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Training Manual

Disease Management of Brackishwater Aquaculture Farming

ICAR - Central Institute of Brackishwater Aquaculture
Kakdwip Research Centre
Kakdwip, South 24 Parganas, West Bengal - 743347

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Training Manual on Disease Management of Brackishwater Aquaculture Farming

Editors:

Dr. Sanjoy Das

Dr. Leesa Priyadarsani

Dr. M. Poornima

Dr. K.P. Jithendran

KAKDWIP RESEARCH CENTRE
ICAR - Central Institute of Brackishwater Aquaculture
Kakdwip, South 24 Parganas, West Bengal - 743347
Tel.: 03210 - 255072, Fax: 03210 - 257030
E-mail: krckakdwip@gmail.com / krckakdwip@yahoo.co.in

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Dr. K.P. Jithendran

Director, ICAR-CIBA, Chennai

Facilitation

Dr. M. Poornima

Principal Scientist, Aquatic Animal Health & Environment Division
ICAR- Central Institute of Brackishwater Aquaculture
Chennai- 600028, India

Conveners:

Dr. Sanjoy Das, *Principal Scientist, KRC of ICAR-CIBA, Kakdwip, India*

Dr. Leesa Priyadarsani, *Scientist, KRC of ICAR-CIBA, Kakdwip, India*

Co-Conveners:

Dr. Debasis De, *Principal Scientist and OIC, KRC of ICAR-CIBA, Kakdwip, India*

Dr. T.K. Ghoshal, Principal Scientist, *KRC of ICAR-CIBA, Kakdwip, India*

Dr. Prem Kumar, *Senior Scientist, KRC of ICAR-CIBA, Kakdwip, India*

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भा. कृ. अनु. पा. - केंद्रीय खारा जलजीव पालन अनुसंधान संस्थान
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Indian Council of Agricultural Research, Ministry of Agriculture and Farmers Welfare, Govt. of India

डॉ. के. पी. जितेन्द्रन, पी. एच. डी.
कार्यकारी निदेशक
Dr. K. P. Jithendran, Ph. D.
Director (Acting)

FOREWORD

Food security in the future world is going to be a big challenge as the population of the world is expanding at very fast pace. In this perspective, the brackishwater aquaculture, which is one of the intensively expanding farming areas, will play a key role in meeting the nutritional requirement of the swiftly growing world population. Thus, for the global food security of the present as well as future generations, the sustainable and environment-friendly growth of this segment with social relevance is imperative. India holds a leading fundamental position in brackishwater aquaculture sector both in terms of production and export. During 2020-21, India earned ₹.4,37,210 million through seafood exports and frozen shrimp is prime commodity with export value of ₹. 3,25,203 million accounting for 74.4% of the total of export earnings. Thus shrimp culture is of paramount importance for both food security and economic growth of the country. However the periodic occurrences of various diseases in brackishwater aquaculture act as major obstacles against sectoral growth due to associated crop losses. Various shrimp diseases, especially White spot disease (WSD), Hepatopancreatic microsporidiosis (HPM), infectious myonecrosis (IMN) etc. caused havoc in shrimp farming at different point of time, due to substantial production losses and consequent economic catastrophes. In India, the gross national productions due to EHP and WSSV diseases were 0.77 and 0.33 M tons respectively. This accounted for corresponding economic loss of ₹. 3977 and ₹. 1670 crores during the year 2018.

Both West Bengal and Odisha hold very important position in the brackishwater aquaculture production, considering vast potential brackishwater areas and the areas under brackishwater farming. The potential brackishwater culture area of West Bengal is around 2.1 lakh hector and presently 25% of the area is under use for brackishwater aquaculture. The shrimp production of West Bengal and Odisha experience a setback due to various diseases, especially WSD and HPM. Further as majority of brackishwater culture area of West Bengal comprises of traditional culture system including bheries, implementation of adoption of

proper biosecurity measures is hard-hitting. Generally it is also observed that there is lack of awareness among brackishwater aqua-farmers on various aspects of diseases management and biosecurity measures as preventive measures of disease outbreaks. Thus, it is essential to educate the brackishwater farmers of these lines of disease management, biosecurity measures and maintenance of appropriate soil and water quality.

