry. The combined loss from shrimp diseases, at the global level from 11 shrimp farming countries for the period 1987–1994, was estimated at 3019 million USD (Israngkura and Sae-Hae, 2002).WSD alone is estimated to cause losses of over US$6 billion since its emergence in 1992. Since 2009, the newly emerging diseases such as early mortality syndrome (EMS), specifically known as Acute Hepatopancreatic Necrosis Disease (AHPND), has caused large losses among shrimp farmers in China, Vietnam, Malaysia and Thailand. Losses due to AHPND are reported to be well in excess of US$ 1 billion per year. Recently in 2013, AHPND was also detected in Mexico with more than 80% mortality in about 50% of its shrimp farms in operation and the loss was estimated to be $ 116.2 million. These case histories clearly reflect the capacity of the lethal disease causing pathogens and the damage caused by the disease outbreaks to the aquaculture sector. Considering the limited therapeutic options available for the control of viral diseases, only timely disease detection using novel diagnostic tools for disease surveillance followed by active response to adopt and practise proper health management approaches would ameliorate the magnitude of the problem.

Aquatic animal health monitoring and aquatic animal disease surveillance

The term aquatic animal health monitoring is nothing but comprehensive collection, analysis and dissemination of information about diseases that are known to occur in the population which is being monitored. It is used to evaluate the frequency and trends of diseases, the risk factors associated with it and its economic impacts. Monitoring programs are used in conjunction with disease control and eradication programs frequently. The term *surveillance* implies an active process in which data are collected, analysed, evaluated and reported to those involved with a goal of providing better control of a disease or condition. It aids in detecting an exotic or new disease within a given population. Disease surveillance programs are designed to argue freedom from specific diseases and should always elicit action in the event of an exotic disease introduction. Important questions often asked as part of the surveillance programs include: (i) is the frequency of the disease remaining constant, increasing or decreasing?; (ii) are there differences in the geographical pattern of the condition?; (iii) does the disease have any impact on productivity and / or profitability?; (iv) is the disease absent from a particular species / strain, region, or nation?; and (v) is a control or eradication program cost-effective?

Due to the wide variety of species cultured, the pathogens and management systems, is necessary to establish surveillance systems; these should be designed to demonstrate freedom of aquatic animals from infectious diseases, taking into account the definition of the

population, including any sub-populations that should be targeted to improve the probability of detecting disease, clustering of disease, documentation of the methodology used, survey design and data analysis procedures, the test or test system being used, the design prevalence or minimum expected prevalence in the presence of disease, sampling approaches, and quality assurance systems. Surveillance has been recognized as one of the key elements of any animal health policy, giving priority to preventive approach, early detection and rapid response.

Approaches to Aquatic Animal Disease Surveillance

The various approaches in performing surveillance programme are General or Passive surveillance and Targeted or active surveillance.

General or Passive Surveillance relies on the use of secondary that is collected from disease records, sales of medicines and production records in the farms. Stakeholders play a critical role in passive surveillance and hence they should be involved in the surveillance programs. Continuous training programs and periodical feedback with results will help in motivating them in their commitment towards such initiatives.

Active surveillance is based on primary data collection encompassing steps through sample collection through data analysis. Active disease surveillance programmes (based on survey techniques which provide representative samples of the susceptible population of interest) are aimed at (i) detecting occurrence of any significant diseases; (ii) may target specific diseases; or (iii) may monitor the progress of specific disease control or eradication efforts. This kind of surveillance provides the data required to prove that the specified populations are free of a specific disease.

A third type of approach to surveillance is the ***"Risk based surveillance (RBS)",*** in which the exposure and risk assessment methods have been applied together with traditional design approaches in order to ensure appropriate and cost effective data collection" (Stärk*et al.* 2006). Risk-based surveillance is useful to support both strategic and operational decision making. The aim of risk-based surveillance is to identify surveillance needs, set priorities and allocate resources effectively and efficiently. Targeted surveillance is a part of RBS, and involves making best use of resources.

Aquatic Animal Disease Surveillance - International scenario

The diseases which are listed by OIE are a major concern at the international level. Codex Alimentarius Commission (CAC) has set standards for various diseases and aquaculture end products. The primary purposes of surveillance is to gather information on the occurrence, course and treatment of aquatic animal diseases and to provide guidelines and set standards for health regulations, including diagnostic techniques which are also applicable to international trade in aquatic animals. The Code describes internationally agreed upon scientific and technical information regarding important aquatic animal diseases, risk analysis procedures and health certification protocols. All the member countries are required to send report on the occurrence of notifiable and other diseases of which are concern to OIE. Through Disease Information System it is disseminated to member countries by OIE. India is a member country of the OIE and its participation in the aquatic disease reporting system contributes to overall aquatic disease surveillance. Many countries have surveillance system in

aquatic animals. Countries like Australia and New Zealand has its own well developed aquatic disease surveillance programme. Moreover Nordic countries have surveillance programme for various fish diseases.

Implications of disease surveillance programme

Diseases surveillance and reporting is considered to be a fundamental component of any national or regional strategy on aquatic animal health programme. Disease surveillance has its own advantages. They form the source of ringing the alarm bell in any emerging disease outbreak in aquatic animal health. Planning and executing aquatic disease control programmes can be done efficiently if an efficient aquatic disease surveillance system is in place. The surveillance programme helps set guidelines / standards in assessing and managing risks of diseases associated with trade. Besides helping to identify the etiology of the disease, the proactive surveillance will also provide the data required for rapid response. The aquatic animal health surveillance directly benefits farmers and stakeholders; certification of exports; international reporting and verification of freedom from diseases of concern. The information obtained from surveillance programme can be used to support contingency planning and monitoring of diseases control measures. Disease surveillance also helps in zoning, which is the process of delineating infected and uninfected populations within a country or group of countries, with regard to specific diseases. Further, the zones defined by appropriate surveillance mechanisms as being free of diseases may be used to facilitate trade and to protect against the introduction of pathogens. Zones defined as having the presence of a specific pathogen may also have unrestricted transfers to zones positive for the same pathogen. Thus, a zone which is positive for a disease is not, necessarily subject to cessation of trade (Subasinghe *et al*., 2004).

India's initiative in aquatic animal disease surveillance

As the disease management is difficult proposition in the aquaculture, the best option available is to have a two pronged approach of prevention and protection against the existing and emerging pathogens through surveillance and monitoring. Better management practices (BMPs) and regulatory framework in the movement of aquatic animals across the farming areas, in par with international standards is required. Timely response and targeted actions are important in the assessment of threat and follow up contingency plans. A good surveillance programme, using novel diagnostics in place can identify the pathogen and prevent the emergence and spread of the diseases.

New national Surveillance programme initiated through a network of fisheries research institutions active in aquatic animal health, with National Bureau of Fish Genetic Resources (NBFGR) and National Fisheries Development Board (NFDB), is a way forward in the aquaculture disease monitoring in India. The project was conceived with the following objectives: (i) Investigate and detect new and exotic infectious disease outbreak in aquatic animals; (ii) Provide evidence of freedom from diseases of concern within a defined geographical area; (iii) Collect information on the distribution and occurrence of diseases of concern; (iv) Assess progress in control or eradication of selected disease pathogens; with a total budget outlay of about `32 crores hopefully would achieve desired results. About 22 leading national

institutions covering fourteen states with passive and active surveillance in more than 100 districts would cover disease problems in shrimp, carp, catfish, tilapia, ornamentals, cold water species, freshwater prawn and molluscs. A Technical Advisory Committee (TAC) has been constituted to oversee the implementation of the project with NACA, Bangkok as a special invitee.

Initiatives byCentral Institute of Brackishwater Aquaculture in disease surveillance

Disease monitoring in brackishwater aquaculture has been a major activity of this institute since its inception. During initial phase of brackishwater aquaculture development during late 80's, soft shell syndrome of shrimp and EUS of finish were plaguing our brackish water sector, where CIBA initiated disease investigation and diagnosis. Scientific shrimp farming took off in India with the DBT-Govt of India pilot project at Nellore, AP. The project with the Andhra Pradesh Shrimp seed production, supply and research centre (TASPARC) with DBT funds injected great impetus and interest on semi intensive shrimp farming. Initially sporadic occurrence of bacterial diseases such as the bacterial septicaemia in grow-out farms caused limited losses, and from 1994 the new white spot syndrome or white spot disease (WSD), caused by one of the lethal and largest DNA viruses showed and it presence causing large scale and massive mortalities, devastating the entire shrimp farming sector. Many brackishwater shrimp farmers shifted to scampi farming in brackishwater ponds considering its ability to carry higher viral load, which also suffered setbacks due to another new viral disease, the white tail disease, caused by an RNA virus, the macrobrachium nodavirus (MrNV). Since 2006, the black tiger shrimp farming continued to face challenges with WSSV, but also with other disease conditions such as loose shell syndrome (LSS), followed by the monodon slow growth syndrome. Since the introduction of the Pacific white shrimp in India in 2009, issues of zoea syndrome in hatcheries and mortality problems during early days of stocking and continuous low level mortalities during the crop have also been reported.

Concluding remarks

Considering the complexities of the aquatic ecosystem, success of aquatic animal disease surveillance programme requires a sustained effort. Surveillance programme is really a challenge in the multifaceted aquatic animal health system. More sophisticated models of the processes underlying disease emergence have to be taken into account, inter-alia, the role of multi-host pathogens and reservoir populations and a greater understanding of the way pathogens are transmitted in aquatic systems are needed to support measures to minimize the number and impact of new diseases. The quantum of both legal and illegal trade in aquatic animals far outstrips that of land-dwelling animals, and it is one of the biggest challenges in preventing the spread of emerging aquatic diseases. In addition, coming few epochs, aquaculture is likely to expand and grow further, such legal/illegal trade is set to increase, and pressures of limited water resources and climate change effects are likely to combine to increase the rate at which diseases emerge and spread. In the face of these combined challenges, the demand for epidemiological approaches to curtail measures to protect aquatic animal health will undoubtedly increase. Sustained efforts by all stake holders in India, the institutional, corporate, farmers level and allied sectors, active in aquaculture need to come together to achieve enhanced aquaculture production, to meet the increasing demand of quality food.

Nutrition and feed management in brackish water aquaculture with special reference to aquatic animal health

Feedmanagementmeansoptimumutilizationoffeedwithminimumwastage by achieving best feed conversion ratio and maximum growth. A very good quality feed can produce poor result if the feed management is poor whereas, a moderate feed can produce very good results under good feed management. The foremost critical factor in feed management is selection of appropriate feeds and planning of optimal feeding regimens. Suitable feed should fulfill the nutritional requirementsof species under culture. Proteins, lipids, carbohydrates, vitamins, minerals and water are the six major classes of nutrients, which are used for building, maintenance of tissues and supply of energy. The requirement for these nutrients varies depending on the species according to their feeding habit, habitat in which they live in and the stage in their life cycle. Our aim should therefore be to produce nutritionally balanced feed with optimum protein energy ratio. It should also ensure that nutrients are not lost in water during the feeding process. Therefore, aquaculture feeds of different formulations are processed using the special technologies to ensure the diet remains intact in water before ingestion, and nutrients are prevented from dissolving. These general categories of feeds used in aquaculture are wet feeds with moisture contents of 50-70 percent, semi moist formulated feed with moisture contents of 20-40 percent and dry pelleted feeds with moisture contents of less than 10 percents. Since problems are associated with the distribution, handling, utilization, storage and quality of wet feeds and moist feeds, more and more dry feedsare manufactured either by steam pelleting or by extrusionpelleting.

Feeding management in shrimp culturesystem

Proper feed management is essential for successful and profitable shrimp farming. As feed alone costs 50-55% of total culture expenditure, strict supervision on feeding is required. Following points should be strictly followed while feeding the shrimp for maintaining good pond hygiene and to reduce wastage of feed and to avoid accumulation in pondbottom.

- Proper feeding guidelines should be followed to fix ration size for shrimp culture pond
- Check tray should be monitored daily
- Time and frequency of feeding should be proper.

Shrimp appetite will vary due to the environmental conditions i.e., water quality, water temperature, sunny/overcast days and physiological conditions such as disease and moulting. Feed should never be given in excess as uneaten feed pollutesthewater.Asshrimpsarethenocturnalfeeder,largerdosesmaybeoffered in the evening and during night. Regular observations and experience helps in mastering the management of feeding in a culture farm. Generally during new moon and full moon moulting of shrimp takes place and they become sluggish and reduce the feed intake. Quantity of feed offered should be reduced at the extent of 30-50 % during thatperiod.

Rationsize

Generally the method of calculating the daily ration is based on the body weight of shrimp (Table 1 and 2). Daily ration should be divided and given 2 to 5 times a day (Table 1, 2 and 3). The feeding activity and quantity of feed consumed may be checked by keeping feed in check trays (size: 80 cm x 80 cm) @ 6 nos./ha in different places in pond. After one month of stocking, consumption of feed shouldbe checked by using check trays. Besides the ration size, the optimal feed particle size also affects the feed intake and growth of shrimp. Feed particle size should varyasperbodyweightofshrimp(Table4).Fee dshouldbebroadcastedevenlyina periphery of about 2 meters from dyke in all sides of thepond.

Table1:Feedingscheduleforfirstfiftydaysofshrimpfarming

Age (Days)	Feed increment per day(g)	No. of meals per day	Feed per day per lakh PL-20(Kg)
1	-	2	2.0
2-10	400	2	2.4-5.6
11-30	600	3	6.2-17.6
31-50	500	4	18.1-27.6

Table 2: Feeding schedule after 50 days of culture based on check tray performance

Days of culture	Expected ABW(g)	% of ABW to be used asfeed	Feed % in Check tray	No. of meals perday
51-55	6-7	5.0-4.8	2.0	4
56-60	7-8	4.8-4.6	2.2	4
61-65	8-9	4.6-4.4	2.2	4
66-70	9-10	4.4-4.2	2.4	4
71-77	10-12	4.2-4.0	2.6	4
78-83	12-14	4.0-3.7	2.7	4
84-90	14-16	3.7-3.5	2.8	4
91-97	16-18	3.5-3.2	2.9	4
98-104	18-21	3.2-2.9	3.0	4
105-110	21-24	2.9-2.7	3.2	4
111-117	24-27	2.7-2.5	3.3	5
118-124	27-30	2.5-2.2	3.5	5
125-131	30-33	2.2-2.0	3.6	5
131-133	33-36	2.0-1.8	3.7	5

Table 3: Feeding Schedule forshrimp

Feed type	Shrimp weight (g)	Time offeeding				
		6.00 AM	11.00 AM	6.00 PM	10.00 PM	2.00AM
Starter	Up to4.0	30%	-	35%	35%	-
Grower	4 –25	25%	15%	30%	30%	-
Finisher	>25	25%	15%	20%	25%	15%

Table 4: Recommended shrimp pellet size

Feedtype	Size of shrimp(g)	Pelletsize
Starter	0-4.0	0.5-1.0 mmcrumble
Grower	4.0-25.0	2 - 2.3 mm x 4 - 5 mm
Finisher	>25	2-2.5 mm x 6 – 8mm

Check traymonitoring:

Quantity of feed to be kept in check tray depends upon pond size and average body weight of shrimp and can be determined using the following formula:

The check trays should be observed after 2 hrs of feeding. Depending on the quantity of feed consumed in the check tray, the next dose should be increased or decreased. Special care should be taken during moulting, low dissolved oxygen and stressed condition due to heavy rain, high temperature, unfavourable pond bottom and waterquality.

Success of feed management depends on the farmer‟s experience and observation on the feeding behaviour and feed intake of shrimp. Following a strict feed management, survivability up to 80 % and average weight of 30 g can be achieved in culture duration of 120 days.

Waterquality

The interrelationships between feeding and water quality in aquaculture is complex. By providing optimal species-specific requirements such as temperature, dissolved oxygen, pH and salinity, adequate feeding to satiation, improved growth and high survival can be ensured. When the water quality parameters fall below optimal levels, the species under culture will be stressed and feeding and growth will be impaired. Accumulation of left over feed together with excretory products is associated with high BOD, NH_3, H_2S, CH_4 and harmful effects of eutrophication. This is a critical issue in management since effluent quality can be linked directly to feeds and feeding practices

and is regulated under water pollution control laws in many countries. Thus, feeding regimes should be designed to minimize the nutrient loss and faecal output and to maximize the nutrient retention and health status of the cultured fishes/shrimps.

Handling and storage of feeds

Optimizing handling and storage procedures on farms is an essential component of good management practice. High quality feed can readily spoil and denature if storedunderinadequateconditionsorfortoolo ngaperiod.Incorrectlystoredfeeds may not only be unappetizing to fish or lacking in essential nutrients but also may contain toxic and antinutritional factors. This can lead to abnormal behavior, poor feeding response and growth. Hence different feed types such as wet feeds, moist feeds and dry feeds must be handled and stored under appropriateconditions.

Disease preventivemeasures

- Always use dry pellet feed, in which the bacteria cannot grow due to low water activity andmoisture.
- Supply nutritionally balanced diet for respective species to avoid nutrition deficiency.
- Avoid dumping more feed in culture pond without monitoring the feed intake as uneaten fed will accumulate

and deteriorate the soil and water quality of pond.

- Feed should be offered in split doses instead of a single dose for better utilization by fish/shrimp and less accumulation of uneaten feed in pond bottom.
- Feed should be stored properly to avoid pest and disease infestation in feed. Freshly prepared good quality feed proven with best potential FCR, could reduce feed waste and disease infish/shrimp.
- Feed with poor water stability, which have lost their nutritional potency and are poorly accepted by the fish or shrimp should berejected.
- Appropriate particle size of the feed should be designed for a particular stage of lifecycle.
- The ration size and feeding Schedules should be regulated with reference to feeding guides, response of fish and environmental conditions.
- At poor feed management, bloom may be formed in pond which causes pH and D.O. fluctuation and cause stress to the fish/shrimp. Water exchange, liming and aeration may be required to reduce the stress offish/shrimp.
- Judicious feed management is important factor in achieving good feed efficiency and reducing feedwastage.

Live feeds and health management in the brackishwater larviculture

Introduction

Aquaculture is one of the fastest growing food producing sectors in the world, accounting for almost half of the total food fish supply. The aquaculture industry prefers the hatchery produced larvae of finfish and shellfish as seed for the farming rather than the wild collected ones. In larviculture of any aquatic species, providing ideal starter feed for the larvae is the challenging task. Many of these larvae are with small mouth gape and therefore the larval feed should be sufficiently small enough to be consumed by the tiny larvae. Suitability of larval feeds are not only valued based on their particle size, but also on their nutritional composition and their ability to preserve the quality of rearing water. Till today, no replacement for tiny plant and animal water creatures commonly called as live feeds has been found to match their range of particle sizes and nutritional quality. Several species of micro algae and plankton crustaceans have been used as classical larval feeds in hatchery production finfishes and shellfisheslarvae.

The development of mariculture and brackishwater aquaculture around the world can be attributed to the development of standard techniques for mass production of live feed. The hatchery production of penaeid shrimp post-larvae depends on the use of live diatoms (*Chaetoceros* spp., *Skeletonema* spp. and *Thalassiosira* spp.) for the early stages and *Artemia* for later stages. Globally the hatchery production of juveniles of marine and brackishwater finfish is achieved by theuseofrotifersand*Artemia*(Fig.1).Microalgaearealsoroutinelyusedinthe, *greenwatertechnique*‟employedinlarvalrearingespeciallyformarinefinfishand crablarviculture.

Importance of live feed is due to several factors such as presence of essential nutrients, broad spectrum composition of food, better intake due to the movement, auto-digestion characteristics, better nutrient assimilation in larvae, stimulation of feeding behavior due to soft texture and attractability and ample scope for enrichment. Live feed organisms are able to swim / disperse in the water column and thus they are constantly available to the larvae. The movement of live feed in water stimulates larval feeding responses. Live feed organisms with a thin exoskeletonandhighwatercontentmaybemorepalatabletothelarvaewhen compared to the hard formulated diets. This is why live feed organisms are commonly called living capsules ofnutrition.

Apart from providing essential nutrients to the larvae, many of the live feed organisms has the potential to prevent many of the diseases in the marine and brackishwater larviculture. Currently many studies are going on all over the world ontheabilityoflivefeeds(mostlymicroalgae)inhealthmanagementinlarviculture and on producing more number of healthy larvae in eachcycle.

Satisfyingthenutritionalrequirement

The live feeds provide most of the essential nutrients to the finfish and shellfish larvae, which are required for growth and development. Among the nutrients,lipids play a crucial role in the larval development, especially the essential fatty acids. There are some microalgae consist of high amount of essential fatty acids, lipids, proteins, vitamins andminerals.

The nutritional value of algal species mainly depends on its cell size, digestibility, production of toxic compounds and biochemical composition. The presence of highly unsaturated fatty acids (HUFA), in particular eicosapentaenoic acid (20:5n-3, EPA), arachidonic acid (20:4n-6, ARA), and docosahexaenoic acid (22:6n-3, DHA), is of major importance in the evaluation of the nutritional composition of an algal species to be used as food for marine and brackishwater larval nutrition. Microalgae are primary producers of n-3 PUFA, and some Chlorophyta species such as *Nannochloropsis* and *Tetraselmis* are excellent sources of EPA, while some Haptophyta species *Isochrysis galbana* and *Pavlova lutheri* are outstanding sources of DHA. Besides being excellent n-3 PUFA producers, microalgae can be mass cultured and their EPA and DHA content can bemodulated. Nutritionally rich microalgae can be used to enrich other live feeds (Rotifers,ArtemiaandCopepods)forbettergrowthandsurvivaloflarvae.

Creating a healthy environment for the larvae

The presence of microalgae in the larval rearing tank will improve and stabilize the water quality in static systems (eg: Remove metabolic by-products and produce oxygen). They can be a direct food source through active uptake by the larvae and the polysaccharides present in the algal cell walls may possibly stimulate the non-specific immune system in the larvae. Apart from that, they can be an indirect source of nutrients for fish larvae (i.e. by enhancing the nutritional value of the live prey organisms in the larval rearing tank). Interestingly the green water system (larval rearing tanks with microalgae-unialgal or mixed algal) may control microbial load in tank water and/or larval gut (FAO fisheries technical paper:361).

There are certain factors, which determine the larval health and survival such as proper environment, suitable feed at right time, absence of pathogens and overallbiosecurity.Allthesefactorsareconnectedeachother.Agreenwater system or the presence of certain microalgae can make all the essential requirementsforbetterlarvalhealth.

Maintaining water quality parameters in the optimal range is an important task in all hatchery operations. Among various water quality measures, ammonia and CO_2 will make more problems to the larvae when their concentrations are above certain limits. Microalgae culture is an effective method for mitigation of ammonia and CO_2 from the larval rearing systems. The experiments showed that a microalgae *Scenedesmus dimorphus* can assimilate up to 98.6% of ammonia from the system, with an ammonia removal rate of 51mg/L/day (Kang and Wen, 2015). The photosynthetic microalgae will use the CO_2 and produceoxygen.