Scientists and staffs of Kakdwip Research Centre of Central Institute of Brackishwater Aquaculture play a pivotal role in the disease monitoring and management of brackishwater aquaculture sectors of Eastern India. The current training programme on 'Disease management of brackishwater aquaculture farming' during 21-26 March is being organized with the objective of creating awareness among brackishwater aquaculture farmers of Eastern region of India on disease management of brackishwater aquaculture farming. This special publication brought out for the training programme is expected to be helpful for the participants. I have every hope that the trainees will make best use of this training manual according to needs in the field of prevention of diseases in brackishwater aquaculture system.

In this regard, I sincerely appreciate the efforts taken by scientists at the Kakdwip Research Centre of CIBA for organizing this training programme considering the immense need of the farmers. I wish all the best to the trainees and a bright future in their career ahead.

17.03.2022

(K.P. JITHENDRAN)
DIRECTOR (ACTING)

Chennai
17th March, 2022

Phone / फ़ोन: 044- 24618817, 24616948 I Fax / फैक्स: 044- 24610311
Email / ईमेल: director.ciba@icar.gov.in; icarciba21@gmail.com I Web / वेब: www.ciba.res.in

काकद्वीप शोध केन्द्र
भाकृअनुप - केन्द्रीय खारा जलजीव पालन अनुसंधान संस्थान
KAKDWIP RESEARCH CENTRE
ICAR - CENTRAL INSTITUTE OF BRACKISHWATER AQUACULTURE ISO 9001:2008 CERTIFIED www.ciba.res.in
(Indian Council of Agricultural Research, Govt. of India), Kakdwip, South 24 Parganas, West Bengal - 743 347

डॉ. संजय दास / Dr. Sanjoy Das
प्रधान वैज्ञानिक / Principal Scientist

डॉ. लीसा प्रियदर्शनी / Dr. Leesa Priyadarsani
वैज्ञानिक / Scientist

PREFACE

Brackishwater aquaculture is very fast-growing food-producing sector, which can meet the food security of future generations. However, occurrence of various diseases, especially the White spot diseases (WSD) and *Enterocytozoon hepatopenaei* (EHP) has paralyzed the shrimp industries of different brackishwater aquaculture producing states of India. In addition to these, other diseases such as Infectious hypodermal and haematopoietic necrosis (IHHN), Loose shell syndrome (LSS), Infectious myonecrosis, White faecal syndrome, White patch disease, etc. very often affect shrimp farms of different regions of India with heavy production loss. At present days, Indian shrimp culture sector is dominated by an exotic species, *Penaeus vannamei*, the broodstock of which needs to be imported. The introduction of the exotic species has again potentiated the risk of transmission of different exotic diseases such as Early mortality syndrome, Taura syndrome, Yellow Head Diseases, etc. The occurrence of different diseases in shrimp culture field can be controlled by good management practices such as use of disease-free post-larvae, maintenance of good culture environment, optimum feeding and thorough disease surveillance. The state West Bengal and Odisha hold very important position in the field of brackishwater aquaculture with vast potential brackishwater aquaculture area. However very often, the occurrence of various diseases in brackishwater aquaculture sector of these two states cause a heavy damage to the sector. Very often, it is observed that lack of awareness among farmers on proper disease management is one of the key factors of occurrence of diseases.

Keeping the above-mentioned facts in the mind, this manual has been designed for educating the farmers of West Bengal and Odisha about different aspects of various diseases of brackishwater aquaculture systems and their management. This manual will be provided to the trainees, who will undergo the training on the area of Disease Management of Brackishwater Aquaculture at Kakdwip Research Centre.

We are extremely grateful to Dr. K.P. Jithendran, Director, ICAR-CIBA for his precious suggestions and guidance in designing this training manual and also for the funding support. We also take the opportunity to thank Dr. M. Poornima,

Principal Scientist, Aquatic Animal Health & Environment Division (AAHED) of ICAR- CIBA for her valuable support for the editorial work. We are also thankful to Dr. Debasis De, Officer In-charge, Kakdwip Research Centre, ICAR-CIBA for his constant support and encouragement preparation of the manual. It is also our proud privilege to thank all the authors of the manual for spending their valuable time for contribution to the manual. Our whole-hearted thanks are also due to our other scientist colleagues Dr. T.K. Ghoshal, Dr. Prem Kumar and Ms Babita Mandal, who gave us moral strengths to complete this uphill task. We are also extremely thankful to Mr. Ramkrishna Patra for providing the technical supports for preparation of the manual. We also sincerely acknowledge the helps received from all the permanent and contractual staffs of Kakdwip Research Centre for various kinds of co-operation in preparation of the manual. I have every hope that this training manual will be very much helpful to the farmers and other related stakeholders in controlling diseases in their farms.