Different algal species used in green water systems in larval rearing has proved to be beneficial for larval

performance among marine finfish species like turbot, Atlantic halibut, sole, sea breams, sea bass, striped mullet, damsels, flounders and crabs (*e.g.* mud crab- *Scylla serrata*). The effect of microalgae in fish larval rearing system is not completely understood. The hypotheses listed in scientific studies include improvement of water quality, direct nutrition through active ingestion, indirect nutrition by enriching the live prey in the rearing tank, micronutrient stimulus for feeding behavior or physiological processes, and regulation of opportunistic bacteria by antibacterial or probiotics action (Muller- Feuga et al., 2003). The selection of suitable algal species for larval rearing is also important.

Antimicrobial property of microalgae

Widespread antibiotic resistance among pathogenic bacteria and the low specificity of these drugs made an urgent need for the development of novel antibacterial agents. Addition of microalgae (green water system) often has a positive effect on the survival rates of finfish and shellfish larvae. Fishes are susceptible to a wide variety of bacterial pathogens which can cause variety of diseases. These bacteria only become pathogenic when fishes are physiologically unbalanced, nutritionally deficient, or under various stress such as poor water quality, overstocking etc. (Anderson, 1995). Antimicrobial activity of microalgae has been studied by many researchers (Austin et al., 1992). Microalgae produce compounds at intracellular and extracellular levels, however, as a large proportion of these compounds is not excreted but remains within the cells (Guedes et al., 2011). Microalgae have been explored for their bioactive compounds with promising applications consisting antibacterial, antiviral and antifungal activities.

Health management by other live feeds

Other live feeds like rotifers, artemia and copepods are also widely used in larviculture in different larval stages of different species. The rotifers and artemia can be enriched with suitable microalgae or commercial enrichment product to get betterlarvalsurvivalandhealth.Thecopepodsarenutritionallysuperiortoartemia and rotifers. The broad range of size (30 µm to 1 mm) of copepods is also suitable for marine larviculture. Unfortunately the usage of copepods in hatchery operations are limited as the single species mass culture of copepods are not yet standardized in many countries and it is verydifficult.

Live feeds asimmune-stimulants

The production of fish larvae is often hampered by high mortality rates, and it is believed that most of this economic loss due to infectious diseases. The immunomodulation of fish larvae has been proposed as a potential method for improving larval survival by increasing the innate responses of the developing animals. Conversely, there is a school of thought that raises the concern of immunomodulating a larvae before its immune system is fully formed as this may adverselyaffectthedevelopmentofanormal immuneresponse.

Microalgae play a crucial role in present shrimp culture management strategies. Algal compounds and their metabolites have been shown to improve the immune system of the shrimp and increase its resistance against pathogens. Someof these compounds have been

known to exhibit either antiviral or antibacterial activities.

Application of transgenicmicroalgae

There are researches going on in the production of transgenic microalgae and its application in aquatic health management. Researchers developed a safer, more effective and less expensive biological bactericide for aquaculture use (Li and Tsai., 2009). A stable transgenic line of *Nannochloropsis oculata,* which has bactericidal activity, was developed. By feeding this transgenic *N. oculata* to a small model fish medaka, significant increase in the survival rate was observed 24 hrs after bacterial pathogenicinfectionwith*Vibrioparahaemolyticus*inthefish‟sdigestivetract.

Conclusion

According to the scientific literature, live microalgae with high nutritive value and appropriate physical properties can provide a healthy rearing environment to the aquaculture system. Finally, a better understanding of the mechanism of *green water systems* both in intensive and extensive culture will aid in optimizing the usage of microalgae in larval culture. A broader range of microalgae species, especially mixtures and including species rich in DHA, should be assessed in green water systems. Further research has to be done on the exact mechanisms on the improvement of growth, survival and health of finfish and shellfish larvae by differentmicroalgae.

Table 1: Commercial algal culture and its applications:

Source:Hemaiswaryaetal.,2011

Species	Use in aquaculture
Nannochloropsis	Growingrotifersandinfinfishhatcheries;veryhighEPAlevel
Pavlova	UsedtoincreasetheDHA/EPAlevelsinoysters, clams,mussels andscallops
Isochrysis	Enrichment of zooplankton such as artemia, used in shellfish hatcheries and used in some shrimp hatcheries, good size for feeding brine shrimp and copepods, oysters, clams, mussels, andscallops
Tetraselmis	Excellent feed for shrimp larvae and contains natural amino acids that stimulate feeding in marine animals, used in conjunctionwith *Nannochloropsis* for producing rotifers, excellent feed for increasinggrowthratesandfightingzoeasyndrome
Thalassiosira weissflogii	Used in the shrimp and shellfish larviculture, considered by several hatcheries to be the single best alga for larval shrimps, also goodfor feedingcopepodsandbrineshrimps
Dunaliella	Used as source of vitamins and pigments for shrimp larval forms.
Chaetoceros	Used as source of vitamins for shrimp larvalforms

CIBA

Application of probiotics and immunostimulants in aquaculture for pond health and disease management

Export of marine products has become one of the major foreign exchange earners in recent years. India exported 10.51 lakh tons of marine products worth ` 33,441.61 crores (USD 5.51 billion) during the year 2014-15. Shrimp constitutes major portions with 3.57 tons worth ` 22,468.12 crores (USD 3.71 billion), while fresh finfish constitutes 3.09 lakh tons worth ` 3,778.50 crores (USD 0.61 billion) (www.mpeda.com). For almost one decade the wild catch has been stagnant and contribution from the aquaculture is growing. Ever increasing population, awareness about the health benefits and the higher demand for aqua food products in international markets has made aquaculture as one of the profitable businesses in developing countries especially India. During the last two decades aquaculture in India has grown many folds, however health management of is one of the major area of concern for the economical sustainability of the industry. Diseases like,white spot syndrome, vibriosis and emerging diseases like Early mortality syndrome, white gut, white muscle, white faeces has necessitated the development of strategies to combat the issues related to disease which are safe for host and environment.

Recent trend of intensification in aquaculture after introduction of *Penaeus vannamei* has posed several challenges both for safety of host and maintaining the quality of environment. Similar trend is observed in almost all the major shrimp producing countries in the world. Recent intensification in aquaculture in India has resulted in deterioration in pond environment exposing the animals to stressful conditions. Such animals are more susceptible to opportunistic pathogensespecially those leading to vibriosis, resulting in severe economic loss. Extensive use of antibiotics for prevention of bacterial infections leads to the development of antibiotic resistance and exportrestrictions.

In an effort to achieve higher production targets various chemicals and biological products are used in hatchery and grow-out culture without clear understanding of the scientific basis. Probiotics and immunostimulants are one such group of products, which are used and abused extensively in Indian aquaculture. Probiotics are single or mixed culture of live microorganisms, which has the beneficial effect on host and the environment. On the other hand, immunostimulants are microbial, animal, plant, chemical or synthetic substances effectively stimulate the immune system to enable the host to fight stress and infections. Though probiotics and immunostimulants are effectively used in human and veterinary medicine, their efficacy and mode of application needs intensive study inaquaculture.

In a recent estimate aqua farmers spend about `15-20 worth probiotics and immunostimulants per kg of shrimp produced which is about 10% of theproduction cost. Clear scientific understanding at the level of scientific community and proper advise to the farming sector will help in effective utilize the beneficial effect of these products in Indian finfish and shellfish culture operations. There is an urgent need to set standards for quality of the

CIBA

probiotic and immune stimulating agents so that genuine products are made available in themarket.

Probiotics in shrimp aquaculture

Generally probiotics for aquaculture applications can be classified as gutprobiotics, water probiotics and soil probiotics. However, some bacteria have demonstrated beneficial effects in both gut and soil. Several mechanisms have been attributed to the beneficial effects of probiotics which include competitiveexclusion of pathogenic bacteria, helps digestion and provide nutrients, utilize organicmatter, enhance immunity and improved water quality. While immune stimulating agents of microbial or plant origin are most commonly used in aquaculturepractice.

Gutprobiotics

Several bacterial species have been reported to have beneficial effect in gut of aquatic animals. However, only few species are extensively used in commercial probiotic products, like, *Bacillus pumilus*, *B. megaterum*, *B. subtilis*, *B. polymyxa*, *B. licheniformis*, *Cellolomonas* spp., etc. Gut probiotics generally colonize the gut and competitively inhibit the pathogenic bacteria in addition to possible release of some bactericidal molecules. These probiotics are applied regularly throughout culture period as feed topdressing.

Waterprobiotics

It has been estimated that almost 70% of the feed in shrimp pond is left unconsumed and accumulates in pond bottom. Build-up of uneaten feed and practice of zero water exchange deteriorates the pond environment causing stress to animals. It is important to note that in line with human medicine, most of the emerging diseases in recent times are due to stress due to pollution. Over a period of culture cycle toxins stockpile in the environment causing stress to animalswhich leads to reduce immune system making animals susceptible foropportunistic pathogens. Stressed animals also face metabolic malfunction and show reduced growth and mortality. *Nitrosomonas* spp. and *Nitrobacter* spp. are the autotrophic bacteria extensively used as water probiotics in aquaculture. These bacteria are associated with nitrogen recycling and help in mitigating the toxic effects of ammonia, nitrite and nitrate in pondwater.

Soilprobiotics

Accumulation of uneaten feed deteriorates the pond bottom leading to black soil due to hydrogen sulphide and other sulphur metabolites. This condition needs immediate attention otherwise leading to releases of toxic gases into water column. Bacteria commonly used in soil probiotics are *Paracoccus* spp., *Rhodococcus* spp., *Rhodobacter* spp. and *Thiobacillus denitrificans*. These are mostly sulphur recycling bacteria establish in the pond bottom and help in converting toxic hydrogen sulphide.

Immunostimulantsinshrimpaquaculture

The stress imposed due to intensive aquaculture practices by the high-density, supress the immune system of animals. Immune compromised animals are susceptible to pathogens ultimately leading to retarded growth, mortality and economic loss. Environmental hazards of using chemicals in aquaculture have prompted the scientists to search for alternative strategies that improve the immune competence of aquatic animals. For development of effective immune stimulatory compounds, it is essential to

understand the basic mechanisms of host physiology and the defencesystem.

Depending on the origin, immunostimulants used in aquaculture can be classified as bacterial, algae-derived, animal-derived, nutritional factors as immunostimulants, and hormones/cytokines. Immunostimulating agents are defined as „naturally occurring compound that modulates the immune system by increasing the host‟s resistance against diseases that in most circumstances are caused by pathogens‟. Unlike vertebrates, shrimp do not have adaptive immune system and depend mostly on innate immune mechanism. Recent interest in immunostimulants as an alternative to the drugs, chemicals and antibiotics for health management in aquaculture is mainly due to the ability of immunostimulating agents to enhance the innate (or non-specific) immune response. The major advantages of immunostimulants are that it can be administered by bathing or orally as feed top-dressing to shrimp. Live and killed bacteria, bacterial cell wall, lipopolysaccharides, peptidoglycan, glucans and chitin/chitosan are some of the extensively studied immune stimulating agents for aquatic species. However, synthetic compounds, polysaccharides, vitamins and animal and plant extracts are also reported to enhance the non-specific immune response in finfish andshellfish.

Logic in method of probiotics and immunostimulantapplication

For probiotics bacteria to colonize the environment and exhibiting the beneficial functions, application of these products in optimum dosage and schedule is very important. Suitable conditions required for multiplication of the probiotic bacteria depends on the concentration and schedule of application, combination of probiotics bacteria and mode of application. It is essential to understand that bacteria in the probiotic products are very sensitive to water parameters like pH, temperature, salinity, DO, nitrite, ammonia etc. Hence it is not necessary that a probiotic product works similarly in all seasons, regions and stage of grow-out culture. Similarly immune stimulating agents reach specific organs in the host and enhance the secretary and differentiating functions for effective mitigation of stress and to encounter the pathogens effectively. Application of these products in appropriate dose and schedule play very important role in expression of beneficial effects on health and growth ofanimals.

Labelling ofproducts

Medicinal products including probiotics and immunostimulants used in human veterinary and aquaculture practice need to be labelled with contents, concentration, dose and indications. However, most of the products do not follow such regulations in Indian aquaculture. Thus the market is full of products without proper labelling and some manufacturers claim effectiveness of the product in removing ammonia, cleaning the pond bottom and improve the growth andsurvival of shrimp. So, farmers should be aware of such products and avoid their applications without scientificbacking.

Conclusion

Though the mechanism behind the beneficial effects of probiotic and immunostimulant application in aquaculture is not unequivocally established, it is definitely an important activity in Better Management Practices (BMPs), both in hatchery and grow-out cultures. It is essential to understand the

composition of products and mode of application, suitable for the use in different types of culture systems. Since these products are prophylactic in nature and not therapeutic agents, hence need regular application. It is essential to understand that, beneficial effects of these products could be realized only when quality products are applied in prescribed schedule and dose. Farmers need to understand the scientific basis behind the application of these products to obtain their intended prophylactic effects and reduce the cost of production.

Pond preparation and management practices for prevention of diseases in brackish water aquaculture

The water and soil quality variables affecting shrimp/fish survival and growth are determining factors for disease outbreaks. Disease is an expression of a complex interaction between host (shrimp/fish), pathogen (bacteria/virus) and environment (pond soil and water quality). Severe alterations in the culture environment deviated from the optimum pose stress on the system leading to reduced immune status of the animal to fight infections. Generally disease does not occur when the culture environment (water and soil parameters) is maintained at optimum and balanced condition. Adverse water quality conditions compromise management and increase stress level thus, making them more susceptible to diseases. Even if the site is good with optimum soil and water characteristics, problems may still crop up by the large quantity of inputs 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 proper management practices during pond preparation and cultureperiod.

Pondpreparation

The main objectives of pond preparation are to provide the animal with a clean pond base and appropriate stable water quality. Pond preparation is generally dealt in two categories viz., newly constructed ponds and existing culture ponds. In newly dug out ponds, the characteristics of the soil have to be understood first, and soil deficiencies should be identified and treated in new ponds instead of waiting until poor bottom soil quality develops later. For example, if soil in a new pond is acidic, it should be limed before initiation of aquaculture. The pond preparation after harvest before initiating next crop is entirely different from that of a newly dug-out pond and comprises of removal of waste accumulated during the previous crop by draining and drying of the pondbottom.

Drying

The pond bottom should be dried for at least 7-10 days for mineralization of organic matter and release of nutrients. Exposure of the pond bottom to sunlight until it dries and cracks, enhances aeration and favours microbial decomposition of soil organic matter. The optimum moisture content for drying is 20%, but it might vary among soils from different ponds. Pond drying certainly enhances the mineralization of organic nitrogen andphosphorus.

Tilling

Tilling bottom soils can enhance drying to increase aeration and accelerate organic matter decomposition and oxidation of reduced compounds. Soil amendments such as agricultural limestone or burnt lime can be mixed into soil by tilling. Accumulation of organic matter and 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 bottom should not be tilled when they are too wet to support tillage machinery. Ruts caused

by machinery will fill with soft sedimentand be likely sites for anaerobic conditions. Depth of tillage usually should be 5 to 10 cm, mould board plow often called turning plow, can be used to turn soilover.

Liming

The reason for liming aquaculture ponds is to neutralize soil acidity and increase total alkalinity and total hardness concentrations in water. This can enhance availability of nutrients in the pond water and improves the conditions for productivity of food organisms and increase aquatic animal production. Either total alkalinity or soil pH may be used to estimate the liming dose. If both are available but values are not in agreement, use the variable that gives the greatest limingdose. Brackishwater ponds with total alkalinity below 60 mg l^{-1} and any pond with soil pH below 7 usually will benefit from liming. Agricultural limestone will not react with dry soil, so when applying over the bottom of empty ponds, it should be applied while soils are still visibly moist but dry enough to walk on. In ponds with highly acidic soil (pH < 6), liming can increase phosphorus availability byincreasing the soil pH. The amount of different lime materials required to raise the pH to7 is given in Table1.

Table1:Amountoflime(tons/ha)toraisethesoilpHto7.0

	Quantity of lime material (tons/ha)		
SoilpH	**Dolomite**	**Agriculturallime**	**Quicklime**
6 to6.5	5.7 to2.8	5.5 to2.8	4.6 to2.3
5.5 to6.0	8.5 to5.7	8.3 to5.5	6.9 to4.6
5.0 to5.5	11.3 to8.5	11.1 to8.3	9.2 to6.9
4.5 to5.0	14.2 to11.3	13.9 to11.1	11.5 to9.2
4.0 to4.5	17.0 to14.2	16.6 to13.9	13.8 to11.5

Agricultural limestone will not react with dry soil, hence when applying over the bottom of empty ponds, it should be applied while soils are still visibly moistbut dry enough to walk on. Generally lime is applied after slight turning over of bottomsoil.

Fertilization and nutrientstransformation

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 hydrolyses to ammonium in pond water. Ammonium may be absorbed by phytoplankton, converted to organic nitrogen, and eventually transformed into nitrogen of fish 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. Organic nitrogen in plankton and in aquatic animal faeces 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.

Soils that are near neutral in pH have less capacity to adsorb phosphorusand a greater tendency to release phosphorus than do acidic or alkaline soils. Phosphate is released from iron and aluminium combination when reducing conditions develop from oxygen depletion. A dynamic equilibrium exists between sediment and overlaying water so that a small amount of phosphorus is maintained in solution. Phosphorus exchange between soil and water can conceivably be influenced by pond management procedures which influence dissolved oxygen concentrations in bottom water, disturb the bottom soil surface, suspend soil particles into the water, mix interstitial water into pond water, influence pH, or alter concentrations of iron, aluminium, and calcium.

Management of pond bottom soil during culture period

Allaquaculturepondssoilbottombecomecoveredwithsedimentandthissediment can be considered as aquaculture pond soil. In describing various physical,chemical and biological processes occurring in the pond bottom, it is convenient to refer to bottom deposit as sediment. The sediment – water interface is an intricate system where complex chemical and microbial changes occur and plays important role in brackish water aquaculture. In the optimum conditions, organic matter presentinthesoilwillbemineralizedbyusingdifferentmicroorganismssuchas autotrophic, heterotrophic microorganisms and it will release the nutrients in the availableform.

Monitoring of soil parameters during culture period

Monitoring of soil quality can be valuable in fish culture pond management. During culture the carbonaceous matter, suspended solids, faecal matter and dead plankton etc. also settle at the pond bottom. Major concerns in pond bottom soil management are low soil pH, highsoil organic matter, loss of the oxidized layer and accumulation of soft sediment. Pond managers should still strive to prevent severe soil quality problems from developing. 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. In order to characterize the soils based on soil type, a pond core sampler fabricated by the Environment Section of CIBA can be used for the depth-wise collection of cores

The low pH of bottom sediment indicates unhygienic condition needs regular check-up. The change in the bottom in terms of increasing organic load should be recorded regularly for the management of the pond bottom. Anaerobic condition can be developed in pond, when input of organic matter exceeds the supply of oxygen needed for decomposition of organic matter. This reducing condition can be measured as the redox potential (E_h). E_h indicates whether the water or soil is in reduced($E_h$with`-vevalue) or oxidized($E_h$with`+'vevalue)condition.In anaerobic sediment, some microorganisms decompose organic matter by fermentation reactions that produce alcohols, ketones, aldehydes,and other organic compounds as metabolites. Other anaerobic microorganisms are able to use oxygen from nitrate, nitrite, iron and manganese oxides, sulfate, and

carbon dioxide to decompose organic matter, but they release nitrogen gas, ammonia, ferrous iron, manganous manganese, hydrogen sulphide, and methane as metabolites.

Some of these metabolites ydrogen sulfide, ammonia and nitrite can enter the water and be potentially toxictofishes.Theredox potential (E_h) of mud should not exceed -200 mV. The oxidized layer at the sediment surface is highly beneficial and should be maintained throughout the culture period. Ponds should be managed to prevent large accumulations of fresh organic matter at the soil surface, or in the upper few mm of soil. Hence, it is extremely important to maintain the oxidized layer at the sediment surface in cultureponds.

Watermanagement

Maintenance of good water quality is essential for both survival and optimum growth of fish. Water treatment is an important step during pond preparation for the maintenance of good water quality at later stage.

Culture of brackishwater species under varying source waters

Shrimp species *P. monodon* and *P. vannamei* and, finfishes Seabass, Mullet and Milk fish are being cultured by farmers in sea, brackish and fresh waters. Though high salinity and clear water with less plankton always causes shrimp stunt, butthis 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 requires closed system to avoid viral diseases as virus carriers grow very fast in fresh water. 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 anddeath. Potassium has an essential role in regulating sodium and therefore fluid balance within the haemolymph. Hence there is a need to supplement potassium as and whenrequired.

Intake watertreatment

Polluted or self-polluted source water through aquaculture causes slow growth, disease outbreak and accelerated mortalities of cultured animals. Direct use of creek or sea water carries the risk of introducing the virus through aquatic crustaceans. There is a need to eliminate these from water before use in culture ponds. 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. Following points should be considered for management of water quality.

- Water from the source is filtered through filter nets of 60 micron mesh/cm^2 to prevent the entry of

parasites and crustaceans that are carriers of diseases.

- Inorganic turbidity (suspended solids) should be removed by providing sedimentation/ reservoir pond before water to be taken into production ponds.
- Chlorination as a means to sterilize the water is practiced by many farmers. To achieve this enough chlorine should be applied so as to overcome the chlorine demand of organic matter and other substances in the water. Water should be taken in reservoir ponds and treated with calcium hypochlorite @ 30 ppm. Permissible level of chlorine residuals in treated water for use in grow-out ponds should be less than 0.001ppm.

Monitoring of water stress parameters during culture period

Salinity

Optimal salinity range of 10 to 35 ppt is considered optimum for growth and proper metabolic processes of tiger shrimp. Researchers indicated that a population of *P. 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).

Temperature

Temperature is one factor controlling the speed of biochemical reactions and regulating the activities of cultured animals. The temperature below and above the optimum range (28 to 32°C) is known to weaken the immune status of the shrimp making it more susceptible to diseases due to *Vibrio*. If shrimp are infected, eitheras 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$_{0}$C, mortality rates increases. Studies show that that the rate of mortality in shrimp infected with some virus 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 andstable.

pH

The pH stress could trigger the disease outbreaks by reducing the immune defense mechanisms of the host. 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 asanincreaseinanionicammonia(NH_3)atp Habove7.Whereasinwaterswith low pH will cause an increase in the fraction of anionic sulphide (H_2S) and the toxicity of nitrite, as well as physiological disorders inshrimp.

DO (Dissolvedoxygen)

In intensive aquaculture practices, dissolved oxygen (DO) is a major limiting factor especially in the bottom layers of shrimp culture ponds. Decomposition of accumulated feed and the animal faeces lead to hypoxic and sometime anoxic conditions particularly at night time. DO levels were above 4 mg/L during day time with aeration, whereas the levels may go less than 2 to 3 mg/L during night time/early morning. DO less than 2.8 mg/l is considered hypoxic condition and it is known to influence growth, survival, feeding, moulting, behavior, osmoregulatory capacity and immune response of Penaeidshrimps.

Transparency and watercolour

CIBA

It reflects the type and density of plankton. The more intense colour 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 than2ppm. Transparency must be maintained at a level of 30-40cm.

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).