(Sanjoy Das) **(Leesa Priyadarsani)**

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THEORY

Brackishwater Aquatic Animal Health Management - Indian Scenario

K.P. Jithendran, M. Poornima and P. Ezhil Praveena

ICAR- Central Institute of Brackishwater Aquaculture, Chennai, India

E-mail of corresponding author: kp.jithendran@icar.gov.in

1. Introduction

Aquaculture is the fastest growing food producing industry with a total global aquaculture production of 112 million tonnes. Shrimp has become the single most successful crop and backbone of the brackishwater coastal aquaculture in India and many Asian countries. Tiger shrimp, *Penaeus monodon*, and Pacific white shrimp *Penaeus vannamei* are the most important farmed shrimp species across the world. Brackishwater farming in India during the past four decades has evolved from age-old traditional systems of 'pokkali' in Kerala, 'bheries' in West Bengal, 'gheris' in Odisha, 'khar lands' in Karnataka and 'khazans' in Goa coasts to modern semi-intensive practice. During the decade of 1990's brackishwater aquaculture development received a huge impetus on semi intensive shrimp farming with the demonstration project by The Andhra Pradesh Shrimp Seed Production Supply and Research Centre (TASPARC) funded by the Department of Biotechnology (DBT), Govt. of India. 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. Giant tiger shrimp became the mainstay of brackishwater aquaculture in India and the area under shrimp farming showed remarkable growth rate till 1995.

However since 1995, the sector has been plagued by viral diseases, especially White Spot Syndrome Virus (WSSV). Although *P. monodon* was dominating species, since 2001, global shrimp aquaculture dramatically shifted to *P. vannamei*, because of the availability of disease free stock. From 2000 onwards, there was a gradual increase in production which reached a maximum of 1,40,000 MT in 2006-07. But in 2007-08 and 2008-09, the production levels reduced drastically and reached the pre-1995 level of 75,000 MT. The introduction of *P. vannamei* in 2009 led to the recovery of the sector, with the total production levels reaching a level of 8,15,000 tonnes from a total area under culture of around 1,08,000 hectares in 2020-21 (Source: MPEDA) marking sea food export valued more than 5 billion dollars. However, farming activity in the country faces problems due to market price fluctuation and disease outbreaks. Hence health management

is the key to success and profitability in aquaculture. The prevalence of the disease in shrimp culture systems is alarming such that there is a need for effective and sustainable control measure of disease outbreak.

2. Diseases in shrimp farming

P vannamei is known to be vulnerable to a wide range of viral diseases, and the reports of mass mortalities and failure of the culture system have also been recorded. The common diseases of economic importance reported to affect *P. vannamei* include, WSD, infectious hypodermal and hematopoietic necrosis (IHHN), Taura syndrome (TS), yellow head disease (YHD) and infectious myonecrosis (IMN).

ICAR – CIBA's investigations have revealed that during the year 2020-21, the prevalence of 18% WSD, 41% hepatopancreatic microsporidosis or *Enterocytozoon hepatopenaei* (EHP) and 30% IMNV in vannamei farms in the major shrimp farming states of Andhra Pradesh and Tamil Nadu. Indian brackishwater aquaculture was free from other OIE listed diseases such as TS, YHD, AHPND and Necrotising Hepatopancreatitis (NHP). Other disease syndromes due to poor farm management such as stunted growth, white faeces syndrome (WFS), white muscle syndrome (WMS), running mortality syndrome (RMS), and black gill syndrome was observed in the shrimp farms. The information generated indicates that WSD still remains the most devastating shrimp disease in India which causes recession in production. The loss of production is high in case of WSSV followed by EHP, an survey conducted by ICAR – CIBA revealed. However the emergence of EHP and its impact due to its accumulation in culture environment since its detection in 2015 in India is alarming. It was estimated that annual revenue loss due to EHP was US $567.62M and WSSV was US $238.33M. Emergence of such new diseases in Indian aquaculture despite stringent screening of brooders at AQF facility of RGCA as post-border measure point to the clandestine use of pond reared brood stock for seed production in the country by a section of operators. Sensitive diagnostics are available to detect any form of these pathogens, however routine diagnosis yet to be adopted by hatcheries and shrimp farmers. It appears that farmers have a preconceived idea that there is no need of screening the SPF vannamei seeds. In this backdrop, ICAR - CIBA initiated the programme of harmonisation of PCR diagnostics across the country as per the guidelines and intense efforts are being taken for capacity building of human resources associated with these laboratories, partnering with MPEDA and CAA.

3. Diseases in brackishwater finfish grow-out culture in India

Presently, the Indian brackishwater aquaculture sector is dominated by shrimp culture due to its high export value. India has a great potential for brackishwater finfish culture and with the support of the aquaculture promoting agencies and R&D institutions, hatchery and farm rearing technologies of new finfish species are already being demonstrated by the farming sector for economic viability. Expansion of brackish / marine finfish aquaculture has been taking place along the coastal states of India especially in the states of Andhra Pradesh, Tamil Nadu, West Bengal, Orissa and Gujarat. As with the case of any live animal rearing issues, disease has been an issue in finfish culture sector also. Although a number of viral and bacterial pathogens have been reported from other finfish farming nations, viral nervous necrosis (VNN), irido viral disease and Tilapia Lake Virus has been reported to be present in India, mainly causing mortalities in the larval stages of seabass. Among the bacterial pathogens, *Vibrio* spp are reported to be associated with diseases in brackishwater finfish by some investigators.

4. Fish Health Management Strategies

An understanding about the environment, biota and biology of the target species along with the in depth knowledge of the disease, pathogen, disease development, diagnostics, epidemiology and control measures are essential factors in management of a disease problem. Hence, Fish health management requires a holistic approach, addressing all aspects that contribute to the development of disease. Outbreak of disease is an end result of negative interaction between pathogen, host and the environment. Hence, management of disease problems must be aimed towards broader ecosystem management with a view to control farm-level environmental deterioration and to take preventative measures against the introduction of pathogens into the aquaculture system. The emphasis should be on better management for prevention, which is likely to be more cost effective than treatment, involving both on-farm management and the management of the environment. Steps must include reducing the use of chemicals and drugs. Regulations with respect to land and water usage, environmental protective measures, inputs that go into the aquaculture systems, farm-wise and region-wise must be put in place by the Government for disease management of aquatic animals and sustainable development of aquaculture at large. In addition, research and development, training programs, extension, and information exchange would help achieve the objective of disease prevention and control in aquaculture effective. The FAO's Code of Conduct for Responsible Fisheries would provide a good base

for the national and international cooperation in harmonizing aquatic animal health management activities.

New generation approaches such as Surveillance techniques, Contingency planning and Import Risk Analysis (IRA) are gaining importance as critical tools in the health management strategies of aquatic animals for quick and effective response to new disease outbreaks. Functioning of a national level (each country) body with necessary responsibility and mandate to implement a 'national health management strategy' or 'health management regulation' on the basis of existing international standards, guidelines or recommendation from FAO, OIE, NACA and WTO must be there in issues related to aquaculture and aquatic animal health management for the region.

5. 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, it 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.

The National Surveillance programme has been initiated through a network of fisheries research institutions active in aquatic animal health since 2013. 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. About 24 leading national institutions covering fourteen states with passive and active surveillance in more than 100 districts are carrying out disease surveillance in farming of 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.

6. Aquaculture biosecurity

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. Implementation of biosecurity practices is an increasingly pressing issue for fisheries and aquaculture managers, considering the importance of this sector in terms of food security and economic development of the people. Biosecurity measures implemented appropriately can be a cost- effective way of managing disease risks. Adopting quarantine measures for broodstock prior to their use, adopting best 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 will help in achieving biosecurity in hatcheries. At farm level, implementing biosecurity plan requires modifying existing farms and management routines. Main preventive measures at pond / farm level include proper pond preparation to eliminate pathogens and their carriers, treatment of water in reservoirs to inactivate free viruses and kill virus carriers, water filtration using fine filters to keep carriers out, closed zero-water exchange systems to avoid contamination from source water. 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.