TAN (Total AmmoniaNitrogen)

The concentration of total ammonia nitrogen (TAN) in intensive grow-out ponds increases as culture progress and levels of more than 1.0 ppm are toxic. The percentage of the toxic form increases as pH and temperature rise during the day and can reach critical levels. In addition to immune response, elevated concentration of TAN affects the growth, moulting, oxygen consumption and ammonia excretion. Increased concentration of TAN decreases the activity of superoxide dismutase responsible for the scavenging of reactive oxygen species (ROS) leading to increase in superoxide anion. Reduced phagocytic activity and clearanceefficiencyleadtoincreasedsusceptibilitytobacterialinfections.

Nitrite

Among the metabolic toxicants nitrite is considered most dangerous as it can accumulate in haemolymph up to 10 fold higher than in water via active chloride uptake mechanism and passive entry. The higher concentration of the nitrite is known to decrease the levels of total haemocyte counts to the reduced prophenoloxidase and phagocytic activities. Nitrite is more toxic in low saline conditions compared to brackish and seawater based culture ponds.

Hydrogensulphide

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/Litre of H_2S may be lethal to aquatic organisms and any detectable concentration is undesirable. Presence of sulphide affects the immune parameters like total haemocyte count, hyaline cells, phenol oxidase activity, phagocyticactivity and clearance efficiency thereby making the shrimp more susceptible to pathogenic infections like,vibriosis.

Waterexchange

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 cyanobacteria and algal blooms by flushing or scooping out the scum facilitates optimum density and prevents sudden die-off of the bloom. Minimization of water exchange will prevent viruses and carriers / bacterial pathogens from entering the ponds and reduce the possibility of disease transmission into culture ponds. But the reduction of water exchange requires closer control of water quality parameter such as pH and ammonia, effective

CIBA

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,whichenablesgoodwaterqualityconditionsinponds,whilereducingthe potential of disease introduction to the farms through intakewater.

Aeration

In a typical black tiger shrimp pond, low rpm (revolution per minute) aerators may suffice but those with high rpm are required for *P. 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 aeratorsisimportanttopreventlocalizeddepositionofsludge.

Feed management and water quality

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. 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 scientificfeeding.

Conclusion

Sustainability of aquaculture depends on the maintenance of a good environment. The two-pronged approach of combining pond management and health monitoring is the key for successful shrimp/finfish production. It is important to know how much biomass 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 well-designed management practices pertaining to soil and water quality should increase the efficiency and productivity by reducing the risk of shrimp / fish health problems, and reduce or mitigate the impacts of farming on the environment. Regular monitoring of environmental parameters and timely mitigation using appropriate biological agents is the key to protect potential losses due to stress and opportunistic bacterial infections. The understanding on ecological process occurring in culture ponds through regular monitoring will help to solve some of the disease issues faced by thefarmers.

Water and soil quality requirements and their management in brackish water aquaculture

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

CIBA

culture ponds through regular monitoring will help us understand and solve someof 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

CIBA

IntroductiontoNucleicAcids

Introduction

The primary aim of this lecture isto givea brief introduction to the basic structure and physical and chemical propertiesof nucleic acids so asto easily understand the in vitro amplificationofDNAthrough Polymerase Chain Reaction (PCR). The knowledge on these basic principleswill helpto manipulate the reaction to suit ones need and effectivelyusethePCRtechnology in shrimp disease diagnosis.Thiswillalsohelpin understanding and solving the various problems one may face during the application of this technology. Therefore, I have avoidedthe cumbersome chemistry of the nucleic acids, but the most relevant principles that are required for the understanding the PCR are discussed and explained.

DNA was isolated way back in 1869

DNAwasfirstisolatedfrompuscellsandfromsalmonspermbyFriedrichMeiescherin 1869.Sinceitwasisolatedfromnucleiitwascallednuclein.DNAfromdifferentcells and viruses vary in their nucleotide sequence, nucleotide ratioandmolecularweight.In fact nucleicacids are the major component ofthe cell. The genomiccontent of the cell varies from 0.01 pg in prokaryotes to 0.3 to 10 pg in higher plants and animals. However, the vast majority of the nucleicacid in cells is present as complexes with proteins. Prokaryotic DNA forms complexes with polyamines and proteins while eukaryotic DNA is associated with histones and variousnon-histone proteins. The amount of DNA in any given species of cell or organism isconstant and can not be altered either by environmental or nutritional or metabolic conditions. The germ cells (sperm/egg) of higher animals possess only one half of the amount of DNA found in somaticcellsofthesamespecies.

Nucleotidesarethebuilding blocks of Nucleic acids

Justastheaminoacidsarebuildingblocksofpeptides(proteins),thenucleotidesare the building blocks for nucleic acids. The monomericunitsofDNAarecalled deoxyribonucleotides.Eachofnucleotidecontainsthreecharacteristiccomponents.(a) a heterocyclic nitrogenous base, derivative of either a pyrimidine or purine. (b) a pentose sugar molecule and (c) a moleculeof phosphate. There are four different deoxyribonucleotideswhichserve as the major building blocks ofDNA macromolecule. They are all similar except for the nitrogenous base. Each nucleotide is named after the base.Thepurinederivativesareadenine(A)andguanine(G)whilethepyrimidine derivatives are cytosine (C) and thymine (T). Similarly, fourdifferent ribonucleotides are the building blocks for the RNA. They are the purine bases adenine and guanine. The pyrimidine bases are cytosine and uracil (U). The pentose sugar isdifferent in DNA and RNA,DNAcontains2-deoxyribosesugar, while RNA contains ribose sugar.

Nucleic Acids exist in different forms

Nucleicacidsexistintwomajortypes,namely DNAandRNA.ThoughDNAexistsin one type they exist in different forms, as linear, circular, singlestranded and double stranded forms. On the contrary mostly RNAexists assingle stranded form. There are three types of RNA present in a living cell- messenger RNA (mRNA), Ribosomal

CIBA

RNA (rRNA)andtransferRNA(tRNA).Eventhough RNAsaresinglestranded,theyform extensivesecondarystructuresasinthecase oftRNAsandrRNAs.MessengerRNAs of eukaryotic cells are uniquethat they contain long stretches of poly (A) sequences at the 3′ ends (the carbon atomsarenumberedbyadding prime to the number, for eg. 2nd carbonatominthesugariswrittenas2′,3rdas3′etc). IngeneralDNAcontainsthe geneticinformation;however,incertainviruses RNAcontainsthegeneticinformation.

DNA/RNAareformed bycovalent links of Deoxy/Oxyribonucleotides

Anucleicacidispolynucleotide- thatisapolymer consistingofnucleotides.The pentose sugar is a cyclic five carbon ribose sugar in caseofRNAand2′deoxyribose sugar in the case of DNA. A purine or pyrimidine base is attached to the 1′ carbon atom of the pentose sugar by an N-glycosidic bond. A phosphate is attached to the 5′ carbon of the sugar by phosphoester bond. It is this phosphate which gives the strong negative charge for the nucleotides and the nucleic acids. This property is used in agarose gel electrophoresisofnucleicacids.Thenucleotidesinnucleicacidsarecovalentlylinked by a second phosphoester bond that joins the5′ phosphate of onenucleotide and the 3′ OH group of adjacent nucleotide. This phosphateplusitsbondstothe3′and5′carbon atomsiscalledaphosphodiesterbonds.

DNA exhibits base equivalence

Edwin Chargaff and his colleagues using quantitative chromatographicseparation methods analyzed the base composition of nucleic acids and proposed the Chargaff's rule for DNA. (1) The base composition of DNAvariesfromspeciestospecies.(2)DNA specimensisolated different tissues ofthe same species have the same base composition. (3) The base compositionofDNAinagivenspeciesdoesnot changewith age, nutritional state or changesin environment. (4) The number of adenine residues is always equal to the number ofthymine residues, A=T. SimilarlyGuanine residues is alwaysequaltocytosineresidues,G=C.(5)DNA extracted from closelyrelated species have similar base composition whereas those of widely different species have widely differentbasecomposition.

Watson and Crick model of DNA

In the year 1953 Watson and Crick proposeda structure for the DNA based on the crystallographic structure. It was an epoch making proposal, which revolutionized the world, for which they got the Nobel Prize. The structure proposed by Watson and Crick explained the long known Chargaff's rule. The DNA exists in a double helix state. The two strands run in opposite direction(antiparallel). ThepolarityoftheDNAstrandis5′ to 3′. These two strands are held togetherby H-bonding between complementary N-bases.AbondswithTandGbondswithC.Phosphateandsugarformbackboneson the outside and hydrophobicN-basesareinside,stacking on top of one another. The doublehelixis20Åindiameter.Thehelixmakesacompleteturnwithin10nucleotides atadistanceof34Å.Theraisepernucleotide is3.4Å.Itisimportant tonotethatnucleic acids have polarity. This means that their ends (termini) are not the same. One end of the polynucleotide chain bears a 5′ phosphate group whereas the other end bears a 3′ hydroxyl group. By convention the sequence of the nucleic acidis written in the 5′-3′ direction,the5′terminusalwaysbeingtotheleft.

DNAcan existindifferentforms

The geometry of the DNA double helix was deduced from X-ray diffraction studies using DNA fibers. Analysis of such patterns revealed the existence of three different conformations of the DNA. Theyare calledas A-DNA, B-DNA andC-DNA. The Watson and Crick model is nothing butthe B-DNA. A-DNA differs from the B structure in the following respects. The base pairs, althoughparallel to one another and spaced by the sameamount(0.30nm)asintheBform,are inclinedbyabout20degreestotheplanes perpendicular to the helix axis. The number of bases per turn is 12 and hence the structureisfatter,butlongitudinallymorecompact. The two grooves that run around the outside helix are approximately the same sizeinA-DNA.Butthegrooves vary in their size in B-DNA, called major and minor grooves. The base pairs overlap in B-DNA such thatthetopviewofthehelixappearsto befullofbasepairsbutA-DNAhasaholedown the middle. C-DNA is a distorted B structure with anon-integral number of bases per full turn and the base pairs are somewhat more inclined to planesparallel to the axis. There is also another form of (synthetic) DNA the Z-DNA. It is left handed. The base pairs are inclined to 7degrees and there are 12 base pairs per full turn. The biological significance ofZ-DNAisstilluncertain.

Secondary structure of DNA is drasticallydifferentfromtheprimarystructure

Linear nature of a DNA sequenceis regarded as theprimary structure. In the case of DNA, secondary structure consists of two independent, covalentlylinked chains coiled arounda commonaxisandformsdoublehelix.In thecaseofRNAmoleculewhichis primarily single stranded, a number of intra-strand H-bonding can be found. This intra strand bending of the helix axisin different directions iscalled the secondary structure. They are termed differently as hairpin loops, hairpin bends, stem loops cruciform etc. Secondary structures of RNA are believed to play important biological roles such as recognitionregionsforcertainenzymes.

DNAisstabilizedbyvariousforces

WhatholdstheDNAdoublehelixtogether? One of the factors isthehydrogenbonding. The Watson-Crick Model isthermodynamically correct. A:T base pairing hastwo hydrogen bonding, while G:C pairing has three hydrogen bonding which is more stable thantheformer.Hydrogenbondingis notthe only stabilizing factor. Hydrophobic interactionsin base stacking interactions between aromatic rings insidethehelixarethe major stabilizing forces against repulsionby negatively charged phosphates. The presenceofcounterionssuchasMg^{2+} andK^{+}alsoplayaroleinstabilizationofDNA doublehelix.

DNA is very stable compared to RNA

Compared to DNA, RNA is less stable. This is primarily dueto the single stranded nature. The nature ofthe pentosesugaralsoplaysaroleinthestability.RNAcan be easilyhydrolyzedbydilutealkali.Thesugarmoietycontains2′hydroxyl group.Dilute sodium hydroxide produces a mixture of nucleosides, 2′ and 3′ phosphates. Cyclic 2′, 3′ monophosphates are the first products of the action of alkali on RNA. Theyare further hydrolyzedbyalkali,whichattackseither oneofthetwoP-O-Clinkagestoyielda

mixtureof2′and3′nucleosidephosphates.SinceDNAhasno2′OHgroupitcannot behydrolyzedbyalkali.

DNAcan be denatured byheatandacidsbutnotbyalkali

Gentle acid hydrolysis of DNA at pH 3.0 causes selective hydrolytic removal of all its purine bases without affecting the pyrimidine deoxyribose bonds or the phosphodiester bonds of the backbone. The resulting DNA derivative, which is devoid of purine bases is called apurinicacid. Selectiveremoval of the pyrimidine baes, accomplished by somewhatdifferentchemicalconditionsproducesapyrimidinicacid.Asmentionedabove alkali cannot hydrolyze theDNA. Nucleases also hydrolyze the nucleic acids. Nucleic acids can be denatured by heating.This process is called melting. When nucleic acid is denatured the stacking is lostandhencetheUV-absorbance increases. This increase is called hyperchromic shift. For total denaturation of the double stranded DNA, the hyper chromiceffectisoftheorderof30%.Thetemperatureatwhichthesolutioncontains 50%denaturedand50%doublestrandedDNAiscalledthemeltingtemperature(Tm).
The value of Tm is the function of the nature of the DNA, ions in the solutions and the ionic strength. Renaturation is not simply the reversal of denaturation. If the denatured DNAsolutionsaremaintained5-10ºCbelowthemeltingtemperature, the complementary strands will slowly re-associate andthe double helical structures will reform. The whole process isconcentration dependent. Thermal denaturation is one of thepropertiesexploitedinpolymerasechain reaction(PCR).

DNAreplicates bysemi-conservativemechanism The most striking feature ofthe Watson-Crick model of DNA, from the genetic point of viewis,thatthetwostrandsofdoublehelicalDNAarecomplementary.Thereplication of each to form new complementary strands results in formation of two daughter duplex DNA molecules, each of which contains one strand from the parental DNA. This process is called semi conservative replication.
Thismodelwasconclusivelyproved by MeselsonandStahlin1957byingeniousexperimentusingbacteria.Thesameistrue in other dividing cells.

DNA polymerase is the keyenzyme in DNA replication

Theenzymaticmechanismbywhichthe DNAisreplicatedwaselucidated by A.Kornberg and his colleagues in 1956. The enzyme involved in this process is DNA polymerase I. Later it was found out that, other enzymes, (Pol II and Pol III) were also involvedinreplication.Now it is shown that Pol III is the major enzyme concerned in the replicationprocess,althoughPolI participates.Thisalso functionsinrepairofDNA.It also has the 3′-5′ and 5′-3′ exonuclease activity. The most striking and the characteristic property of DNA polymerase is that it requires the presence of some pre-existing DNA called primer, in the absence of which thepurified enzyme will not be able to make any DNA at all.

Pre-existingDNAprimerandtemplate is essential for DNA replication

Okazaki and his colleagues discovered that nascent DNA occurs in short pieces, called Okazaki fragments. These fragmentsarefoundinviral,bacterialandeukaryoticcells during DNA replication. Replication of DNA in short steps is a device that permits replicationofbothstrandsofDNAbyDNApolymerase that replicates only in 5′-3′

direction. These short pieces are quickly joined bycovalent bonds. It was also shown that the purified DNA polymerase cannot utilize the native DNA strand as a primer. Therefore, DNA replication is preceded bythe formation of a short strand of RNA complementary to a section of double strandDNA.ThisprimingRNAisgenerated bya DNA directed RNA polymerase. Once the primingRNAstrandhasbeenmade,DNA polymerase begins to add nucleotides to formDNA from 5′-3′ direction. This is the principle that is involved in the necessity of primers for the PCR.

RNA too can act as a template for DNA synthesis.

There are many viruses whose genetic material is made of RNA. These viruses replicate their genome via synthesis of DNA.Thisismediatedby an enzyme called reverse transcriptase (RT). This enzyme was first isolated by Temin and Baltimore. It is primarily an RNA dependent DNApolymerase. Such enzymes are purified from RNA tumor viruses. This enzyme is used in molecularbiologytosynthesizecompliment ary DNA(cDNA)frommRNAs.Theyarealsoused to amplify RNA viral genes through RT- PCR. This technology is used for detectingthepresenceofRNAviruses.

Polymerase Chain Reaction (PCR) is the resultofsuccessfulexploitationofthe propertiesofDNAanditsreplication.

DNApolymeraseusessinglestrandedDNA asatemplate forthesynthesisofa complementarynewstrand. These single stranded DNA templatescanbeproducedby simplyheatingdoublestrandedDNAtotemperaturesnearboiling.DNApolymerase also requires a small section ofdoublestranded DNA to initiate (prime) synthesis. Therefore the starting point for DNA synthesis can be specified by supplying an oligonucleotide primer (a small piece of DNA with 15-40 nucleotides) that anneals to the template-DNA at that point. This is the first important feature ofthe PCR- that DNA polymerase can be directed to synthesize a specific region of DNA. Both DNA strands canserveastemplatesforsynthesis,provided an oligonucleotide primer is supplied for each strand. For a PCR, the primers are chosen to flank the region of DNA that is to be amplified so that the newlysynthesized strands of DNA, starting at each primer, extend beyond the position ofthe primer on the opposite strand. Therefore, new primer binding sites are generated on each newly synthesized DNA strand. The reaction mixture is again heated to separate the original and newly synthesized strands, which are then available for further cycles of primer hybridization, DNA synthesis and strand separation.The net result ofa PCR is the amplification of the DNA-molecules in geometric proportion. This is all possible due the DNA polymerase from a heat stable bacteria*Thermus aquaticus* (Taq). Using this technology it is now possible to synthesizeDNAinthepurestform,whichisthe basic requirement for any genetic manipulations.PCRtechnologynowformsthe most preferred, rapid, sensitive and specificdiagnostictoolbothinhumansandanimals.

Conclusion

The basic knowledge on the structure and function of nucleic acid is the basis of many revolutionary developments in biology.PCR is one such technologythat has revolutionized molecular biology. The deeperunderstanding of the chemistry of nucleic acids will help not only to understand the

noveltechnologiesbutalsotodevelopnew
technologiesin future.

Viruses, structure, biology – viral replication, infection, variations / methods

Biology of virus

A virus is a submicroscopic small infectious agent that replicates only inside the living cells of other organisms. Viruses can infect all types of life forms, from animals and plants to microorganisms, including bacteria and archaea. The true infectious part of any virus is its nucleic acid, either DNA or RNA but never both. The nucleic acid encodes the genetic information unique for each virus. The infective, extracellular (outside the cell) form of a virus is called the virion. It contains at least one unique protein synthesized by specific genes in the nucleic acid of that virus. In virtually all viruses, at least one of these proteins forms a shell or capsid around the nucleic acid. Certain viruses also have other proteins internal to the capsid; some of these proteins act as enzymes, often during the synthesis of viral nucleic acids. Viruses are depend on the host cell for almost all of their life-sustaining functions. The virion capsid has three functions: (1) to protect the viral nucleic acid from digestion by certain enzymes (nucleases), (2) to furnish sites on its surface that recognize and attach (adsorb) the virion to receptors on the surface of the host cell, and, in some viruses, (3) to provide proteins that form part of a specialized component that enables the virion to penetrate through the cell surface membrane or, in special cases, to inject the infectious nucleic acid into the interior of the host cell. Viroids(meaning "viruslike") are disease-causing organisms that contain only nucleic acid and have no structural proteins.

The amount and arrangement of the proteins and nucleic acid of viruses determine their size and shape. The nucleic acid and proteins of each class of viruses assemble themselves into a structure called a nucleocapsid. Some viruses have more than one layer of protein surrounding the nucleic acid; still others have lipoprotein membrane (called an envelope), derived from the membrane of the host cell, that surrounds the nucleocapsid core. Penetrating the membrane are additional proteins that determine the specificity of the virus to host cells. Most viruses vary in diameter from 20 nanometres to 250–400 nm. Animal viruses exhibit extreme variation in size and shape. They may be icosahedrons , helical , polygonal and elongated cylinders either naked or enveloped. The nucleic acids of virions are arranged into genomes which may be segmented or non segmented. They may be single or double stranded and some have circular genomes.

Viral classification

The International Committee on Taxonomy of Viruses established the viral classification scheme in 1966. From the broadest to the narrowest level of classification, the viral scheme is: Order, Family, Subfamily, Genus, Species and Strain/type. For the purposes of classification, however, three criteria are paramount. These criteria are the host organism or organisms that the virus utilizes, the shape of the virus particle, and the type and arrangement of the viral nucleic acid.

Structure of Viruses

Structure of viruses

- Virion
- Capsid
- Capsomere
- Genome
- Nucleocapsid
- Envelope

www.nsf.gov/od/lpa/news/03/pr0328_images.htm

Structure of Viruses

Capsomere subunit, Capsid, Nucleic acid, Nucleocapsid, Spike — Naked Virus

Glycoprotein, Envelope, Capsid, Nucleic acid, Nucleocapsid — Enveloped Virus

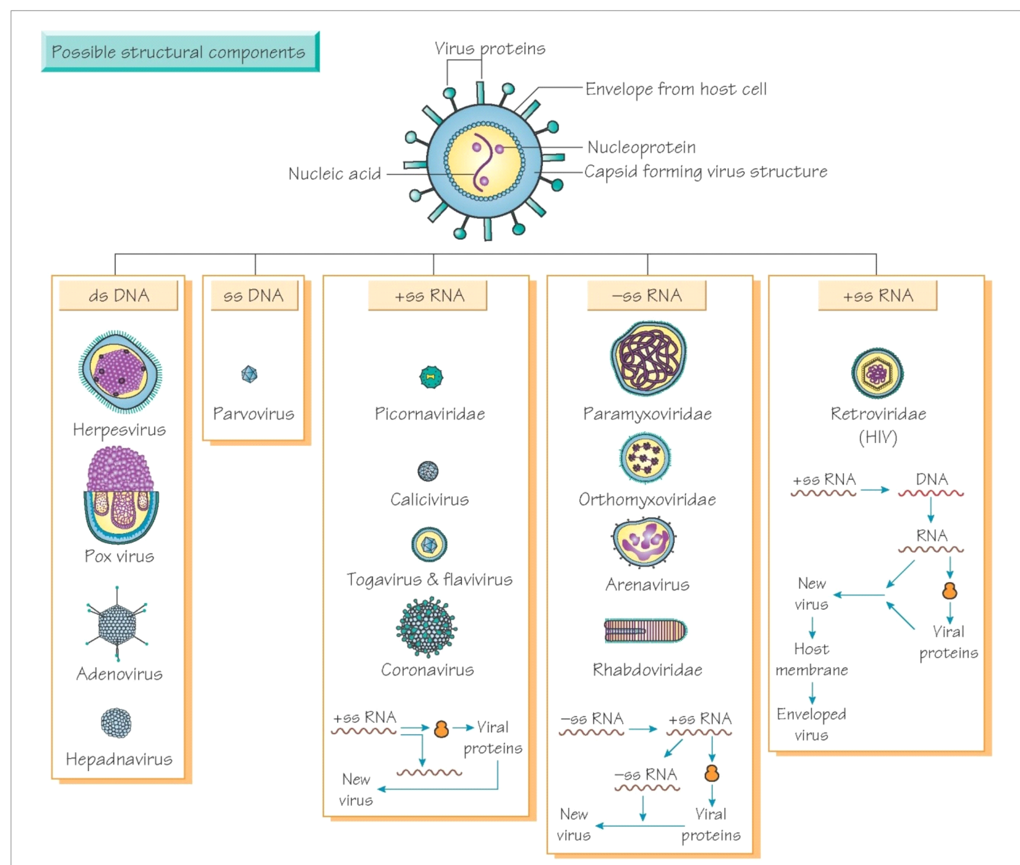
DNA containing Viruses

Hepatitis B virus HBV | *Human Papiloma virus* HPV | *Adenovirus* | *Parvovirus*

RNA containing Viruses

Rota virus | *Paramyxo virus* | *Entero virus* | *Influenza virus*

Source: Viruses; http://virology-online.com/viruses



VIRUS REPLICATION

The following steps take place during viral replication

- Adsorption - virus binds to the host cell.
- Penetration - virus injects its genome into host cell.
- Viral Genome Replication - viral genome replicates using the host's cellular machinery.
- Assembly - viral components and enzymes are produced and begin to assemble.
- Maturation - viral components assemble and viruses fully develop.
- Release - newly produced viruses are expelled from the host cell

1. Adsorption

The virus becomes attached to the cells, and at this stage, it can be recovered in the infectious form without cell lysis by procedures that either destroy the receptors or weaken their bonds to the virions. Viruses typically attach to cells via specific cell surface receptors. Cells lacking specific receptors are resistant. Many different molecules are used as receptors by different viruses, including cell surface glycoproteins, components of the extracellular matrix and receptors involved in cell signalling and activation. A number of viruses also use coreceptors to gain entry to cells

2. Penetration

Penetration rapidly follows adsorption, and the virus can no longer be recovered from the intact cell. Following attachment, the virus must traverse the lipid bilayer surrounding the cell, without killing the cell. Once inside the cell, the virus must the disassemble itself in such a way that (1) its genetic information and any associated enzymes remain intact and (2) the viral nucleic acid and associated enzymes are directed to the appropriate cellular compartment . The process of virus entry can proceed via several pathways. Some virus able to fuse directly with the host cell membrane at neutral pH, via the action of the viral fusion protein (F). This results in uncoating of the viral genome at the cell membrane.

Receptor-mediated endocytosis. Other viruses do not fuse directly with the host cell membrane, but in stead undergo uptake into endocytotic vesicles.

Endosome fusion. In some cases, the virus takes advantage of the natural process of endosome acidification. the HA (hemagglutininneuraminidase) protein of influenza virus, which undergoes a low-pH induced shape change, such that the protein enters a fusion-competent state. This allows the virus to fuse with the host cell membrane, bringing the virus particle into the cell.

Endosome lysis. Non-enveloped viruses lack the lipid membranes found in enveloped viruses. As a result, these viruses cannot enter cells via a simple process of membrane fusion between the virus envelope and the host cell membrane. One strategy employed by non-enveloped viruses is virus attaches immunoglobulin-like molecule, and it then binds to cellular integrins. Following binding, the integrin and virus are internalized, and the virus then begins to disassemble in the mildly acidic environment of the early endosome (~pH 6). As the endosome acidifies further, is thought to trigger endosome lysis, thereby mediating the escape of the partially disassembled core particle into the cytosol. Pore formation. Some non-enveloped viruses, such as picornaviruses, form pores in the cell membrane.

3. Uncoating

A key step in uncoating is the acidification of the content of the endosome to a pH of about 5. The low pH causes rearrangement of coat components, which then expose normally hidden hydrophobic sites. They bind to the lipid bilayer of the membrane, causing the extrusion of the viral core into the cytosol.

4. Viral Nucleic Acid Replication

Virulent viruses, either DNA and RNA, shut off cellular protein synthesis and disaggregate cellular polyribosomes, favouring a shift to viral synthesis.

DNA viruses replicate their genetic material by one of three modes:

- Bidirectional replication from a circular substrate. This process may proceed via a "theta-form" intermediate (eg, papillomaviruses), or in some cases via a "rolling circle" mechanism that results in the generation of concatemeric (head-totail) viral genomes.
- Replication from a linear substrate. In this case, synthesis of new DNA strands is not simultaneous. Rather, it occurs sequentially (ie, first one strand is made in its entirety & then the next strand is made). Examples include adenoviruses.
- Replication via an RNA intermediate. Hepadnaviruses (hepatitis B virus) are unique since they contain a partially dsDNA genome that must be converted into an RNA form by the virion enzyme reverse transcriptase during the virus life cycle

Replication of RNA Viruses. RNA viruses have a variety of modes of replication. Three important points about their replication are as follows:

1. The viral RNA genome can act as its own message (positive strand viruses) OR the complementary strand can be the mRNA (negative strand viruses).

2. All RNA viruses except retroviruses encode an RNA-dependent RNA polymerase. In the negative strand RNA viruses this polymerase is part of the virion, and it must enter the cytosol along with the viral genome. This is necessary in order for the virus to generate mRNAs from its genome.

3. All RNA viruses replicate in the cytoplasm except orthomyxoviruses (influenza A & B), borna disease virus, hepatitis delta virus and retrovirses. Additional feature of orthomyxoviruses: these viruses have segmented genomes (influenza A has 8 separate strands of genomic RNA)

4. Retroviruses are unique. These viruses have a positive sense RNA genome which must be converted into a dsDNA form by the virion enzyme reverse transcriptase (an RNA-dependent DNA polymerase). This double-stranded DNA is then integrated (at random sites) into the host cell chromosome by the viral integrase enzyme. Upon integration into the host chromosome, the viral DNA can then be transcribed by cellular RNA polymerase II, to produce new genomic RNA molecules.

Viral variation

Genetic variation is an essential feature of all living organisms. It provides the resource for natural selection and for the progressive adaptation of the population to a changing environment. Viruses face continuous environmental change as they pass from host to host. Evasion of the host defences is a central feature of the survival strategy of all viruses. However, allelic variations in host genes present a changing environmental landscape that can determine susceptibility to infection or efficiency of replication.

Viral variation can be generated by a number of mechanisms. Major rearrangements in genome structure and genetic recombination, gene duplications, gene exchanges and gene adoptions. However, the most common form of variation is mutation by nucleotide substitution. This occurs as a consequence of polymerase error in reading the template during replication. As viruses replicate rapidly and prodigiously, viral variation has significant implications for diagnosis and epidemiology.

Genetic variation in RNA and DNA viruses

From a genetic perspective, viruses can be classified as genome comprises RNA or DNA. RNA viruses are inherently hypervariable as RNA polymerases, which replicate the viral genome, lack proof reading and error editing functions resulting rate of nucleotide misincorporation in RNA viruses . Some mutations are lethal. However, many mutations result in viable genomes that continue to replicate and contribute to the virus population. In this way, RNA viruses continually refine their genetic structure to accommodate the changing environment. Some RNA viruses may also undergo genetic rearrangements that allow exchange of corresponding genes or gene segments during mixed infections . These recombination and reassortment events allow the most efficient and environmentally adapted combinations of genes to emerge from the available genetic pool, increasing the potential for viral survival.RNA viruses known to infect farmed shrimp include Taura syndrome virus (TSV), yellow head virus (YHV), gill-associated virus (GAV), lymphoid organ virus (LOV) and rhabdovirus of penaeid shrimp (RPS). Each of these viruses is likely to replicate with a high mutation frequency. Some may also have a capacity for genetic recombination.

In DNA viruses, the mutation rate is usually far lower than in RNA viruses . DNA polymerases, both cellular and viral, do employ proof reading and repair functions to reduce the intrinsic error rate. However, some small DNA viruses (eg. parvoviruses) appear to produce factors that suppress the repair function, generating an error rate similar to that of RNA viruses. DNA viruses have also been reported to produce sequence duplications and insert host DNA sequences into the viral genome.DNA viruses known to infect farmed shrimp include white spot syndrome virus (WSSV), monodon baculovirus (MBV), baculoviral midgut necrosis virus (BMNV), infectious hypodermal and haematopoeitic necrosis virus (IHHNV), spawner mortality virus (SMV) and hepatopancreatic parvovirus (HPV). A recent study of WSSV DNA from sources in different geographic locations has suggested little sequence variation between isolates.

Genetic variation in viral detection and disease diagnosis

Observations of genetic variability in viruses proclaim the need for care in the use of molecular methods for disease diagnosis. Mutations in the nucleotide sequence can prevent binding of PCR primers to target sequences, cause primers to bind non-specifically to non-target sequences, or prevent PCR extension. Sequence insertions or duplications can generate size variations in the PCR product. In each case, the result may appear falsely negative. At the protein level, mutations and other variations in sequence can affect the binding of diagnostic reagents. Variations can also cause closely related strains to

have significantly different biological properties such as pathogenicity, tissue tropism or host range. An understanding of these factors is important for accurate interpretation of data obtained for disease diagnosis, epidemiolgical investigation or screening for disease-free certification.

Virological Methods

The most commonly used laboratory methods for the detection of viruses and virus components in biological samples can be– visualization of virus particles , measurement of virus infectivity, viral serology and molecular methods.

Direct visualization of virus particles: Visualization of virus particles requires the use of the electron microscope (EM). The EM accelerates electrons to high energy, and focuses them using a magnet. The high energy of the electrons gives them a short wavelength, which is much smaller than that of viruses. it is necessary to coat or stain samples for EM analysis. This process is called “shadowing” and it involves the deposition of a thin layer of a heavy metal such as osmium on the surface of the sample to be analyzed. One particularly useful aspect of EM analysis is that it allows one to directly count the number of virus particles within a sample – regardless of whether or not those particles are actually infectious. One problem of the shadowing process is that it tends to result in sample desiccation, and in some loss of structural information – particularly for enveloped viruses. As a result, the procedure of cryoEM analysis was developed. In this case, the virus sample is rapidly frozen onto an EM grid in such a way that it becomes embedded in a thin sheet of vitreous ice (ice that does not contain any large crystals). This process retains some moisture in the virus, and it results in enhanced preservation of viral structures. The virus particles are then visualized in the microscope at very low temperature, to prevent thawing.

Measurement of Virus Infectious Units To study and quantitate virus infectivity in the laboratory, animal viruses must be propagated in cells and cell lines. One very widely used assay is the plaque assay. This method is very similar to the procedure used to titer bacteriophages, and it relies upon (1) the use of confluent monolayers of cells which are susceptible to the virus, (2) the induction of a visible cytopathic effect by the virus and (3) the use of a semisolid overlay of agar which prevents virus diffusion from one infected cell to other nearby cells. As a result, small round plaques (clear areas) form in the cell monolayer as the virus replicates. These plaques can be visualized by staining the cells with a vital dye like crystal violet or neutral red.

Endpoint dilution assay can be used to determine titers of many viruses. In this case, add serially diluted aliquots of virus to cultured cells, which are typically plated into multiwell plates. Allow the cells to grow for fixed period of time, after which the wells are scored for the presence of viral replication. This is most often done by analysis of virally induced cytopathic effect. Endpoint dilution assays provide a widely used basis for the calculation of virus titers. Typically, the 50% infectious dose (TCID50 or tissue culture 50% infectious dose) is calculated using a mathematical analysis of the data. Popular methods include that of Reed and Munch.

Reed-Münch formula $I = \frac{\text{(\% of w ells infected at dilution above 50\% - 50\%)}}{\text{(\% of wells infected at dilution above 50\% - \% of wells infected at dilution below)}}$

50% endpoint titer = $10^{\text{log total dilution above 50\% - (I x log h)}}$

I = Interpolated value of the 50% endpoint h = dilution factor

Table 1: Classification of known shrimp virus

Name	Approximate Virion size	Nucleic Acid	Probable Classification	Reference
White spot syndrome virus (WSSV)	80-330 nm	dsDNA	Whispovirus	Wongteerasupaya*et al*, 1995
Infectious hypdermal and hematopoietic necrosis virus (IHHNV)	22 nm	ssDNA	Parvovirus	Lightner*et al*, 1983
Monodon baculovirus (MBV)	75X300 nm	dsDNA	Baculovirus	Lightner*et al*, 1983
Hepatopancreatic parvovirus (HPV)	22-24 nm	ssDNA	Parvovorus	Lightner and Redman, 1985
BaculovirusPenaei (BP)	50-75X300 nm	dsDNA	Baculovirus	Couch, 1974
Baculoviralmidgut gland necrosis (BMN)	75X300 nm	dsDNA	Baculovirus	Sano *et al*, 1981
Yellow head virus (YHV)	44X173nm	ssRNA	Rhabdovirus	Flegel*et al*, 1995
Taura syndrome virus (TSV)	30-32nm	ssRNA	Picornavirus	Lightner*et al*, 1995
Infectious myonecrosis virus (IMNV)	40 nm	dsRNA	Totivirus	Poulos *et al*., 2006
Laem-Singh virus (LSNV)	30nm	ssRNA	Luteovirus-like (unclassified)	Srisala et al., 2010
covert mortality nodavirus (CMNV),	32 nm	ssRNA	Noda virus	Zhang et al., 2014
Reo like virus	70nm	dsRNA	Reo-like virus	Tsing and Bonami, 1987
Lymphoid organ vacuolization virus (LOVV)	30XSSnm	ssRNA	Toga-like virus	Bonami*et al*, 1992
Rhabdovirus of penaeid shrimp (RPS)	75X125nm	ssRNA	Rhabdovirus	Nadala*et al*, 1992

CIBA

Viral diseases and their management with special reference to white spot disease and Indian shrimp farming

Introduction

Aquaculture is a rapidly expanding industry, which augments cheap protein to a large portion of world population. In terms of value, shrimp is considered as the single largest seafood commodity because of its high export value. More than 75% of shrimp production is in the form of aquaculture and several modifications are being constantly carried out to increase the production. However, adoption of high stocking density, increase use of inputs and lack of proper biosecurity has resulted in the outbreak of diseases. All most all kinds of microorganisms have been foundto be capable of producing some forms of disease to shrimp. However, the emergence of viral diseases since 1981 has made the situation more complicated and the industry is constantly facing huge loss due to mortality and crop failure. So far more than 20 viruses have been reported from shrimp and new viruses are frequently being added up to the existing list. Amongst all the viruses, white sot syndrome virus (WSSV) many times acts as a single most causative agent threatening the sustainability of shrimp aquacultureindustry.

White spot syndrome virus(WSSV)

WSSV is a rapidly replicating and highly virulent shrimp virus that has wide spread presence throughout the world. Originating from Taiwan in 1992, it spread to Japan during 1993 and subsequently very quickly to all other Asian countries. By 1995, it had already spread to North America and further by 1999 to South America. The quick spread of this virus to different regions and simultaneous investigation by different scientists speculated the same agent to be different ones and thereby called them in different names such as Systemic Ectodermal and Mesodermal Baculovirus (SEMBV), rod shaped nuclear virus of *Penaeus Japonicus* (RV-Pj), Hypodermal and Haematopoietic Necrosis Baculovirus (HHNBV), third *Penaeus monodon* non-occluded virus (PmNOB III), penaeid rod shaped DNA virus or white spot baculovirus. On subsequent investigation and data analysis when it was known that all these names are for the same agent, it was unanimously called as white spot syndrome virus. Other than the penaeid shrimps which serve as host for this virus, a large number of other crustaceans serve as carrier and therefore it has been impossible to eradicate this virus from the culture system. The typical clinical symptoms of WSSV infection are the formation of circular whites spotson the carapace. However, there can be many other reasons for this and therefore the infectionshouldgetconfirmedbyvariousdi agnosticprotocols.

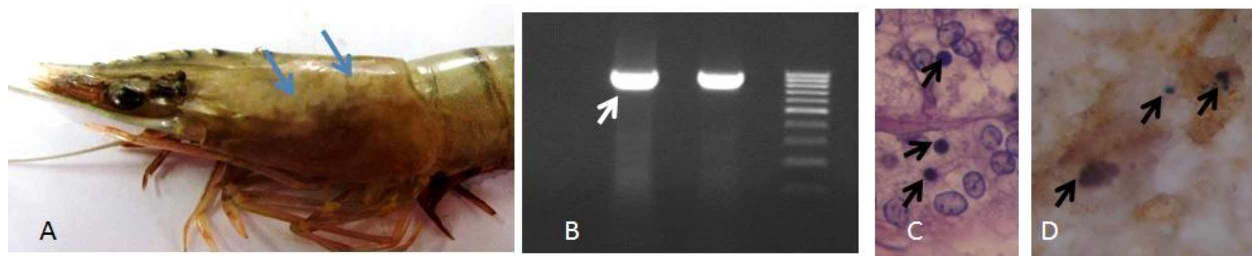


Fig. 1: Typical (circular white spot on the carapace) clinical sign of WSSV (A); The detection of the virus by PCR (B); Histopathology (C) and *in situ* hybridization (D)

Prevention andcontrol

- **Virus exclusion and preventing the entry**
- **Geneticselection**

Wherever possible, specific pathogen free (SPF) and specific pathogen resistant (SPR) animals should be stocked to avoid the disease occurrence. After determining the disease resistance markers, animals can be selected for the production oftotallydiseaseresistanceprogenies.Wherecompleteresistancestrainproductionis not possible, care can be taken to produce at least pathogen free stocks as has been tried for *Penaeus vannamei*. As in this case the seed will be free from major disease causing agents such as WSSV and subsequentbettermanagement practices can be followed to get a successfulharvest.

- **Screening oflarvae**

Stress test should be carried out to choose the healthy larvae. Further, the larvae should be screened by PCR for the presence of virus. Early, accurate and sensitive detection of pathogens are important and at least a nested PCR protocol should be carried out to rule out the presence of WSSV inlarvae.

- **Better management practices (BMPs)**

Once the larvae are shifted from the hatchery to the pond, it is subjected to enormous stress. Stress is the main factor to initiate disease and this can be overcome to a large extent through BMP. Soil and water qualities are theimportant aspects for health management. Ponds should be prepared adequately to ensure it as pathogen free. Sufficient gap should be there between the culture cycles to adequately prepare the ponds. WSSV can remain viable and infectious for considerabletimeperiodintheponds.Therefore,pondsshouldbedriedsufficiently beforetherealpreparation.

- **Biosecurity**

As has been already mentioned, WSSV is a highly virulent virus and has a wide host range. Utmost care should therefore be taken to prevent the spread of the causative agents. Source water, seed, equipment, workers and invasive organisms are the main source of virus spread. Step by step protocols and precautions should be taken from the beginning to avoid the entry of the pathogens to culture system.If strict biosecurity measures are adopted, disease can be avoided to a large extent. Adoption of proper sanitation protocols, development of a reservoir pond, bird fencing and crab fencing are some of the common biosecurity measures to avoidthe spread ofWSSV.

- **Development of recirculatory/zero water exchange culture system**

Water can be an important source of infection as far as WSSV is considered. Care should be taken to avoid the virus from the intake water. Recirculatory/zero

CIBA

water exchange will ensure the prevention of pathogen entry into the system. Culturing the virus free larvae and adopting better management practice, it will then possible to prevent the entry of WSSV into the system.

- **Prevention of the spread**

Once a disease outbreak occurs, it becomes a rich source of pathogen to contaminate the nearby brackishwater fishery resources. Therefore, it is necessary to confine the infected pond and do immediate treatment to ensure complete pathogen killing. Sufficient biosecurity and emergency planning should be in hand to prevent the spread through carrier aquatic organisms orbirds.

- **Boostingtheimmunityandincreasing diseaseresistancecapacity**

- **Immunostimulants**

Non-specific immune system of shrimp is the primary defence against a wide range of pathogens. This system can be stimulated through various microbial and plant based products to provide better protection. Lipopolysaccharide (LPS) from gram negative bacteria and peptidoglycan/beta glucan from gram positive bacteria / yeast are widely used immunostimulants. Different plant based products with proven medicinal properties have been successfully used as immunostimulants to provide partial or full protection. In addition to providing protection against diseases,theseimmunostimulantshavealsob eenfoundusefultoprovidebetter growth. Because of the lack of memory in shrimp, it is necessary to apply these immunostimulants very frequently during a culture period for successful protection. Similarly, the shrimp larvae can also be treated through immersion or feedtohavebetterprotectionduringtheinitial criticalperiodofculture.

- **Adoption of Biofloctechnology**

This recently developed eco-friendly technology where beneficial microorganisms are multiplied in an ecosystem through the manipulation of carbon and nitrogen ratio has been found to be useful in boosting the immune system and thereby increasing the disease resistance capacity. Similar to probiotics, the microbes produced here maintain the water quality and at the same time stimulate the immune system of shrimp when taken as feed. Through this technology, the wastes generated are efficiently utilized and uses of many additional inputs are also avoided. Therefore, this technology has several added advantages to avoid disease problems, particularly from that ofWSSV.

- **Use ofprobiotics**

Probiotics are a group of "good bacteria", which are proved to improve the host immune system and thereby provide good health when consumed. These bacteria go and colonize in the gut. In this way they occupy the space and do not allow the pathogenic bacteria to settle down. They also produce specific molecules which has ability to stimulate the host immune system. Based on its application, probiotics in aquatic system can be of two types. Gut probiotics does the usual function in replacing the pathogenic bacteria and stimulating the immune system. Whereas the water probiotics helps in increasing the diversity of good bacteria in water and thereby do not allow the multiplication of pathogenic bacteria. These bacteria are also known to secret extracellular products that has inhibitory effect against harmfulbacteria.

A number of probiotics products, consisting of several bacteria species such as *Lactobacillus* spp., *Bacillus* spp., *Pseudomonas* spp., etc either as single

species or as consortium, are available for the use both in fish and shellfish culture. Experimental evidences regarding precipitation of WSSV by secondary bacterial infection has been generated. Therefore, during this period, application of probiotics will prevent the multiplication of pathogenic bacteria and thereby prevent the quick precipitation ofdisease.

- **Controlmeasures**
- **Preventingtheprecipitation**

There are evidences where farms can continue with WSSV and have successful harvest.Thisismainlybypreventingtheprecipitation.Theviruscanremainina latent/dormant stage and cannot cause disease. This is mainly through good water quality maintenance, BMP and other precautionarymeasures.

- **RNA interference(RNAi)**

Experimentally, this technology has been proved to be very useful for the control of several pathogens including WSSV. It has been particularly very effective for the control of many viral diseases where treatments through medicines are not possible. Specific virulence genes of the pathogens are targeted to develop short RNA fragments (either single or double stranded) and this is either injected or supplied through oral route after modification. This brings degradation of the pathogen through post translational modification. The RNAi system has been found to be functional in shrimp. This method is particularly look promising for the treatment of viral diseases of shrimp where neither any treatment methods nor any vaccines are available. Several experiments have been carried out targeting some of the common virulent genes of WSSV such as VP28, VP19, VP15, rr1, rr2 etc. Unfortunately, many of these protections have been demonstratedthrough injection which is not a practical method during culture practice. However, the injection method to produce viral fee larvae will be helpful for some of the penaeid shrimps like Penaeus monodon where development of SPF stocks has not been possible.

- **Developmentofanti-WSSVmedicine**

A lot of medicinal plants have shown to have antiviral molecules and have been effectively used against a wide range of animal and human viral pathogens. Such types of screening from plants for the development of anti-WSSV molecules should also be tried seriously. Similarly many of the marine macro and microorganisms are also a good source of antiviral molecules and effort should be there for their screening. The newly developed concept, “synthetic biology” should also be applied tosyntheticallydevelopdifferentmoleculesalreadyproventohaveanti-viraleffect.

• Conclusion

WSSV is a highly virulent pathogen that can bring mass mortality within a few days of time period. It can be a single most important factor in threatening the sustainability of shrimp aquaculture practice. Though, it has been more than two decades after its emergence, no solution to this problem has been possible to develop. In this context, general management practices to prevent the entrythrough viral exclusion will be a better mechanism. However, further research shouldbecarriedouttodevelopaneffectivemedicineforcontrolmeasure.