7. Aquatic animal quarantine

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, broodstock and other live organisms. Presently two amphibian, ten fish, seven molluscan and eight crustacean 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. India established its first state of the art AQF in the year 2009 at Chennai to facilitate *P. vannamei* broodstock import in India. However, India needs to establish separate AQFs for quarantine needs of various aquatic species such as ornamental fish, candidate finfishes such as seabass, corals, being proposed by the private sector for import. These facilities can be created under PPP mode, where central agencies, state Govt., research institutions and the private players can play a harmonised role. The import risk analysis (IRA) of CIBA clearly revealed that the aquatic quarantine at the importers' facilities was highly risky and recommended low-risk options to the Govt. of India to i) establish quarantine facilities under public sector with restriction on the culture practices and ii) establishment of SPF broodstock multiplication centre cum quarantine under public-private partnership with restricted permits for culture, as is presently being done with Pacific white shrimp. At large, the Quarantine and Biosecurity must be vested with the Govt agencies and Institutions as done in all other developed nations for effectiveness and delivery as per the regulations of the country.

8. Aquaculture Certification

Fish is one of the most highly traded food commodities globally. According to FAO, global trade of fish and fishery products reached $136 billion in 2013. EU, US and Japan together account for about 70% of international fish imports and most fish exporting countries are trying to access these markets. Import requirements in these countries are very stringent in terms of quality, safety and fair trade practices. The World Trade Organisation (WTO) Sanitary and Phytosanitary (SPS) and Technical Barriers to Trade (TBT) agreements provide a framework for international trade including that

of fish and fishery products. These are based on principles of (a) sovereignty, (b) harmonization, and (c) equivalence. According to these agreements, WTO member countries have the right to take measures to protect animal health and consumer health, based on a scientific risk assessment performed according to internationally accepted practices. The member countries are expected to harmonise their standards by those adopted by international organisations. For food safety, Codex Alimentarius Commission standards and for animal heath, OIE standards have been recognized in the SPS agreement. Issues related to food safety such as food hygiene, maximum permissible limits for contaminants, residues of veterinary drugs are generally part of national regulations. Examples include EU regulations and USFDA regulations. In India, the Food Safety and Standards Authority of India (FSSAI) are responsible for developing and implementing national food safety related standards. Fish processing establishments, that already implement the mandatory HACCP based quality and safety management programme, are obliged to get certified by private certifying bodies like the British Retail Consortium (BRC), International Featured Standards (IFS), Food Safety System Certification (FSSC) 22000, Safe Quality Food (SQF) Institute certification and others. The EU requires that the national regulatory requirements are harmonized and equivalent to EU requirements. The US and Japan follow different procedures and it was the responsibility of the importing company to ensure that the operators in the producing country meet the USFDA / Japanese Ministry of Health requirements. India has been successful in accessing all these major markets in addition to other regional markets as it is evident by growing exports of Indian seafood. Keeping this growth trend requires sustained efforts to maintain quality standards as required by the importing countries. The Export Inspection Council of India although specifies the requirements of traceability for fish and fishery products, it is essential that traceability to be established at all stages of production, process & distribution to get the confidence of the food business operators. However, considering costs involved in aquaculture certification which has to be borne by the producer (farmer), it is essential that India develops its own aquaculture certification programme for the benefit of the sector, keeping in view more than 80% small aqua farmers, where institutions such as CIBA, MPEDA and CAA could play the lead role.

9. Concluding remarks

While Indian aquaculture sector is booming after the introduction of exotic specific pathogen free Pacific white shrimp, trans-boundary diseases could limit this growth and affect sustainability. Maintaining strict biosecurity in

aquaculture is a challenge, but is not an impossible task. Aqua farmers have to be educated on biosecurity principles and adoption of best management practices on a regular basis to maintain sustainable productivity. While India has shown its capacity in establishment and successful operation of AQF for *P. vannamei* broodstock since the year 2009, there is a need to establish similar separate AQFs for quarantine of various aquatic species proposed for import by the private sector time to time to safeguard India's aquatic biosecurity. This will be driven by various factors like production profitability, investment and development issues, and new threats of emerging health problems, resource protection, food security, trade, consumer preference for high quality and safe products. For sustaining international trade, aquaculture certification needs to be evolved within India based on the FAO Technical Guidelines for Aquaculture Certification as the benchmark.

10. Further readings

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Viral Diseases in Farmed Shrimp and their Management

M. Poornima, P. Ezhil Praveena, Leesa Priyadharshini and *Sanjoy Das
ICAR- Central Institute of Brackishwater Aquaculture, Chennai, India
**Kakdwip Research Centre of ICAR-CIBA, Kakdwip, West Bengal, India*
E-mail of corresponding author: sanjoy125@yahoo.co.in

1. Introduction

Shrimp farming is one of the promising enterprises in the current scenario providing employment opportunities to many. This sector is gaining impetus day by day and many are involved in shrimp aquaculture. As the intensification of shrimp aquaculture begins there are also so many adversities it has to encounter. Of which the major one is diseases, and it does not give any time for the farmer to think and thereby it is causing loss to the farmer. In this article, brief outlines on important shrimp viral diseases which can have implications on shrimp aquaculture are discussed. The viral diseases of importance in *Penaeus vannamei* shrimp culture are

- White spot disease (WSD)
- Infectious hypodermal haematopoietic necrotic disease (IHHN)
- Infectious myonecrosis (IMN)
- Taura syndrome viral disease (TS)
- Yellow head viral disease (YHD)
- Infection with Decapod Iridescent Virus 1 (DIV1)
- Viral covert mortality disease (VCMD)
- *Penaeus vannamei* Nodavirus (*Pv*NV)

2. White spot disease (WSD)

This virus is the most serious threat facing the shrimp farming industry. WSSV was first reported in farmed *P. japonicus* from Japan in 1992/93, but was thought to have been imported with live infected PL from Mainland China. WSD has been identified from crustaceans in China, Japan, Korea, South-East Asia, South Asia, the Indian Continent, the Mediterranean, the Middle East, and the Americas. All penaeid shrimp species are highly susceptible to infection, often resulting in high mortality. Crabs, crayfish, freshwater prawns, spiny lobsters and clawed lobsters are susceptible to infection, but morbidity and mortality consequence of infection is highly variable. Prevalence of WSSV is reported highly variable, from <1% in infected wild populations to up to 100% in captive populations.

2.1. Causative agent of WSD

WSD is caused by one of the largest animal viruses. WSSV is a large double-stranded DNA virus of 120-150 by 270-290 nm size, assigned to a new virus family, whispoviridae. WSSV had several names such as Chinese baculovirus (CBV), White spot syndrome baculovirus complex (WSBV), Shrimp Explosive Epidermic Disease (SEED), Penaeid Rod-shaped DNA Virus (PRDV), Japan's Rod-shaped Nuclear Virus (RV-PJ) of *P. japonicus*, Thailand's Systemic Ectodermal and Mesodermal Baculovirus (SEMBV) of *P. monodon*. WSSV can infect a wide range of aquatic crustaceans including marine, brackish and freshwater penaeids, crabs and crayfish. All decapod crustaceans from marine and brackish or freshwater sources are susceptible host species.

2.2. Clinical symptoms of WSD

WSSV affects most organs derived from ectodermal and mesodermal origin, including the cuticular epithelium, connective tissue, nervous tissues, muscle, lymphoid organ and haematopoietic tissues. The virus also severely damages the stomach, gills, antennal gland, heart and eyes. During later stages of infection, these organs are destroyed and many cells are lysed. The shrimp then show reddish discolouration of the hepatopancreas and characteristic 1-2 mm diameter white spots on their carapace, appendages and inside surfaces of the body. Affected shrimp show lethargic behavior. Cumulative mortality typically reaches 100 percent within two to seven days of infection.

Shrimp infected with WSSV displaying white spots on carapace

Carapace of WSSV infected shrimp with white spots

2.3. Diagnosis of WSD

It can be visually diagnosed through the presence of the characteristic white spots, which can be seen in advanced stage of infection. However, white spots may not be always present in infected shrimp. WSSV can be detected by using PCR, or with probes for dot-blot and *in situ* hybridization tests. PCR detection efficiency can be increased by exposure to stressful conditions (e.g. eye-stalk ablation, spawning, moulting, changes in salinity, temperature or pH, and during plankton blooms). WSSV can be confirmed histologically (particularly in asymptomatic carriers) by the presence of large numbers of Cowdrey A-type nuclear inclusions and hypertrophied nuclei in H&E-stained sectioned tissues, or simply by rapid fixation and staining of gill tissue.