Update on Acute hepatopancreatic necrosis disease (AHPND)

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 lossof 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.

CIBA

Diseases in shrimp aquaculture-Current scenario in India: white faeces/gut syndrome (WFS), growth variation / retardation, white muscle syndrome, running mortality syndrome (RMS) and Hepatopancreatic microsporidiosis

Aquaculture continues to be the fastest growing food production sector with an annual average growth rate exceeding 6 % (FAO, 2014) and having enough potential to meet the growing demands for aquatic food. Aquaculture delivers not only economic income and high quality food products, but also employment to both skilled and unskilled workers. Over the last three decades, shrimp farming has been one of the most rapidly growing aquaculture sectors throughout the world. Shrimp continues to be the largest single seafood commodity by value, accounting for 15% of all internationally traded fishery products. Farm raised shrimp is comprised of 55 % of global shrimp production and this is entirely dominated by two species – the black tiger shrimp (*Penaeus monodon*) and the white Pacific white shrimp (*Penaeus vannamei*) (FAO 2014). In India, shrimp aquaculture started as a traditional practice in natural water bodies such as bheries or pokkali fields and subsequently transformed to commercial industry during 90"s. Initially it was dominated by a single species, *Penaeus monodon* and the production of which reached a maximum of 1.44 lakh tons in 2006-07 (www.mpeda.com). Due to disease outbreak and other social issues, the farmers felt it difficult to continue further with this species and thereafter an exotic species; the Pacific white shrimp

P. vannamei was introduced into the brackishwater aquaculture system of India. Because of its SPF status, fast growth rate and culture feasibility in wide salinity range, this got readily accepted by the farmers and subsequently became the dominant culturedspecies.

The increasing trend in intensification and commercialization has exacerbated the epidemics of diseases and become a major constraint for the sustainability of this industry. Severe disease related mortality and thereby economic loss due to different viral agents such as monodon baculovirus (MBV) in Taiwan, infectious hypodermal and hematopoietic necrosis virus (IHHNV) in the Americas, yellow head virus (YHV) in Thailand and Taura syndrome virus (TSV) in the Americas during the different periods have been reported. In addition to all these, the major disease outbreak due to White Spot Syndrome Virus (WSSV) has also been reported from all parts of the world. During the recent past, the havoc createdbyEarlyMortalitySyndrome(EMS)indifferentSouthEastAsiancountries has also been found to have severe economic impact on shrimp industry of these regions. Further, a number of other diseases by unidentified etiologies are constantly being associated with shrimp culture practice which has been responsible either for direct mortality or growth reduction and thereby bringing loss to farmers. Shrimp hatcheries are

also prone to losses either due to disease outbreak or other unknownfactors.

Some of the problems associated with current shrimp culture practice in India are discussed below. In addition to the already existing WSSV problems, the industry at present is going through a very critical phase due to several uncharacterizedfactors.

Currently prevailing diseases in Grow-outsystems

- **White faeces syndrome(WFS)**

White faeces syndrome reported since last decade, has recently been noted as seriousproblemfor*P.vannamei*throughou ttheworld.However,thisdiseasehas been reported from both cultured black tiger shrimp and pacific white shrimp. White faeces syndrome usually occurs after 60 days of culture (DOC) and it may be accompanied by high shrimp mortality. Ponds affected with white faeces syndrome show white faecal strings floating on the pond surface while the shrimps show white/golden brownintestine, reduced feed consumption, growth retardation and often associated with loose shell. The disease can cause moderate to severe economic loss by reducingthe shrimp survival by 20–30 percent when compared to normal ponds. While investigating the aetiology of WFS this disease has been associated with presenceof vermiform like gregarine bodies, vibriosis, *Enterocytozoan hepatopenaei*, bluegreen algae and loose shell syndrome. Sriurairatana et al (2014) revealed that 96% of the pondsexhibitingWFSpresentedvermifor mbodiesresemblinggregarines.Whenthe contents of the gut or faecal strings were examined in squash mounts with the light microscope, they consisted of masses of vermiform bodies that superficially resembled gregarines. Bacteriological results showed that total bacteria and *Vibrio* spp. found in haemolymph and intestine were significantly higher in diseased shrimp than in healthy shrimp. Six species of fungi(*Aspergillusflavus,A. ochraceus, A. japonicus, Penicillium* spp., *Fusarium* spp., and *Cladosporium cladosporioides*) were isolated from shrimp naturally infected with white faeces syndrome. Histopathological examination revealed diffused haemocyte encapsulation and dilated hepatopancreatic tubules accompanied by necrosis. Tangprasittipap et al., 2013 revealed that the microsporidian newly found in *P. vannamei* is nonspecific with previously described *E. hepatopenaei* and it is not causally associated with WFS. Sriurairatana et al. (2014) concluded with Transmission electron microscopy (TEM) study that vermiform structures superficially resembling gregarines and commonly found now in the HP of Asian cultivated shrimp are not independent organisms but result from the transformation, sloughing and aggregation of microvilli from the HP tubule epithelial cells themselves and the denuded epithelial cells subsequently undergo lysis, can lead to the phenomenon called white faeces syndrome (WFS) and transformed microvilli (ATM) in very severe cases they may retard shrimp growth and may predispose shrimp to opportunistic pathogens. Furthermore it has been estimated that the Thai production losses due to WFS in 2010 were 10–15%. The cause of white faeces syndrome and treatment is uncertain. However reduced stockingdensity,properwaterexchangeto getherwithbettermanagementpractices

willbehelpfulinevadingWhiteFaecessyn drome(WFS).

Running Mortality Syndrome(RMS)

Since 2011, a new syndrome has brutally affected the shrimp industry and causing substantial mortality. The disease has been loosely termed as Running Mortality Syndrome (RMS) by the farming community. The affected ponds show different mortality patterns with unusual symptoms, no relation to any known diseases anda slow mortality rate (e.g. <1%/day), but the cumulative loss over phase will be high. Some farmers have lost up to four crops, with mortality percentage reaching 70% in most of the cases. Generally mortalities start after a month or 40 days of culture (DOC) but a portion of shrimp continue to survive and can grow to fully harvestable size. Investigations (conducted in ICAR-CIBA) revealed no associationof Running Mortality Syndrome (RMS) with known shrimp viral infection. Further, bacteriological examination of haemolymph samples of RMS affected shrimp indicated predominance of *Vibrio* spp., such as *Vibrio parahaemolyticus* and *Vibrio azureus*. The population of anaerobic bacteria in the gut of RMS affected shrimp ranged from 72 - 252 x 10^{14} cfu mL^{-1} and were identified based on16S rRNA gene analysis as *Enterococcus faecium*, *E hirae*, and *Lactobacillus plantarum*. Bacterial diversity of RMS affected shrimp gut examined by Denaturing Gradient Gel Electrophoresis (DGGE) revealed a number of uncultured bacterial sequences. Histopathological examination of the hepatopancreas was largely normal. However, some samples showed karyomegaly and increased inter hepatopancreatic tubular space with haemolymph infiltration, muscle necrosis, loosened LO tubule cells and constricted lumen. Bioassay experiments carried out by feeding RMS affected shrimp tissue to healthy 13-14 g shrimp did not elicit any disease in the experimental shrimp. All the experimental animals were healthy and active even after 44 hrs of feeding RMS affected shrimp tissue like that of control animals. RMS affected shrimp showed recovery and appeared healthy and active after 155 hrs of transferring to wet lab in water with optimal parameters. Co-habitation experiment with healthy shrimp and the infected animals also failed to induce RMS. All shrimp appeared healthy and active. Relatively few studies done on Running Mortality syndrome, and still the causative agents or aetiology of RMS areunknown.

Size variation/ Growthretardation

More recently shrimp farmers have been reported several cases of size variation / growth retardation in *P. vannamei* grow-out cultures. It is reported that viruses,viz., infectious hypodermal and haematopoietic necrosis virus (IHHNV), lymphoid organ vacuolization virus (LOVV), monodon baculovirus (MBV), hepatopancreatic parvovirus(HPV)andLaem-Singhvirus(LSNV)areassociatedwithslo wgrowthand size variation in shrimp. In India, Madhavi et al. (2002) recorded

multiple viral infections in shrimp with stunted growth. Rai et al. (2009) observed IHHNV, MBV and HPV associated with slow growth shrimp and stated that IHHNV could be oneof the causes of slow growth in cultured *P. monodon*. In the event of white faeces syndrome affected animals there is a decrease in feed consumption and growth rates were reduced as revealed by average daily weight gain (ADG) for the whole crop operation of less than 0.1 g/day compared to 0.2 g/day in normal ponds. Feed conversion ratios (FCR) ranged from 1.7 to 2.5 when compared to 1.5 or less for normal ponds (Sriurairatana et al, 2014). Recently *Enterocytozoan hepatopenaei* found to be associated with size variation/growth retardation (Tangprasittipap et al.,2013).

White gutdisease

This disease of *P. vannamei* is mostly caused by different species of *Vibrio* and is very much prevalent in Andhra Pradesh and Tamilnadu. Vibrios are normally present in water bodies. But sometimes stressed environmental factors such as sudden change of environment and salinity, low DO, mechanical injury, higher stocking density, etc. caused rapid multiplication of this organism in the gut and hepatopancreas. Vibriosis caused red discolouration and melanization of appendages (red disease), necrosis of tail, broken antennae, etc. Six species of *Vibrio* viz. *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus* and *V. splendidus* are generally associated with the diseased shrimp. The diagnosis of the disease can be done by isolation of organism by plating haemolymph on the TCBS agar followed by identification of the species of *Vibrio* by biochemical tests or 16S rRNA genesequencing.

Hepatopancreatic microsporidiosis

Hepatopancreatic microsporidiosis (HPM) is caused by *Enterocytozoon hepatopenaei* (EHP). It was first reported as an unnamed microsporidian from growth retarded giant or black tiger shrimp *Penaeus monodon* from Thailand in 2004. It was subsequently characterized in detail and named in 2009. It also has much smaller spores (approximately 1 µm in length) and is currently known to infect both *P. monodon* and *P. vannamei*.It has been found that EHP can be transmitted directly from shrimp to shrimp by cannibalism and cohabitation. *E. hepatopenaei* (EHP) is confined to tubule epithelial cells of the shrimp HP and shows no gross signs of disease except retarded growth. It is likely that other penaeid shrimp and/or other crustaceans or even other marine or brackish water species in the region may also be susceptible to infection. For example, some samples of polychaetes and mollusks have tested positive by PCR, but it is still not known whether they are infected or passive carriers. More recently, samples of frozen A*rtemia* mass has been reported to test positive for EHP by PCR , but again, it is not known whether *Artemia* is susceptible to EHP infection or just a mechanical carrier. It is urgent that these possibilities be explored in order to improve control measures.Although EHP does not appear to cause mortality in *P. monodon* and *P.*

vannamei, information from shrimp farmers indicates that it is associated with severe growth retardation in *P. vannamei* . Thus, farmers and hatchery operators monitor *P. vannamei* and *P. mondon* for EHP in broodstock, PL and rearing ponds. The best approach for maturation and hatchery facilities to avoid EHP is not to use wild, captured, live animals (e.g., live polychaetes, clams, oysters, etc.) as feeds for broodstock. Better would be pasteurization (heating at 70°C for 10 minutes) . Another alternative would be to use gamma irradiation with frozen feeds. Alternatively, polychaetes could be selected and tested for freedom from shrimp pathogens and then reared as broodstock feed in biosecure settings designed to maintain their freedom from shrimp pathogens (i.e. SPF polychaetes).

Conclusion

Aquaculture is now integral to the economies of many countries. Growing demand for seafood and limitations on production from capture fisheries will inevitably lead to the increased intensification in commercialization of shrimp aquaculture. This in turn increases the number of diseases and leads to emergence of new diseases. The emergence and spread of infectious disease is usually the result of a series of linked events involving the interactions between the host (including the physiological, reproductive and developmental stage conditions), the environment and the presence of pathogens. Focusing efforts on producing high quality seed, better pond manage to reduce stress and risk of infection, following routine farm biosecurity, responsible trade practices, response to disease outbreak, and improved better management practices shall aid in preventing the epidemics of diseases. Further health management is a shared responsibility, and each stakeholder‟s contribution is essential to the health managementprocess.

Shrimp Diseases: An overview

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 which 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. Risk of diseases is inherent with transboundary movement of live aquaculture species and 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 diseases and preparedness for health management are being discussed.

WHITE SPOT DISEASE (WSD)

Described elsewhere in this manual

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 knownthat 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.

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.

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.

***PENAEUS VANNAMEI* NODAVIRUS (PVNV) INFECTION**

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 *Macrobrachiumrosenbergii*. It is not highly virulent in lab challenges. Not associated with major on-farm mortality.

Thegross 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.

COVERT MORALITY DISEASE (CMD)

The disease first reported from China. In China since 2009, continuous mortality of shrimp especially peaked at 60-80 days with cumulative losses reaching up to 80%.T he new disease was named as Covert mortality disease (CMD) because affected shrimp died at the bottom of the pond and farmers would initially be unaware of the mortality. The causative agent was identified and named as covert mortality nodavirus (CMNV). The virus cause gross signs of muscle whitening, similar to that caused by infectious myonecrosis virus (IMNV) and Penaeus vannamei nodavirus (PvNV). Histopathologically enlarged nuclei in the shrimp hepatopancreas together with coagulative muscle necrosis observed . An RT-PCR detection method was developed and virus was detected in affected shrimp such as *Penaeus (Fenneropenaeus) chinensis* and *Penaeus (Marsupenaeus) japonicas*. Experimental studies conducted at Thailand showed injection of extracts derived from tissue homogenates of RT-PCR positive shrimp into naïve shrimp at 32°C resulted in conversion of the injected shrimp to CMNV-positive status, but was not associated with any gross signs of disease or mortality.

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.

General strategy for disease prevention and control

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.

PartII

Laboratory Experience for Aquatic Animal Health Management

CIBA

Requirements for Aquatic Animal Disease Diagnostic Laboratory

The requirements for the the aquatic animal disease diagnostic laboratory here is for Level III diagnosis including Clinical examination and epidemiological description (level-I), simple laboratory tests such as microscopic examination of blood or tissue samples (level-II) and sophisticated laboratory tests for confirmatory diagnosis (Level-III). The major components of the aquatic animal disease diagnosis include molecular diagnostic work, histopathological work and microbiological work, and hence the aquatic animal disease diagnostic laboratory must have facilities that include (i) molecular biology laboratory for isolating nucleic acids and performing polymerase chain reaction (PCR) test, (ii) histology laboratory, and (iii) microbiology laboratory. Additionally wet lab facility for pathogen challenge tests, bioassay or any other test as per the requirement would add value.

A. Molecular biology lab

Polymerase Chain Reaction has been one of the sensitive methods for detection of microorganisms (pathogenic or other importance). There should be one dedicated room for molecular biology related work. Initially only PCR related work can get started and it should have scope for future expansion for any other molecular biology works. One of the main problems in PCR work is contamination that can lead to false results. The entire PCR work should be divided and performed in three parts (preferably in different locations).

Pre-PCR Area: Pre-PCR area will receive samples and the samples will be prepared for diagnosis. This step involves dissection of animals and DNA / RNA extraction. Animal dissection materials include scissors, forceps, microfuge racks, micropipettes of different capacities, etc., besides consumables such as chemicals for buffer preparation and sample collection, gloves, tissue paper, tips for the micropipettes, microcentrifuge tubes (1.5 ml and 2.0 ml capacity), sample containers, etc.

PCR Area: The PCR part will involve setting up of the PCR reaction. This will involve use of various molecular biology chemicals and reagents. Extreme care should be taken not to contaminate any of these components.

Post-PCR area: Post PCR will involve electrophoresis of amplified product and detection of the PCR product using a UV transilluminator, photographic facility for imaging gels, and computer and software for analysis of gel images.

As far as possible, it is advisable to have three separate compartments in different locations for each of the above activities (Pre-PCR, PCR and Post-PCR).

The facilities required for the PCR laboratory include major equipment such as refrigerated centrifuge, water bath, freezer (-20°C and -80°C), ice flaking machine, PCR work station, thermocycler, gel documentation system with a computer, electrophoresis apparatus (Power pack, gel casting tray, combs, etc.), and minor equipment such as dedicated micropipettes (10 ul to 1000 ul capacity), pH meter, etc will be required. Besides, consumables for PCR chemicals, Cryobox

to hold temperature sensitive chemicals, PCR tubes / Strips / plates, filter tips, tissue paper, gloves, etc will be also required.

B. Histology Laboratory

The histology laboratory will involve various activities that include collection of samples, dissection, fixation, processing, sectioning, staining and observation. The major equipments required would be (i) Hot air oven to keep paraffin wax at a particular temperature (for manual processing), chemical hood to work with acids / formaldehyde, tissue processing by manual method, staining etc., (ii) microtome for cutting sections, (iii) histoembedder (optional, tissue embedding can be done manually also), (iv) automatic tissue processor (optional, if the number of samples handled is very large, this equipment is advisable, Otherwise, the processing can be done in coupling jars manually). And finally, a good microscope is required for observation of the slides. Minor equipment and consumables for histology laboratory include micropipettes, water bath chemicals for tissue processing and fixing, preparation of stains, sample containers, gloves, tips, glass slides, coverslips etc, coupling jars, slide holder, slide rack etc.

C. Microbiology Laboratory

This lab will involve routine isolation of microbes (bacteria and fungi) and their identification. The major equipments required would include laminar flow for sample inoculation and processing, autoclave for sterilization, incubator for incubation of culture plates / tubes at a particular temperature, hot air oven for the sterilisation of glassware, microscope for routine observation of microorganisms. Besides, minor equipment such as micropipettes, water bath, test tube racks, dissection apparatus etc, consumables such as different microbiological media, chemicals, cotton, gloves, tissue paper, test tubes, petri plates, microcentrifuge tubes, tips, spreader, inoculating loops, glass slides, conical flasks, measuring cylinders, beakers etc. will be required.

D. Wet Lab Facility

This lab will be required to rear experimental animals and carry out challenge tests or bioassay experiments to determine virulence of microorganisms. This facility can also be used for any other purposes as per the requirement. The wet laboratory would require FRP tanks of different capacities to hold aquatic animals as required, clean / filtered sea water, aerators /blower, aerator tubes, stones, etc as required.

Investigating disease in brackishwater aquaculture: Overview

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

Gross observations of clinical signs in shrimp can be easily made at the farm or pond side. Although, in most cases, such observations are insufficient for a definite diagnosis, such information is essential for preliminary compilation of a strong “case description” (or case history). Accurate and detailed gross observations also help with initiation of an action plan which can effectively reduce losses or spread of the disease, *e.g.*, destruction or isolation of affected stocks, treatments or alterations to husbandry practices (*i.e.*, feeding regimes, stocking densities, pond fertilization, *etc.*). These can all be started while waiting for more conclusive diagnostic results.

Behaviour

Abnormal shrimp behaviour is often the first sign of a stress or disease problem. Farmers or farm workers, through daily contact with their stocks, rapidly develop a subconscious sense of when “something is wrong”. This may be noticeable changes in feeding behaviour, swimming movement or unusual aggregations. Even predator

activity can provide clues to more "hidden" changes such as when fish or shrimp-eating birds congregate around affected ponds. Record-keeping can provide valuable additional evidence that reinforces such observations and can indicate earlier dates when problems started to appear. It is important that farmers and workers on the farm, as well as field support staff, get to know the "normal" (healthy) behaviour of their stocks. Since some species and growing environments may demonstrate or evoke subtle differences in behaviour, these should be taken into account, especially if changing or adding species, or when information gathered from a different growing environment is used. Where any change from normal behaviour affects more than small numbers of random individuals, this should be considered cause for concern and warrants investigation. Some clues to look out for in shrimp stocks include:

- Unusual activity during the daytime – shrimps tend to be more active at night and stick to deeper water during the day.
- Swimming at or near pond surface or edges - often associated with lethargy (shrimp swimming near the surface may attract predatory birds).
- Increased feed consumption followed by going off-feed.
- Reduction or cessation of feeding.
- Abnormal feed conversion ratios, length/ weight ratios.
- General weakening - lethargy (note: lethargy is also characteristic in crustaceans when the water temperature or dissolved oxygen levels are low, so these possibilities should be eliminated as potential causes before disease investigations are started).

Mortalities

Mortalities that reach levels of concern to a farmer should be examined for any patterns in losses, such as:

- Relatively uniform mortalities throughout a system should be examined immediately and environmental factors determined.
- Apparently random or sporadic mortalities may indicate a within-system or stock problems. If the following conditions exist - (a) no history of stock-related mortalities,

(b) all stock originate from the same source, and (c) there have been no changes to the rearing system prior to mortality problems - samples of affected and unaffected shrimp should be submitted for laboratory examination, as appropriate, and supported by gross observations and stock history.

- Mortalities that spread suggest an infectious cause and should be sampled immediately. Affected shrimp should be kept as far away as possible from unaffected shrimp until the cause of the mortalities can be established.

Feeding

Abnormalities in feeding behaviour and lack of feed in the gut are good indicators of potential problems. Daily gut content checks can be made on shrimp caught in feeding trays or bowls (where used) or, less frequently, from samples taken to determine growth. Ideally examination of feeding behaviour should be made every 1-2 weeks, even in extensive farming systems. Feeding behaviour is most easily checked by placing feed in a tray or bowl and seeing how quickly the shrimp respond, ideally after the shrimp has not

been fed for at least a few hours. It is important that the feed used is attractive to the shrimp as poorly formulated, old or badly stored feeds may not be attractive to the shrimp. Gut contents can be checked by holding the shrimp against a light to show the gut in the tail segments. If these are empty, especially just after providing feed, it may indicate either of the following conditions: i) underfeeding, or ii) onset of cessation of feeding (anorexia). Feed records should be maintained to determine normal feed consumption patterns (*i.e.*, feeding activity by healthy shrimp), which can be compared with "suspect" feeding activity. In many cases of chronic loss, daily feed consumption patterns may remain stable or oscillate over periods of several weeks. These can be detected by making a graph of daily feed consumption or by comparing daily feed consumption in the record book over an extended period (*e.g.* 3-4 weeks).

Colonisation and Erosion

Colonisation of the shell (cuticle) and gills of a crustacean is an on-going process that is usually controlled by grooming. The presence of numerous surface organisms (*e.g.* "parasites" - which damage their host; or "commensals" - that do not adversely impact their host) suggests sub-optimal holding conditions or a possible disease problem. Apparent wearing away (erosion) of the cuticle or appendages (legs, tail, antennae, rostrum), or loss of appendages, with or without blackening (melanization) are also highly indicative of a disease problem. Breakage of the antennae is an early warning sign. In healthy penaeid shrimp, these should extend approximately 1/3 past the length of the body (when bent back along the body line). Likewise, erosion or swelling of the tail (uropods and telson), with or without blackening, is an early sign of disease.

Cuticle Softening, Spots and Damage

Softening of the shell, other than during a moult, may also indicate the presence of infection. Damage or wounds to the shell provide an opportunity for opportunistic infections (mainly bacterial and fungal) to invade the soft-tissues and proliferate, which can seriously impact the health of the shrimp. Certain diseases, such as White Spot Disease, directly affect the appearance of the shell; however, few changes are specific to a particular infection. In the case of white spots on the cuticle, for example, recent work has shown that bacteria can produce signs similar to those produced by White Spot Disease and Bacterial White Spot Syndrome.

Colour

Shrimp colour is another good indicator of health problems. Many crustaceans become more reddish in color when infected by a wide range of organisms, or when exposed to toxic conditions, especially those that affect the hepatopancreas. This is thought to be due to the release of yellow-orange (carotenoid) pigments that are normally stored in the hepatopancreas. This red colour is not specific for any single condition (or groups of infections), however, so further diagnosis is needed. Yellowish coloration of the cephalothorax is associated with yellowhead disease and overall reddening can be associated with gill associated virus infections, white spot disease or bacteria, as described above, or bacterial septicemia. In some cases, the colour changes are restricted to extremities, such as the tail fan or appendages and these should be examined closely. It should be noted that some shrimp brood stock, particularly those from deeper waters, can be red in colour (thought to be due to a carotenoid-rich diet). This does not appear to be related to health and its normality can be

established through familiarization with the species being grown. Under certain conditions, some crustaceans may turn a distinct blue colour. This has been shown to be due to low levels of a carotenoid pigment in the hepatopancreas (and other tissues), which may be induced by environmental or toxic conditions. Normal differences in colouration (light to dark) within a species may be due to other environmental variables. For example, *Penaeusmonodon* grown in low salinities, are often much paler than *P. monodon* grown in brackish-water or marine conditions. These variations do not appear to be related to general health.

Soft-Tissue Surfaces

A readily observable change to soft tissues is fouling of the gill area, sometimes accompanied by brown discoloration. This can be due to disease and should trigger action since it reduces the shrimp's ability to take up oxygen and survive. Shrimp with brown gills or soft shells (or a representative sub-sample), should be transferred to a well aerated aquarium with clean sea water at the same salinity as the pond from which they came. They should be observed every 1-2 hrs over 1 day. If the shrimp return to normal activity within a few hours, check environmental parameters in the rearing pond(s). Removal of the shell in the head region of shrimp allows gross examination of this region, particularly the hepatopancreas. In some conditions, the hepatopancreas may appear discoloured (*i.e.*, yellowish, pale, red), swollen or shrunken, compared with healthy shrimp. If the hepatopancreas is gently teased out of the shell, the mid-gut will become visible and permit direct examination of colour (dark - feeding; light/white/yellow - mucoid, empty or not feeding). This information is useful for determining the health of the shrimp and if infectious disease agents are present.

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.

Environmental Parameters

Environmental conditions can have a significant effect on crustacean health, both

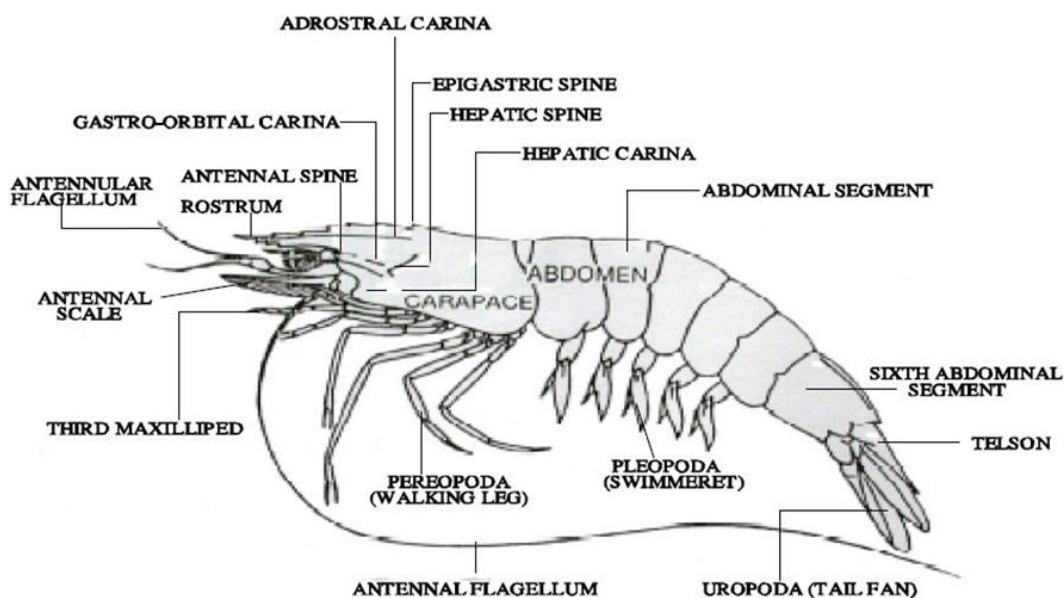
directly (within the ranges of physiological tolerances) and indirectly (enhancing susceptibility to infections or their expression). Examples include changes to dissolved oxygen levels and/or pH which may promote clinical expression of previously latent yellowhead disease and white spot disease or the effect of salinity on the expression of necrotising hepatopancreatitis. This is especially important for species grown under conditions that bear little resemblance to the wild situation. Water temperature, salinity, turbidity, fouling and plankton blooms are all important factors. Rapid changes in conditions, rather than gradual changes, are particularly important as potential triggers for disease. Therefore, the farm manager and workers, should attempt to keep pond rearing conditions within the optimum range for the species *and* as constant as possible within that range. High stocking rates are common in aquaculture but predispose individuals to stress so that even minor changes in environmental conditions may precipitate disease. In addition, many small changes do not affect shrimp health. However, when several of these small changes occur simultaneously, results can be far more severe.

CIBA

Shrimp anatomy, dissection, tissue preservation, sampling, preservation/fixation and transport of samples for disease investigation

Scientific classification:

Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Crustacea
Class: Malacostraca
Order: Decapoda
Suborder: Dendrobranchiata
Superfamily: Penaeoidea
Family: Penaeidae



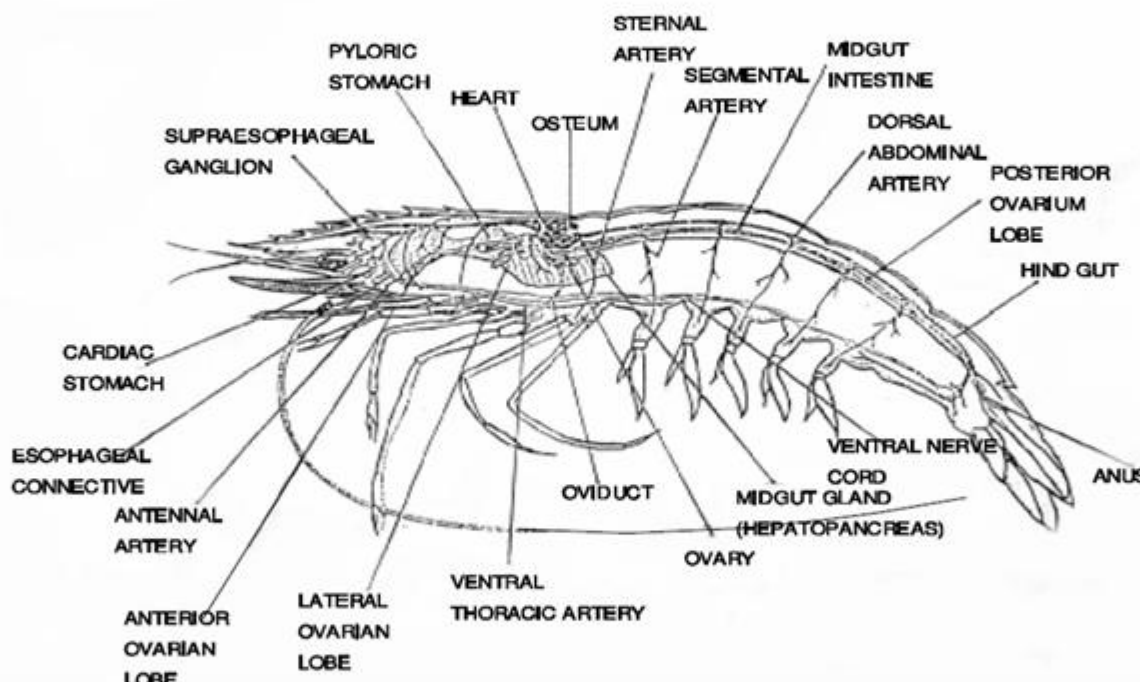
Morphology of shrimp

External Morphology

The entire shrimp can be divided into two parts – Head and abdomen. The head region containing the chest part is called cephalothorax. The head is covered by a hard cuticle called carapace. The carapace again contains different spines and one "S" shaped organ in the front called rostrum. Towards the upper end of head and on both sides, two compound eyes are present. Other important organs in the frontal part includes antenna, antennule and maxilliped. The down part of the head contains 5 pairs of walking legs called as periopods. The abdominal part is

divided into six segments. At the end of the abdominal segment, a apine like structure called telson and flnaking to this two fan like structures called as uropods.
.

In the down part of first 5 abdominal segment, a pair of leg present called as pleopods or swimming leg



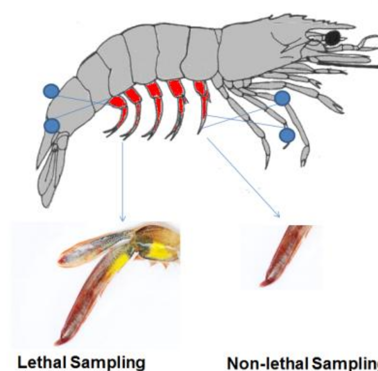
Anatomy of shrimp

Internal Anatomy

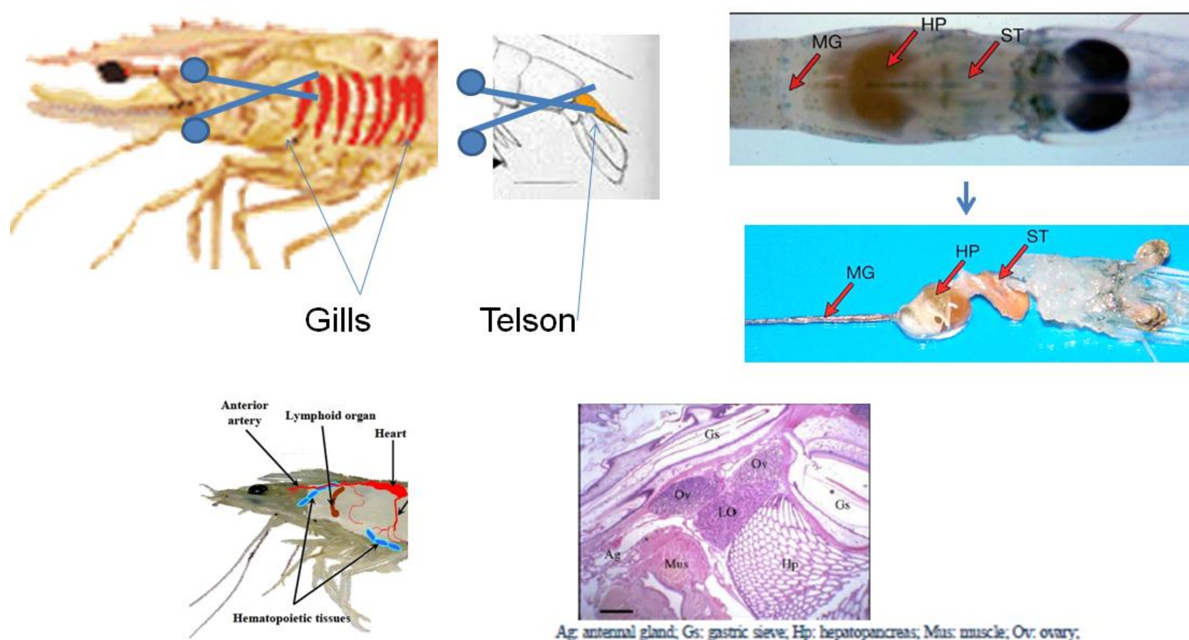
Major internal organs of shrimp include hapatopancreas, heat, digestive system, lymphoid organ, nerves and arteries. The hepatopancreas is an important part which contains different enzymes and functions both as liver and pancreas. Heart of shrimp contains hemolymph which becomes green in colour because of a copper containing pigment called haemocyanin. Digestive system includes mouth, mandibles, cardiac stomach, pyloric stomach, midgut, hindgut and anus.

Dissection for sampling

Depending upon the situation, lethal or non-lethal sampling can be done. For valuable animals lie broodstocks or fully grown cultured shrimps, non-lethal sampling is preferred where only tip of the pleopod is cut and taken for testing. At the same time, if lethal sampling has to be done, the entire pleopod can be cut and taken as sample.As lethal sampling, many of the other organs can be taken as sample after dissecting out the animal. This depends on the type of pathogen to be analysed. For example: for WSSV and IHHNV, gill can be taken as a sample. If IMNV has to be detected, telson is preferred as a sample. For HPV, MBV, AHPND and EHP, hepatopancreas,



stomach, midgut etc can be taken as samples. Lymphoid organ is a preferred sample for many of the shrimp viruses.



Ag: antennal gland; Gs: gastric sieve; Hp: hepatopancreas; Mus: muscle; Ov: ovary;

Depending upon the requirement, samples can be collected either on alcohol, RNAlater or Davidson's fixative.

Propersamplingprocedureisverymuchi mportantforappropriatediagnosis of fish and shrimp diseases. Improper sampling may result in wrong disease diagnosis leading to incorrect interpretation. Sampling should be carried out in such a way as to provide the best possible likelihood that the sample will be representative for the population. For proper sampling, the actual objective of the surveillance programme should be considered. The principal objective of sampling from a population is to select the subsets units from the population, which is representative of that particular population. The simple random selection ofsample is called as probability sampling and is one of the most common sampling techniques. When this is not possible, the sampling should provide the best practical generating optimal inferences about disease patterns in the target population. Usually, the disease survey is done with the objective of exhibiting presence or absence of a certain factor (e.g. Disease) or to estimate the prevalence of anydisease.

Sampling methods for disease diagnosis of shrimps

The target organs in case of crustaceans depend upon the disease or pathogen to be tested, age and size of the animals and objectives of testing i.e. diagnosis of overt disease, detection of sub-clinical pathogens or carriers or sampling for targeted surveillance to show absence of specific diseases. For collection of shrimp samples, the proper record of sample is very much important and the sample should be properly labeled preferably with a sample code and proper record should be noted against the sample code. The record should include name and address of the farm, contact information, cultured species along with stocking

density, seed source, water exchange information, days of culture (DOC), date and timeof commencement of disease symptom, details of samples (organs, collection media, etc.), water source, feed used, number of ponds, sampling pond number, water salinity, time of collection of samples and date of submission to the laboratories. When any specific disease is suspected based on symptoms and history, special consideration should be given targeting that particulardisease.

General precaution in microbiology laboratory

Working in microbiology lab needs some sorts of special precaution which everyone working in microbiology lab should understand. The general precaution in microbiology lab should be:

- Keep microbiology lab clean and wear a lab coat during the microbiological work.
- Do not eat or drink anything in the microbiology laboratory.
- Report all accidents even though you consider them as minor – such as cuts, pricks, abrasion to the scientist or incharge of the laboratory.
- Place all the solid wastes (Cotton, discarded plate) in a container provided for the purpose.
- Smear desk top with mercuric chloride (1: 1000), or alcohol (70%) prior to and after plating of cultures to reduce contamination.
- Wash your hands with soap before and after work.
- Plan and organise your work in advance, as microbiology work needs a lot of preliminary arrangement.

Sampling

The first step in disease diagnosis is sampling. The sample for microbiology work should be collected in aseptic condition and better to be processed immediately or otherwise should be kept on ice or at 4^0C. The sample for molecular biology work should be preserved in 95% ethanol and for histopathological work in 10% neutral buffer formalin. Apart from fish and shrimp, water and soil sample should also be collected from a disease affected pond.

1. **Water sampling**: Using a sterile container, 100 ml – 500 ml of water sample should becollected keeping at least 10% of air space to allow adequate space for mixing the sample prior to analysis. Care must be taken to avoid sample contamination during collection. To make a representative sample within pond water should be collected from at least 3 places. For best results, the sample should be inoculated within 6 hours after collection and should be maintained at or below 10 °C, but not frozen.

2. **Soil sampling**: Using a sterile container, soil sample should be collected from at least fiveplaces within a pond. Then the sub-sample should be mixed to make a composite sample. Sample should be collected in a sterile sample container. Before use the sample should be homogenised.

3. **Sampling of shrimp**

For bacteriologicalanalysis

Live shrimp is the most suitable sample for bacteriological analysis. The moribund shrimp samples (preferably minimum three in number) can be collected in double polypack with oxygen. The bags should be kept in styrofoam or thermocol boxes to keep the temperature cool. While sending to long distance, either ice pack

or gel pack should be kept inside the boxes. The equal number of normal shrimp may also be collected and sent to the laboratory in similar condition for comparison study. However, if live shrimps are not available, the dead shrimp samples for bacteriological analysis can be dispatched onice.

Alternatively, the haemolymph from the moribund shrimp may be drawn with the help of syringe and 1-2 drops of haemolymph may be spread on pre-dried Thiosulfate citrate bile salt sucrose (TCBS) agar or Zobell marine agar (ZMA). After inoculation, the plates should be incubated at 30 ºC for 18-24 hrs. When thesample has to be collected from parts with necrosis or blisters, the area should be cleaned thoroughly with sterile normal saline. Then the sample can be collected from infected area with the help of a sterileswab.

For collection of sample from internal organs: After carefully dissecting the shrimp aseptically, the sample can be collected from hepatopancreas with an inoculating loop or swab. Similarly, other organs can also becollected, homogenized in 0.1 % peptone water and inoculated into culture broth or solid culture plate. The particular species of bacteria can be identified by Gram‟sstaining, different biochemical tests or different molecular methods such as PCR, Real time PCR, hybridization with DNA probe, 16S rRNA gene sequencing,etc.

For molecular diagnosis by PCR

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. The collected samples may be transported in 95 % ethyl alcohol. If the samples are intended for detection of RNA viruses, then it can be preserved in any RNA preservation media (e.g. RNA later). For PCR analysis, the sample to be transported in frozen or chilled condition preferablyindryice.

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

Letters in bold indicates preferred tissues for PCR

Forhistopathologicalanalysis

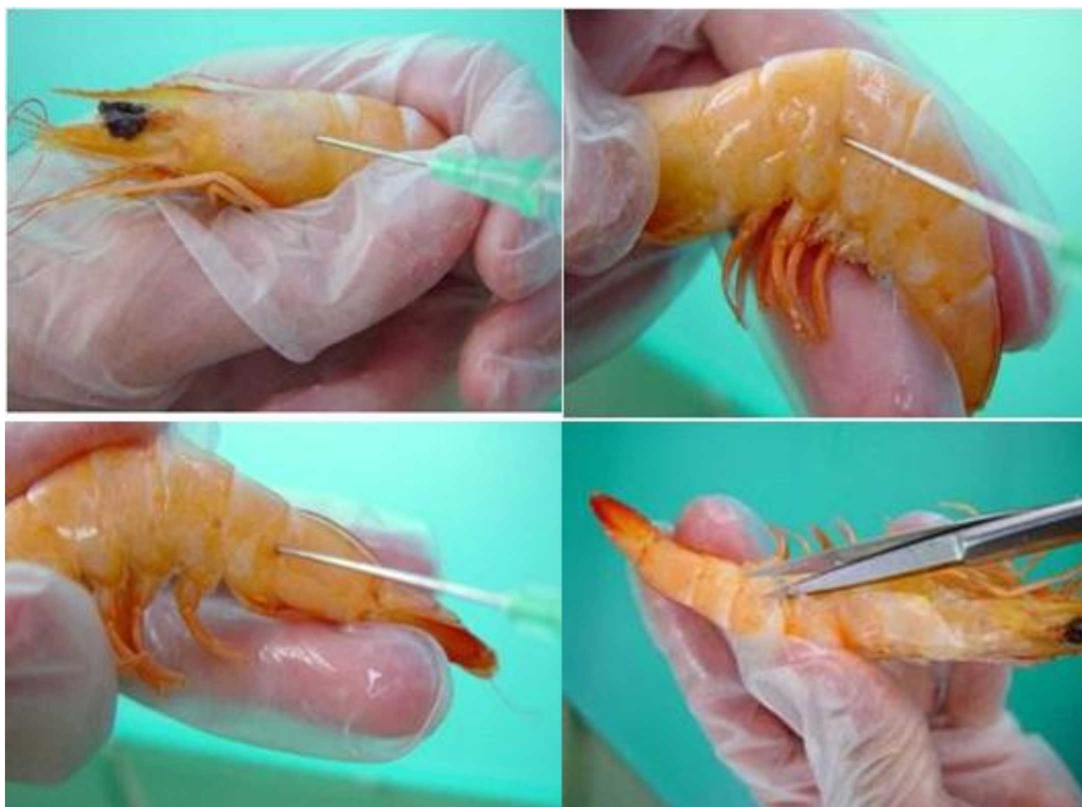
For histopathological analysis, the moribund shrimp should be selected. Theshrimp should have preferable the clinical symptom and is about to die. Davidson's fixative is the most suitable media for preservation of shrimp for histopathology.

Davidson'sfixative:
Formalin: 220ml
Ethanol (95 %): 330ml
Distilled water: 335ml
Glacial acetic acid: 115ml

The shrimp should be injected with Davidson‟s fixative at the rate of 10 % of the bodyweight.Iftheweightoftheshrimpis20g,then2mlofDavidson'sfixativeis required. At first, the fixative should be directly injected into hepatopancreas to ensure rapid fixation. Then the remaining portion of the fixative should be injected at different parts of body of theshrimp.

Then the cuticle of the shrimp should be silted along the midline to ensure penetration of fixative. Then the sample should be placed in a container with 10 volumes of fixative. If the size of shrimp is 20 g, then requirement of fixative will be 200 ml. Now, the sample in this condition is ready to be transported to laboratory.

When diagnosis has to be done for specific disease, the target sample will vary depending upon the disease suspected. The appropriate sample to be collected for specific disease has been summarized in Table1.



Fixing of shrimp with Davidson's

Table 1: Samples to be collected for different diseases

Disease	Causativeagent	Preferredsample
	Shrimpdiseases	
Early mortality syndrome	Specific strain of *Vibrio parahaemolyticus*	• For bacteriology: Live/ moribund shrimp • For histopathology: Shrimp fixed in Davidson's fixative.
Vibriosis inshrimp	Different species of Vibrio	• Haemolymph aspirated directly from heart on TCBS or ZMA media for isolation and identification of *Vibrio* colony • Gut and hepatopancreas for isolation ofbacteria • Finding of luminescence in case of luminescent *Vibrio*(e.g. *V.harveyi*)underdark

White spotdisease	White spot syndrome virus(*Whispovirus*)	• Gills and cuticular epithelium for microscopy and PCRanalysis • Haemolymph: For demonstration of aggregates of WSSV virions
Infectious hypodermal and haematopoietic necrosis (IHHN) disease	Viral infection caused by *Brevidensovirus* (Parvoviridaefamily)	Forhistopathology: Ectodermal and mesodermal tissues including gill, cuticular epithelium, connective tissue, haematopoietictissue, lymphoid organs, antennal gland,etc.
Black gilldisease	Bacteria (*Flavobacterium*spp., *Cytophaga* spp.) and parasite(e.g. *Zoothamnium*spp.)	Gills for observing parasites directly under the microscope and also for isolation of specific bacteria.
Hepatopancreatic parvovirus (HPV) infection	*Brevidensovirus* (Parvoviridaefamily)	Hepatopancreas of the affected shrimp
Yellow HeadDisease	Yellow head virus (*Okavirus* under Roniviridaefamily)	For histopathology: Cephalothorax tissueof moribund shrimp forhaematoxylin-eosinstaining. Light microscopy of tissues of ectodermal and mesodermal origin for finding of cytoplasmic inclusion bodies.
Taurasyndrome	Taura syndrome virus (*Aparavirus* under familyDicistroviridae)	Cuticular epithelium, appendages, gill, hindgut and subcuticular connectivetissue
Loose shellsyndrome	Unknownaetiology	For histopathology: Hepatopancreas, lymphoid organs andmuscle.

Proper sampling is one of the pre-requisites for disease diagnosis. The aquatic health experts should take care at each and every steps of processing of samples for correct diagnosis of every disease. Every aspects of the sampling such as sample size, objective of the surveillance programme, choice of proper fixatives (as in case of histopathology), choice of proper media (as in case of microbiological analysis), proper incubation temperature should be taken into consideration for sampling procedure of both finfish andshrimp.

CIBA

Bacteriological methods for disease investigation: Sampling, isolation and identification of bacteria

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 surfaceof the agar plate, steak and incubate
- *Enteric infections*: excise the hepatopancreas, midgut and foregut and touch the exposedinner 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.

Aseptic techniques

Maintenance of aseptic conditions during all steps of microbiology is the first step for successful microbiological investigation. For ensuring this, all glassware, plasticware, media, solutions etc. are sterilized before use. Personnel care should be taken to wash the hands with 70% ethanol before start of any culture work. Sterilization methods employed for different kinds of materials used in the laboratory are given in Table 1.

Table 1. Common sterilization methods

Sl. No.	Methods of sterilisation	Condition	Materials
1	Dry sterilization by hot air oven	160^0C for two hour or 180^0C for one hour	All types of glassware like pipettes, tubes, flasks, petridish etc. and lipid like paraffin oil
2	Autoclaving	121^0C, 15 lb pressure for 15 min	Bacteriological media, discarded media, plasticware, steel items, corks, rubber materials, filter pads, distilled water, buffers, solutions and also glassware
3	Filtration	0.22 µm or 0.45 µm pore size membrane filter	Tissue culture media, antibiotics, sera, heat sensitive solutions like carbohydrate, amino acids etc.

Autoclaving: This is used for the sterilisation of media. Usually, 15 minutes at 15 lb

CIBA

steampressure which gives the temperature of about 121°C is sufficient for sterilisation of media.

Precaution

1. Media containing sugar should not be sterilised for more than 15 minute since prolonged heating hydrolyse sugars.
2. Flask containing media should be placed with enough spacing in the autoclave. This will facilitate the steam circulation uniformly.
3. Cotton plugged flask, tubes should be covered with heavy paper or aluminium foil before autoclaving. This prevents cotton becoming saturated with moisture.
4. Before raising pressure in the autoclave, make sure that all the air in the autoclave has been displaced by steam. This can be done by keeping the valve open for 5 minutes after the autoclave starts steaming.
5. At the close of sterilisation do not release the pressure suddenly. This results in the plugs being blown out or getting soaked by the boiling media.

Preparation of culture media

Media refers to any substance that will enable the microorganisms to grow and multiply. It may be liquid media (broth) or solid medium. Both, liquid or solid media must be sterile before use.

Procedure

- Take known volume of distilled water (1 liter) in an Erlenmeyer flask. Add the required amount of dehydrated agar and stir with a glass rod to prevent lumping. The quantity required/ liter is always written over the media bottle.
- Set the flask on an electric hot plate to completely dissolve the media. Then, close it with a cotton plug.
- Autoclave at 15 lb pressure for 15 minutes.
- After taking out from autoclave, allow the media to cool to about 50°C (the agar should be warm and melted, but not too hot to handle in its flask). Then after quickly pour the melted, sterile agar into a series of petri dishes to about one-third capacity. Replace each petri dish top as the plate is poured. When the plates are cool (agar solidified), invert them to prevent condensing moisture from accumulating on the agar surfaces.
- Place inverted agar plates and tubes of sterilized nutrient broth at 37°C in incubator for at least 24 hours to ensure they are sterile.

Isolation of pure culture of bacteria

Isolation of pure culture of bacteria is necessary to characterize it further by cultural, biochemical, molecular methods. A number of methods are used for this purpose. These are:

1. Streak plate technique (streaking onto solid media)
2. Pour plate technique (Incorporation into molten semi-solid media)
3. Spread plate technique

Streak plate method

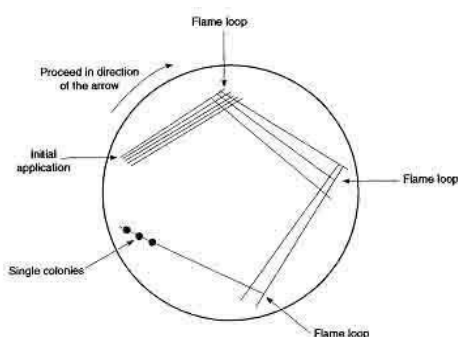
This is the most commonly employed technique for obtaining pure culture of specific bacterium from mixed bacterial populations.

Procedure

1. Inoculate the infected larvae/ affected tissues/ haemolymph/water sample on the culture plates with the help of

sterile bacteriological loop and streak the inoculum to get isolated colonies.

2. Incubate the inoculated agar plates at optimal temperature (28°C - 30°C) for 24-48 h and observe for development of bacterial colonies.
3. Examine cultural characteristics of the bacterial colonies as given in the subsequent sections and record.
4. Obtain pure culture of bacteria by picking up morphologically distinct colonies with the help of a sterile bacteriological loop and subculture on ZMA for further characterization.



Pour plate technique

The pour plate method requires only small volume of samples like 1 ml, 0.1 ml, and 0.01 ml or 0.001 ml of sample.. These dilutions are prepared by pipetting 1 ml of undiluted sample into 9 ml of buffered dilution water. The TSA medium is preferable for counting total heterotrophic bacterial population and 15-18 ml of the medium is poured to each dilution and mixed well and allowed to set with inoculums. After solidification, the plates are incubated upside-down at 35° C for 48 hours.

Spread Plate technique

The sterile TSA plates are inoculated and spread uniformly with 100µl of 10^{-1} to 10^{-7} dilutions of samples (depending upon the type of sample and amount of bacteria present) in duplicate and the plates are incubated upside-down at 35° C for 48 hours. Between spreading operations of each plate, the glass rod used for spreading is sterilized by dipping in alcohol and flaming. After 48 hours of incubation, the colonies developing in the each plate are counted using Quebec colony counter. The sample volumes and dilutions are selected to get the total number of colonies on a plate between 30 and 300. The number of samples to be plated at any one time is limited so that not more than 20 minutes (preferable 10 minutes) elapse between the dilution of the first sample and the pouring of the last plate. The sample container is mixed thoroughly before performing dilutions (approximately 25 times) and sterile pipette is used with sterile microtips for each sample. TPC i.e. Colony-Forming Units (CFU)/g of sample is calculated as follows.

CFU/g sample = Average count x Dilution factor.

In the case of colony counts from spreaders, the average count has to be doubled before calculation of TPC. The spread plate technique is commonly used for TVC with the same procedure except using TCBS agar.

Identification of bacteria

Bacterial cells are very small in size, usually in the range of 0.5µ to 20µ (1 µ = 10^{-3} mm = 10^{-6} m). Limit of vision of human eye is just 100 µ, so they are not visible to naked eye. Hence a microscope is used for observing bacterial cell. But bacterial cells are semi-transparent, so, suitable dyes are required to stain them to obtain better contrast. Staining and microscopy represents the first stage in microbial identification and provides the crucial information with regards to shape, size, capsule, spore etc.

Staining

Dyes used for staining purpose of living organisms are called stain. There are many stains available, and depending on our requirement, suitable ones are selected. Based upon staining principal, staining techniques are broadly classified into two categories. These are:

1. Simple staining
2. Differential staining

Simple staining: Staining of bacterial cells by the use of a single staining solution is calledsimple staining. Ex: Methylene blue staining, Carbol fuchsin staining, Crystal violet staining. Simple staining is done by flooding the staining solution on fixed bacterial smear over a slide. It reveals the shape and size of bacteria and distinguishes bacterial cells from non living material.

Differential staining: It involves staining of bacterial cells by more than one stainingsolution which may be applied separately or mixed and applied in single step. It is used to differentiate between bacterial cells or parts of bacterial cells. Ex: Grams staining, Acid-fast staining, spore staining, capsular staining.

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.

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

- Prepare serial ten fold dilutions of the inoculum (10^{-1} to 10^{-7})

- Place 0.1 ml of the inoculum on the agar surface and the evenly spread the entire surface using a sterile bent glass rod.

- For each dilution one plate is inoculated as described above.

- All the inoculated plates are incubated at 36°C for 24-48 hours.

- After incubation the plates are observed for bacterial growth and colonies are counted. In the higher dilutions discrete colonies can be observed.

CIBA

- 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.

Culture and General Tests

- Check plates at 12 to 18 hours for luminescent colonies as luminescence tends to fade within 24 hours after incubation
- 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:
- Rapid Identification Test Kits – Biolog, API NFT strips
- 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).
- Antibiotic sensitivity of the bacteria.

Application of molecular diagnostics in disease management

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.

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.

Extraction and quantification of nucleic acids

Isolation of the genomic DNA and RNA from shrimp tissues is prerequisite for diagnosis of pathogenic virus and bacteria of shrimp using PCR. Hence expertise on isolation and quantification of the nucleic acids from shrimp tissue is basic requirement for PCR .

Equipments and Materals

Refrigerated centrifuge, Deep freezer, Ice flaker, Shaking water bath, Sphectrophotometer, Vortex mixer, Autoclaved Milli Q water, Adjustable mocropipettes, Micro-centrifuge tubes,Homogenizer, Disposable gloves

Sample material: Specific tissue from shrimp or bacterial colony for DNA isolation.

DNA extraction

Principle

Good quality DNA is an essential requirement for molecular biology work. DNA extraction protocols comprise of the basic steps of disruption of the cell and nuclear membrane to release the DNA into solution followed by precipitation of DNA while ensuring removal of other biomolecules such as the proteins, polysaccharides, lipids, phenols and other secondary metabolites. In addition to conventional DNA extraction protocols, many others modifications protocols to suit a particular tissue type or downscaling applications are available. In addition to these basic protocols, DNA isolation kits based on either anion exchange chromatography or silica gel membranes are available commercially. While using any kit, the instruction given by the manufacturer should be followed step wise to extract DNA.

1. Extraction of nucleic acid

a. DNA

Method I : DNA extraction by lysis method

Lysis buffer

10mM Tris Hcl pH 8.0 0.15g

25mM EDTA 0.93g

0.5% SDS 0.5g

100mM NaCl 0.58g

Make up to 100ml with dd water

Procedure

- ✓ Lysis: Add 0.2ml The lysis buffer to the tissue and homogenize. Make up to 0.5ml with lysis buffer. Add 5 µl Proteinase K to the homogenate. Boil for 10 min in water bath.
- ✓ Centrifuge at 12,000 g for 10 min at 4^0C
- ✓ Take out 300 µl of supernatant in to fresh tube.
- ✓ Add 600 µl Phenol – Chloroform (1:2)
- ✓ Centrifuge at 12,000 g for 10 min at 4^0C.
- ✓ Take out supernatant in to fresh tube
- ✓ Add equal volume of isopropanol
- ✓ Centrifuge at 10,000 g for 10 min at 4^0C.
- ✓ Wash the pellet with 70% ethanol at 8,000 g for 5 min at 4^0C.
- ✓ Discard supernatant and air dry the pellet

Method II : DNA extraction by CTAB method

Chemicals required:

a) NaCl b) Hexadecyltrimethyl-Ammonium Bromide (CTAB) c) Tris HCl

d) Na2- EDTA e) Proteinase K f) RNase A g) β-mercaptoethanol h) Phenol i) Chloroform

j) Iso-amyl alcohol k) Iso-propyl alcohol l) Sodium acetate

Preparation of Buffers:

CTAB buffer:100mM Tris-HCl, pH 8.01.4M NaCl 20mM EDTA2% w/v hexadecyl-trimethyl-ammonium bromide (CTAB) (1.4 M NaCl, 2% (w/v) CTAB, 0.1 M Tris-Base, 0.015 M EDTA)
This buffer stabilizes nucleic acids and aids in the separation of organic molecules.

1x TE buffer: (10 mM Tris, pH 8.3, 1 mM EDTA)

For 100 ml 1x TE buffer combine 1 ml 1 M Tris (pH 8.3) and 200 µl 0.5 M EDTA (pH 8.0) and adjust the volume to 100 ml with de-ionized water. Autoclave and store at room temperature.

Phenol:Chloroform:Iso-amyl alcohol (24:24:1):

For 100 ml phenol:chloroform:iso-amyl alcohol (24:24:1) combine 49 ml phenol, 49 ml chloroform and 2ml iso-amyl alcohol. Mix properly and store at room temperature under the fume hood.

Method:

1. DNA was extracted from 20-50 mg of sample material. 500 µl extraction buffer is added to 1.5 ml micro-centrifuge tube containing the sample DNA. To that add 2µl of proteinase-K to dissolve the protein constituent and 2 µl RNase-A to remove RNA from the DNA extract and add 25 µl of β-mercaptoethanol to the mixture. This Keep it in the water bath for 1 hr at 60°C.

2. After 1 hr incubation add 500 µl of phenol:chloroform:iso-amyl alcohol (24:24:1) to the mixture in micro-centrifuge tubes to precipitate the cell debris and protein present in the crude extract. The micro-centrifuge tubes should be mixed gently by inverting for 5 min and kept for centrifugation at 10,000 rpm for 10 min at 4°C temperature .

3. After centrifugation, pipette out 250 µl of the upper phase and transfer to new micro-centrifuge tube. To this, add25 µl of 3M sodium acetate and 250 µl of iso-propyl alcohol. The mixture gently swirl. In this step, the DNA get precipitated and appears like a white coil in the solution, and keep it at -20°C for 1 hr.

4. Then centrifuge the tubes at 10,000 rpm for 10 min at 4°C. Discard the supernatant. A pure DNA appears as translucent pellet on the wall of the tube. The tubes were kept upside down on tissue paper to air dry samples at RT overnight.

5. Warm TE buffer at 50°C and add 200 µl to the DNA pellet and incubated for 10 min at same temperature. Solubilize DNA by tapping gently and stored at -20°C until further use. DNA in TE should be suitably diluted before use in PCR reactions. DNA can also be resuspended in H2O

Quantification of DNA

The concentration of DNA and RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of DNA purity with respect to contaminants that absorb UV light, such as protein. The A_{260}/A_{280} ratio is influenced considerably by pH. For accuracy, pure DNA has an A_{260}/A_{280} ratio of 1.8–2.0 alkaline buffer (e.g., 10 mM Tris·Cl, pH 8.5)

For quantification of DNA, take 10µl of DNA and dilute in 990µl of TE buffer (1:100 dilutions). Take the O.D at 260nm and the concentration of DNA to be calculated as follows.

1 O.D of double stranded DNA at 260nm = 50µg/ml.

Therefore DNA concentration (µg/µl) = *OD X Dilution factor X* 50

1000

Storage of DNA

Purified DNA should be stored at –20°C or –70°C under slightly basic conditions (e.g., Tris® Cl, pH 8.0 or TE buffer) because acidic conditions can cause hydrolysis of DNA. Avoid repeated freeze-thawing as this will lead to precipitates. Diluted solutions of nucleic acids (e.g., dilution series used as standards) should be stored in aliquots and thawed once only.

Isolation of RNA

Principle:

RNA (Ribonucleic acid) is a polymeric substance present in living cells and many viruses. RNA is used in all the steps of protein synthesis in all living cells and carries the genetic information for many viruses. The isolation of RNA with high quality is a crucial step required to perform various molecular biology experiment.

TRIzol Reagent is a ready-to-use reagent used for RNA isolation from cells and tissues. TRIzol works by maintaining RNA integrity during tissue homogenization, while at the same time disrupting and breaking down cells and cell components. Addition of chloroform, after the centrifugation, separates the solution into aqueous and organic phases. RNA remains only in the aqueous phase. After transferring the aqueous phase, RNA can be recovered by precipitation with isopropyl alcohol. Total RNA extracted by TRIzol Reagent is free from the contamination of protein and DNA. This RNA can be used in Northern blot analysis, in vitro translation, poly (A) selection, RNase protection assay, and molecular cloning.

Stabilization of RNA in biological samples

Traditionally, samples harvested for RNA analysis are immediately frozen in liquid nitrogen and stored at –80°C until processed. Stabilization reagents, available from commercial suppliers, can alternatively be used to stabilize RNA in biological samples.

Preventing measures of RNase contamination:

RNases can be introduced into the RNA preparation during the isolation procedure. To reduce this, Always wear disposable gloves. Use sterile, disposable plastic ware and automatic pipettes reserved for RNA work to prevent cross-contamination.

Isolation of RNA using TRIZOL method

Reagents

- TRIZOL® Reagent
- Chloroform
- Isopropyl alcohol
- 75% Ethanol (in DEPC-treated water)
- RNase-free water or 0.5% SDS solution

DEPC inactivates the RNases by the covalent modifications of the histidine residues. To prepare RNase-free water, draw water into

RNase-free glass bottles. Add diethylpyrocarbonate (DEPC) to 0.01% (v/v). Let stand overnight and autoclave.

Procedure

1. *Tissues* Homogenization

Homogenize per 50-100 mg of tissue samples in 1 ml of TRIZOL® Reagent tissue using a homogenizer. Following homogenization, remove insoluble material from the homogenate by centrifugation at 12,000 × *g* for 10 minutes at 4°C. Transfer the clear homogenate solution to a fresh tube and proceed with chloroform addition and phase separation.

2. Phase Separation

Incubate the homogenized samples for 5 minutes at room temperature (15 to 30°C) to permit the complete dissociation of nucleoprotein complexes.

Add 0.2 ml of chloroform per 1 ml of TRIZOL® Reagent. Shake tubes vigorously by hand for 15 seconds and incubate them at for 2 to 3 minutes.

Centrifuge the samples at 12,000 × *g* for 15 minutes at 4°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.

3. RNA Precipitation

Transfer the aqueous phase to a fresh tube. Precipitate the RNA from the aqueous phase by mixing with 0.5 ml of isopropyl alcohol. Incubate samples at 15 to 30°C for 10 minutes. Centrifuge at 12,000 × *g* for 10 minutes at 4°C. The RNA precipitate, often invisible before centrifugation, forms pellet on the side and bottom of the tube.

4. RNA Wash

Remove the supernatant. Wash the RNA pellet once with 1 ml of 75% ethanol. Mix the sample by vortexing and centrifuge at 7,500 × *g* for 5 minutes at 4°C.

5. Re-dissolving the RNA

Air dry the RNA pellet. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Dissolve RNA in RNase-free water and incubating for 10 minutes at 55 to 60°C. RNA can also be redissolved in 100% formamide (deionized) and stored at -70°C. The RNA precipitate can be stored in 75% ethanol at 2 to 8°C for at least one week, or at least one year at –5 to -20°C.

Determining RNA quality

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. For accurate ratios, it is recommend measuring absorbance in a low-salt buffer with slightly alkaline pH (e.g., 10 mM Tris·Cl, pH 7.5). Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1.

Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. To ensure significance, readings should be between 0.15 and 1.0. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per

ml (A_{260} = 1 → 44 μg/ml; based on a standard 1 cm path length).

Example of RNA quantitation
Volume of RNA sample = 100 μl
Dilution = 10 μl RNA sample + 490 μl of 10 mM Tris·Cl, pH 7.0 (1/50 dilution)
Measure absorbance of diluted sample in a 1 ml cuvette (RNase-free)
A_{260} = 0.2

RNA concentration
= 44 μg/ml x A_{260} x dilution factor
= 44 μg/ml x 0.2 x 50
= 440 μg/ml

Total amount of RNA
= concentration x volume of sample in ml
= 440 μg/ml x 0.1 ml
= 44 μg RNA

Principles and practice of polymerase chain reaction

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. The basic principles of replicating a piece of DNA using primers were described by H. G. Khorana in 1971 but the progress was limited by primer synthesis and polymerase purification issues. The DNA was amplified by Kary Mullis during 1983 who was awarded Nobel Prize in Chemistry in 1993 for his contribution in PCR**.** Several molecular methods, particularly PCR has become inevitable tool and being widely used for shrimp disease diagnosis. PCR derives its name from one of its key components, a DNA polymerase used to amplify a piece of DNA by *in vitro* enzymatic replication. As PCR progresses, the DNA thus generated is itself used as template for replication which is further exponentially amplified. 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).

Components and reagents

1. Buffer solution

It provides a suitable chemical environment for optimum activity and stability of the DNA polymerase.

2. Mg^{2+} concentration

The Mg^{2+} ion forms complex with dNTPs, primers and DNA template. It plays major role in the yield of PCR product. So the optimal concentration of $MgCl_2$ has to be selected for each reaction. Too few Mg^{2+} ions result in a low yield of PCR product, and too many increase the yield of non-specific products, and promote misincorporation and multiple bands to appear. Lower Mg^{2+} concentrations are desirable when fidelity of DNA synthesis is critical. The concentration of $MgCl_2$ should be selected empirically, starting from 1 mM and increasing in 0.1 mM steps, until a sufficient yield of PCR product is obtained. If the DNA samples contain EDTA or other chelators, the $MgCl_2$ concentration in the reaction mixture should be raised proportionally.

3. dNTPs

The concentration of each dNTP in the reaction mixture is usually 200 μM. Inaccuracy in the concentration of even a single dNTP dramatically increases the misincorporation level. So it is very important to have equal concentrations of each dNTP (dATP, dCTP, dGTP, dTTP). When maximum fidelity of the PCR process is crucial, the final dNTP concentration should be 10-50 μM.

4. Primers

PCR primers are usually 15-30 nucleotides in length. Longer primers provide higher specificity. The CG content in primers should be 40 – 60% and distributed uniformly throughout of the primer. To avoid nonspecific priming, more than three G or C nucleotides at the 3'-end of the primer should be avoided. The primers should have neither self-complementarity nor inter-complementarity in order to avoid

CIBA

primer-dimer and hairpin formation. The melting temperature of flanking primers should not differ by more than 5°C, so the GC content and length must be chosen accordingly. The annealing temperature should be approximately 5°C lower than the melting temperature of primers.

5. Distilled Water

Autoclaved distilled water is used to make up the desired volume for the reaction.

6. DNA template

It contains the DNA region (target) in question to be amplified.

7. DNA Polymerase

In 1957, Arthur Kornberg identified the first DNA polymerase and was awarded the Nobel Prize in 1959 for the same. In 1969, Thomas Brock reported the isolation of a new species of thermophilic bacterium, *Thermus aquaticus* from which Taq DNA polymerase was isolated in 1976. The enzyme, Taq DNA polymerase is world widely used in PCR reaction. It should be stored at -20°C in a non-frost free freezer, typically in 50% glycerol. The tubes should never be allowed to reach room temperature and gloves should be worn when handling to avoid contamination. Before opening a new tube of enzyme, it is spun briefly as there is often enzyme in the cap. When pipetting enzyme from a stock tube, the end of the tip is just plunged far enough into the enzyme to get what is needed to avoid excessive adherence of enzyme to the peripheral tips. Enzyme should never be added to unbuffered water to avoid its denaturation. Usually 0.5-1.0 U of *Taq* DNA polymerase is used in 50 µl of reaction mix. Higher *Taq* DNA polymerase concentrations may cause synthesis of nonspecific products.

The Cycling Reaction

There are three major steps in a PCR, which are repeated for 30 or 40 cycles depending on the product size. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

1. Initial Denaturation

The initial denaturation should be performed over an interval of 1-3 min at 95°C. This interval may be extended up to 10 min for GC-rich templates. If the initial denaturation is no longer than 3 min at 95°C, *Taq* DNA polymerase can be added into the initial reaction mixture. If longer initial denaturation or a higher temperature is necessary, *Taq* DNA polymerase should be added only after the initial denaturation, as the stability of the enzyme dramatically decreases at temperatures over 95°C.

2. Denaturation

During the denaturation, the double strand melts open to single stranded DNA which is important in PCR reaction. Incomplete denaturation of DNA results in the inefficient utilization of template in the first amplification cycle leads to a poor yield of PCR product. Usually denaturation for 0.5-2 min. at 94-95°C is sufficient. If the amplified DNA has a very high GC content, denaturation time may be increased up to 3-4 minutes. Alternatively, additives such as glycerol (up to 10-15 vol.%), DMSO (up to 10%) or formamide (up to 5%) may be used to facilitate DNA denaturation. In the presence of such additives, the annealing temperature should be adjusted experimentally, since the melting temperature of the primer-template DNA duplex decreases significantly when these additives are used. The amount of enzyme in the reaction mix should be increased since DMSO and formamide, at the suggested concentrations, inhibit *Taq* DNA polymerase by approximately 50%.

CIBA

3. Annealing

Usually the optimal annealing temperature is 5°C lower than the melting temperature of primer-template DNA duplex. Incubation for 0.5-2 min is usually sufficient. The annealing temperature should be optimized by increasing it stepwise by 1-2°C to avoid nonspecific PCR products. The primers are jiggling around, caused by the Brownian motion and ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer which does not break anymore.

4. Extension

Usually the extending step is performed at 70-75°C. The rate of DNA synthesis by *Taq* DNA polymerase is highest at this temperature. Recommended extending time is 1 min.for the synthesis of PCR fragments up to 2 kb and may be further increased by 1 min. for each 1000 bp. Primers that are on positions with no exact match don't give an extension of the fragment. The polymerase adds complementary dNTP's to the template from 5' to 3', reading the template from 3' to 5'. Because both strands are copied during PCR, there is an exponential increase of the number of copies of the gene. But, polymerization is not strictly doubling the DNA at each cycle in the early phase. The number of PCR cycles depends on the amount of template DNA in the reaction mix and on the expected yield of the PCR product. Usually 25-35 cycles are sufficient.

6. into a plasmid or the genetic material of another organism.

5. Final Extension

After the last cycle, the samples are usually incubated at 72°C for 5-15 min to fill-in the protruding ends of newly synthesized PCR products. Also, during this step, the terminal transferase activity of *Taq* DNA polymerase adds extra A nucleotides to the 3'-ends of PCR products.

The PCR can be standardized and optimized either by changing the denaturing time, annealing time and temperature, Mg^{2+} ion concentration, extension time and temperature, and or the individual quantity ingredients in reaction mixture. The final product is visualized in transillumination to know the amplification of the product of the interest.

Application of PCR

1. PCR is used in diagnostic application in pathology for the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific sequence.

2. Different PCR-based methods are used in genetic fingerprinting to identify the extremely small amounts of target of interest.

3. Used to identify genetic and evolutionary relationships between living organisms.

4. Used to identify beneficial and pathogenic microbiota at strain level by techniques such as ribotyping, randomly amplified polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE) of rare-cutting restriction fragments, and amplified fragment length polymorphism (AFLP).

5. Recombinant DNA technologies involving the insertion of a DNA sequence

7. PCR may also be used in the analysis of ancient DNA that is thousands of years old.

CIBA

8. Larger quantities of DNA are required for Southern or northern hybridization and DNA cloning. PCR augments these techniques with high amounts of pure DNA, enabling analysis of DNA samples even from very small amounts of starting material.

9. DNA sequencing.

PCR diagnosis of some important OIE listed viruses and bacterial pathogens of shrimp

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.Polymerase Chain Reaction (PCR) is very often used in molecular biology for selectively amplifying a particular segment of NA.

Equipments and Materials

PCR machine, Electrophoresis apparatus.Adjustable micropipettes, Micro-centrifuge tubes, UV transillumunator, Vortex mixer, spinner, Adhesive tape.Disposable gloves.

Methods

All the chemicals and reagents should be of molecular biology grade.Sterilized condition should be well maintained in all operations as a preliminary requisite.

PCR reaction set up

All the necessary reagents and enzymes are to be 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

Sl. No.	Pathogen	Primer Sequence 5' to 3'	Amplicon size First step	Nested
1	WSSV , Kimura et al (1996)	F: ATCATGGCTGCTTCACAGAC R: GGCTGGAGAGGACAAGACAT Fn: TCTTCATCAGATGCTACTGC Rn: TAACGCTATCCAGTATCACG	982 bp	570 bp
2	IHHNV , OIE, 2014	F:TCCAACACTTAGTCAAAACCAA R: TGTCTGCTACGATGATTATCCA	309 bp	-
3	MBV, OIE, 2014	F:CGATTCCATATCGGCCGAATA R:TTGGCATGCACTCCCTGAGAT F:TCCAATCGCGTCTGCGATACT R:CGCTAATGGGGCACAAGTCTC	533 bp	361 bp
4	HPV, OIE, 2014	F:GCATTACAAGAGCCAAGCAG R:ACACTCAGCCTCTACCTTGT	441 bp	-
5	IMNV, OIE, 2014	F:CGACGCTGCTAACCATACAA R:ACTCGGCTGTTCGATCAAGT F:GGCACATGCTCAGAGACA R:AGCGCTGAGTCCAGTCTTG	328 bp	139 bp
6	YHV, OIE, 2014	F:CCGCTAATTTCAAAAACTACG R:AAGGTGTTATGTCGAGGAAGT	135 bp	-
7	TSV, OIE, 2014	F:AAGTAGACAGCCGCGCTT R:TCAATGAGAGCTTGGTCC	231 bp	-
8	NHPB, OIE, 2014	F:CGTTGGAGGTTCGTCCTTCAGT R:GCCATGAGGACCTGACATCATC	379 bp	-
9	AHPND Sirikharin et. al, (2014)	F:ATGAGTAACAATATAAAACATGAAAC R:GTGGTAATAGATTGTACAGAA	336 bp	-
10	EHP Tangprasittipap et al. (2013)	F:CAGCAGGCGCGAAAATTGTCCA R:AAGAGATATTGTATTGCGCTTGCTG F:CAACGCGGGAAAACTTACCA R:ACCTGTTATTGCCTTCTCCCTCC	779 bp	176 bp

F: Forward primer R: Reverse primer Fn: Nested Forward primer Rn: Nested Reverse primer

Thermocycling

The tubes are to be arranged in the thermal cycler. Care should be taken to close it properly to avoid evaporation. Cyclic conditions for the amplification of some important pathogens of shrimp are listed in Table given below:

Sl. No.	Pathogen	PCR protocol
1	WSSV , Kimura et al (1996)	Cycle 1: 94ºC for 5 min Cycle 2: 94ºC for 40sec 58ºC for 40sec 72ºC for 90sec Go to cycle 2 repeat 30 cycles Cycle 3 72ºC for 7 min
2	IHHNV , OIE, 2014	Cycle 1: 95ºC for 5 min Cycle 2: 95ºC for 30sec 55ºC for 30sec 72ºC for 1 min Go to cycle 2 repeat 35 cycles

CIBA

		Cycle 3: 72ºC for 7 min
3	MBV, OIE, 2014	Cycle 1: 96ºC for 5 min Cycle 2: 94ºC for 30sec 65ºC for 30sec 72ºC for 1 min Go to cycle 2 repeat 40 cycles Cycle 3 72ºC for 7 min
4	HPV, OIE, 2014	Cycle 1: 95ºC for 5 min Cycle 2: 95ºC for 30sec 60ºC for 30sec 72ºC for 60sec Go to cycle 2 repeat 40 cycles Cycle 3: 72ºC for 7 min
5	IMNV, OIE, 2014	Cycle 1: 60ºC for 30 min 95ºC for 2 min Cycle 2 95ºC 45 sec 60ºC 45 sec Go to cycle 2 repeat 39 cycles Cycle 3: 60ºC for 7 min
6	YHV, OIE, 2014	Cycle 1: 94ºC for 2 min Cycle 2: 94ºC for 30sec 58ºC for 30 sec 72ºC for 30sec Go to cycle 2 repeat 40cycles Cycle 3: 72ºC for 10min
7	TSV, OIE, 2014	Cycle 1: 94ºC for 2 min Cycle 2: 94ºC for 45 sec 60ºC for 45sec 72ºC for 45sec Go to cycle 2 repeat 40cycles Cycle 3:72ºC for 7 min
8	NHPB, OIE, 2014	Cycle 1: 95ºC for 2min Cycle 2: 94ºC for 30sec 60ºC for 30sec 72ºC for 30sec Go to cycle 2 repeat 25cycle Cycle 3:72ºC for 7min
9	AHPND Sirikharin et. al, (2014)	Cycle 1: 94ºc for 5min Cycle 2: 94ºC for 30sec 53ºC for 30sec 72ºC for 40sec Go to cycle 2 repeat 35 cycles Cycle 3:72ºC for 5 min

10	EHP Tangprasittipap et al. (2013)	Cycle 1: 94ºC for 5min Cycle 2: 94ºC for 20sec 58ºC for 20sec 72ºC for 45sec Go to cycle 2 repeat 35 cycles Cycle 3:72ºC for 5min Nested Cycle 1: 94ºC for 5min Cycle 2: 94ºC for 20sec 64ºC for 20sec 72ºC for 45sec Go to cycle 2 repeat 35cycles Cycle 3:72ºC for 5min

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 5 to 10 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 at voltage of 80 - 120. Continue the electrophoresis until the dye migrates to the appropriate distance in the gel.

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.

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.

Trouble shootings in PCR

Observation	Possible Cause	Solution
SEQUENCE ERRORS	Low fidelity polymerase	• Choose a higher fidelity polymerase
	Suboptimal reaction conditions	• Reduce number of cycles • Decrease extension time • Decrease Mg^{++} concentration in the reaction
	Unbalanced nucleotide concentrations	• Prepare fresh deoxynucleotide mixes
	Template DNA has been damaged	• Start with a fresh template • Try repairing good DNA template • Repair Mix • Limit UV exposure time when analyzing or excising PCR product from the gel
	Desired sequence may be toxic to host	• Clone into a non-expression vector • Use a low-copy number cloning vector
INCORRECT PRODUCT SIZE	Incorrect annealing temperature	• Recalculate primer Tm values using the
	Mispriming	• Verify that primers have no additional complementary regions within the template DNA
	Improper Mg^{++} concentration	• Adjust Mg^{++} concentration in 0.2–1 mM increments
	Nuclease contamination	• Repeat reactions using fresh solutions
NO PRODUCT	Incorrect annealing temperature	• Recalculate primer Tm values • Test an annealing temperature gradient, starting at 5°C below the lower Tm of the primer pair
	Poor primer design	• Check specific product literature for recommended primer design • Verify that primers are non-complementary, both internally and to each other • Increase length of primer
	Poor primer specificity	• Verify that oligos are complementary to proper target sequence
	Insufficient primer	• Primer concentration can range from 0.05–1

CIBA

Observation	Possible Cause	Solution
	concentration	µM in the reaction. Please see specific product literature for ideal conditions
	Missing reaction component	• Repeat reaction setup
	Suboptimal reaction conditions	• Optimize Mg^{++} concentration by testing 0.2–1 mM increments • Thoroughly mix Mg^{++} solution and buffer prior to adding to the reaction • Optimize annealing temperature by testing an annealing temperature gradient, starting at 5°C below the lower Tm of the primer pair
	Poor template quality	• Analyze DNA via gel electrophoresis before and after incubation with Mg^{++} • Check 260/280 ratio of DNA template
	Presence of inhibitor in reaction	• Further purify starting template by alcohol precipitation, drop dialysis or commercial clean up kit • Decrease sample volume
	Insufficient number of cycles	• Rerun the reaction with more cycles
	Incorrect thermocycler programming	• Check program, verify times and temperatures
	Inconsistent block temperature	• Test calibration of heating block
	Contamination of reaction tubes or solutions	• Autoclave empty reaction tubes prior to use to eliminate biological inhibitors • Prepare fresh solutions or use new reagents and new tubes
	Complex template	• Use High-Fidelity DNA Polymerases • . Include the appropriate GC enhancer. • For longer templates, use specific high fidelity DNA polymerase
MULTIPLE OR NON-SPECIFIC PRODUCTS	Premature replication	• Use a hot start polymerase • Set up reactions on ice using chilled components and add samples to thermocycler preheated to the denaturation temperature
	Primer annealing temperature too low	• Increase annealing temperature

CIBA

Observation	Possible Cause	Solution
	Incorrect Mg^{++} concentration	• Adjust Mg^{++} in 0.2–1 mM increments
	Poor primer design	• Check specific product literature for recommended primer design • Verify that primers are non-complementary, both internally and to each other • Increase length of primer • Avoid GC-rich 3´ ends
	Excess primer	• Primer concentration can range from 0.05–1 µM in the reaction. Please see specific product literature for ideal conditions.
	Contamination with exogenous DNA	• Use positive displacement pipettes or non-aerosol tips • Set-up dedicated work area and pipettor for reaction setup • Wear gloves during reaction setup
	Incorrect template concentration	• For low complexity templates (i.e. plasmid, lambda, BAC DNA), use 1 pg–10 ng of DNA per 50 µl reaction • For higher complexity templates (i.e. genomic DNA), use 1 ng–1 µg of DNA per 50 µl reaction

Histopathological techniques in shrimp disease diagnosis

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

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

The processed slides are ready for examination under microscope

CIBA

Collection, preservation and despatch of aquatic animal sample to a diagnostic laboratory / referral laboratory for disease diagnosis

Accurate disease diagnosis is an important aspect for health management. Since disease investigation facilities may not be available in many shrimp farming areas, it would be essential that the samples will have to be sent to diagnostic laboratories or the referral laboratories for diagnosis of the diseases. For investigation of disease in aquaculture, background information on the disease and appropriate samples are essential.

For investigating disease, moribund sample (infected animals those are about to die) are best samples for diagnosis. However, individuals from the same pond may also be collected fixed appropriately and provided to the diagnostic laboratory. Different ways of collection and despatch of samples to the diagnostic laboratory is given below.

Live shrimp: Aquatic animals in live / moribund state will be best samples for diagnosis. Sending correct number of samples is important. If samples are collected from a pond, it should represent the entire pond and therefore samples should be collected from 4 corners and from middle by cast net. The number of samples to be collected from a pond or hatchery tank depends on the stock (Table, Sample sizes needed to detect at least one infected host in a population of a given size, at a given prevalence of infection. Assumptions of 2% and 5% prevalences are most commonly used for surveillance of presumed exotic pathogens, with a 95% confidence limit). Affected and control animals should be packed separately in polypacks with oxygen, placed in a Styrofoam box and additionally some ice/gel pack should be placed to control temperature. All the samples should be labelled properly and then sent by courier. Such live moribund samples are ideal for collection of haemolymph samples and microbiological investigation.

Freshly dead: Freshly dead samples will be ok for PCR. Effort should be taken to send these as quickly as possible in ice. However, these samples will not be suitable for bacteriological and histopathological analysis.

Fixed samples: Samples for molecular biology and histopathological analysis can be fixed and sent for laboratory for analysis. Affected moribund samples and apparently healthy looking samples can be fixed by a variety of fixatives (Davidson's fixative is highly desired for shrimp samples) for histopathology. For molecular diagnostics, dissected tissues should be fixed in 95% ethyl alcohol for detection of DNA viruses and in RNA later for detection of RNA viruses. Shrimp larval samples can be fixed in these fixatives whereas bigger animals have to be fixed in a proper manner (described elsewhere in this manual). For histopathology and PCR analysis, individual organs can be dissected out and fixed in the fixative. Samples should be packed separately in tightly capped containers and have proper labelling. Leakage of fixative solution (which generally contain some alcohol or acid), may affect the labelling of the container when marked by a marker pen. Therefore, pencil marking is preferred. All the containers should be sealed, put in a box

and sent to laboratory in shortest possible time. Fixed samples are not suitable for bacteriological or other microbiological analysis.

Water sample: Water samples are generally preferred for microbiological analysis. Sample collected from a pond should be representative one. This should contain samples from 4 corners and from the centre of the pond. About 300-500ml sample should be sufficient for analysis. This should be collected in a plastic container with tightly packed, labelled properly and send on ice. The container should be sterile (commercially available). If any heat resistant containers are available, these can be boiled for 20-30 minutes, cool it and use for sample collection.

Some of the essential background information is required to be recorded provided to the diagnostic laboratory. History of the events prior to disease outbreak and information on the clinical signs (appearance, colour change etc.), behaviours (feeding pattern, swimming pattern, active / lethargy etc) will help arriving at diagnosis.

General Precautions:

1. Accurate fixatation protocol should be followed.
2. Samples should be tightly packed and sealed properly.
3. Accurate and detailed labelling of the samples is very important. Label the samples with pencil. Containers marked with permanent markers will erase due to the fixatives.
4. Avoid using glass containers as these may break during transport.
5. The sample should be accompanied with the proformae as required by the diagnostic laboratory.

Proforma for sample collection:

1. Name and address of the farm
2. Contact numbers and Email
3. Date of collection
4. Types of sample – Water/animal
5. Species used for culture practice (animal)
6. Culture history – Days of culture, area of pond, water source
7. History of disease occurrence

Composition of the fixatives

1. Davidson's fixative 1000ml
   37% formaldehyde (Formalin): 220 ml
   95% ethanol: 330 ml
   Distilled water (Tap water if distilled water not available): 335 ml
   Glaceal acetic acid: 115 ml
2. Neutral Buffered formalin 1000ml
   37% formaldehyde (Formalin): 100 ml
   Distilled water (Tap water if distilled water not available): 900 ml
   Sodium phosphate monobasic monohydrate: 4.0 g
   Sodium phosphate dibasic anhydrous: 6.5g
3. 95% Ethyl alcohol 1000ml
   Absolute alcohol: 950 ml
   Distilled water: 50 ml

Table, Sample sizes needed to detect at least one infected host in a population of a given size, at a given prevalence of infection.

Population size	Prevalence (%)					
	0.5	1.0	2.0	3.0	4.0	5.0	10.0
50	46	46	46	37	37	29	20
100	93	93	76	61	50	43	23
250	192	156	110	75	62	49	25
500	314	223	127	88	67	54	26
1000	448	256	136	92	69	55	27
2500	512	279	142	95	71	56	27
5000	562	288	145	96	71	57	27
10000	579	292	146	96	72	57	27
100000	594	296	147	97	72	57	27
1000000	596	297	147	97	72	57	27
>10000000	600	300	150	100	75	60	30

Central Institute of Brackishwater Aquaculture (CIBA) is a referral lab for shrimp disease diagnosis. Samples can be sent to **Director, Central Institute of Brackishwater Aquaculture, 75 Santhome High Road, R.A. Puram, Chennai 600 028**

.

CIBA

Aquatic Animal Health and Environment Division
Central Institute of Brackishwater Aquaculture
Indian Council of Agricultural Research
(Ministry of Agriculture)
#75, Santhome High Road, R.A. Puram, Chennai, Tamil Nadu. 600 028

Research on aquatic animal health was initiated at the Central Institute of Brackishwater Aquaculture since 1990. Since then it has considerably grown in terms of expertise, manpower and laboratories. Presently, the Aquatic Animal Health and Environment Division or the AAHED in short, has scientists of relevant disciplines, such as Microbiology, Pathology, Parasitology, Biotechnology and Molecular Diagnostics, Soil Chemistry, water chemistry, Environment and Aquaculture. The AAHED has well established laboratory facilities for carrying out hi-tech research in molecular biology in addition to aquatic animal health and environment management including diagnostics, prophylactics and health management in brackishwater aquaculture. The advanced facilities have been developed with funding support from ICAR, National Agricultural Research Project (NARP), World Bank, National Agricultural Technology Project (NATP), Department of Biotechnology and National Fisheries Development Board with dedicated efforts of scientists. A well designed wet lab is also in place for carrying out live aquatic animal experiments and evaluating Koch's and River's postulates.

The AAHED, CIBA has the mandate to carry out research on (a) economically impacting diseases of brackishwater culture species and develop technologies for rapid diagnosis, prophylaxis and control; (b) brackishwater environment and develop mitigatory measures as required; and (c) provide technical and policy support to the Government on matters pertaining to aquatic animal health and environment management to improve productivity.

The AAHED of CIBA was the first to commercialise a white spot syndrome diagnostic kit to a premier Biotechnology company in the year 2002. The AAHED also produced kit for diagnosis of white tail disease in scampi (2004). AAHED has the expertise and capacity to carry out proposed levels of Diagnostics of all the OIE listed Brackishwater pathogens.

The environmental section has the laboratory and expertise to look into all aspects of abiotic parameters. Novel methods have been developed for the bioremediation and environmental monitoring of the brackishwater rearing systems, including hatchery and farms. The unit also has expertise in climate related studies, and has developed climate smart solutions for brackishwater farming systems. Environment section has the expertise for the environmental impact assessment and monitoring studies, carrying capacity assessment of source waters for optimisation of Brackishwater aquaculture development.

AAHED, CIBA has published over 70 research publications in peer reviewed national and international journals, produced 12 Ph.Ds, who are currently employed in key positions in various Institutions in India and abroad.

Director: Dr. KK Vijayan
Microbiology & Virology: Dr SV Alavandi, Dr M. Poornima, Dr. P. K. Patil, Dr. Sanjoy Das, Dr. T. Bhuvaneshwari, Dr. N. Lalitha
Biotechnology,Molecular Diagnostics & Aquaculture: Dr. S.K. Otta;Dr. Satheesha Avunje
Parasitology & Pathology: Dr.K.P.Jithendran, Dr. Ezhil Praveena, Mr.T. Satheesh Kumar, Mrs. R. Vidya
Aquatic Environment (Soil & Water Chemistry): Dr. M. Muralidhar, Dr. R. Saraswathy, Mr. P. Kumararaja

Contact: Central Institute of Brackishwater Aquaculture, Indian Council of Agricultural Research (Ministry of Agriculture), #75, Santhome High Road, R.A. Puram, Chennai, Tamil Nadu. 600028
Email:director.ciba@icar.in; director@ciba.res.in