2.4. Transmission of WSD

The mode of transmission of WSD around Asia was believed to be through exports of live PL and broodstock. The infection can be transmitted vertically, horizontally by cannibalism, predation, etc. and by water-borne routes. Dead and moribund animals can be a source of disease transmission. However, some studies have shown that disinfection of water supplies and the washing and/or disinfection of the eggs and nauplius is reported to be successful in preventing its transmission from positive broodstock to their larvae. It is generally believed that the virus sticks to the outside of the egg, and if it gains entry to the egg, it is rendered infertile and will not hatch. Thus, using proper testing and disinfection protocols, vertical transmission can be prevented in the hatchery. It has now become clear that the presence of WSSV in a pond does not always lead to disaster. Outbreaks are usually triggered from latent carriers due to environmental changes,

probably related to osmotic stress through changes in salinity or hardness or rapid temperature changes. Fluctuations in temperature have been shown to induce mortalities of infected shrimp.

2.5. Prevention / control of WSD

Broodstock should be PCR screened before breeding. PL should also be PCR screened before stocking into ponds, as this has been proven to result in a higher percentage of good harvests. Washing and disinfection of eggs and nauplii has also been shown to prevent vertical transmission of WSSV from infected broodstock to larval stages. Feeding with fresh crab and other crustaceans to broodstock should be avoided. Polyculture techniques with mildly carnivorous fish species (such as Tilapia spp.) has also proven effective at limiting the virulence of WSSV in ponds, as the fish can eat infected carriers before they become available to the live shrimp. The white spot virus only remains viable in water for 3-4 days, so disinfection of water used for changes and fine screening is effective in preventing transmission. Formalin treatment (70 ppm) has been shown to prevent transmission and not cause any harm to shrimp. In addition, all effluent from farms or processing plants should be disinfected with formalin or chlorine prior to discharge.

3. Infectious hypodermal and haematopoietic necrosis (IHHN)

IHHN was first discovered in *P. 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 *P. vannamei* culture in the Americas during the 1990s.

3.1. Causative agent of IHHN

IHHNV is caused by a small (20-22 nm) single-stranded DNA-containing parvovirus.

3.2. Clinical symptoms of IHHN

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) (Fig 2), slow growth, poor FCR and a greater size variation at harvest, contributing substantially to reduction in profits. These effects are typically more pronounced when the shrimp are infected at larval stages. Hence 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. 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.

Shrimp infected with IHHNV displaying size variation and slow growth

3.3. Diagnosis of IHHN

IHHNV can be diagnosed using methods such as DNA probes in dot blot and *in situ* hybridisation and PCR techniques (including real-time PCR) as well as histological analysis of H&E-stained sections looking for intracellular, Cowdrey type A inclusion bodies in ectodermal and mesodermal tissues such as cuticular epithelium, gills, foregut, hind gut, lymphoid organ and connective tissues.

3.4. Transmission of IHHN

Transmission of IHHN is known to occur rapidly by cannibalism of weak or moribund shrimp. It can also be transmitted through waterborne route and cohabitation. Vertical transmission from broodstock to larvae is common

and has been shown to originate from the ovaries of infected females (whilst sperm from infected males was generally virus-free). IHHNV may be also transmitted through vectors such as insects, which have been shown to act as mechanical carriers for the disease.

3.5. Prevention / control of IHHN

IHHNV is reported to be highly resistant to all the common methods of disinfection including chlorine, lime and formalin. One of the big problems with IHHNV is its eradication in infected facilities. Complete eradication of all stocks, complete disinfection of the culture facility and avoidance of restocking with IHHNV-positive animals may bring down incidences of IHHNV infections.

4. Infectious myonecrosis (IMN)

Infectious myonecrosis is an emerging *P. 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*.

4.1. Causative agent of IMN

IMN is caused by a virus, a putative totivirus. IMNV particles are icosahedral in shape and 40 nm in diameter.

4.2. Clinical symptoms of IMN

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.

Shrimp infected with IMNV displaying white muscle

4.3. Diagnosis of IMNV

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.

4.4. Transmission of IMNV

IMNV has been demonstrated to be transmitted through cannibalism. Transmission via water and vertical transmission from broodstock (transovarium 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 *P. vannamei* in regions where IMNV is enzootic.

4.5. Prevention / control of IMN

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). Fallowing and restocking of affected farms or entire culture regions with IMNV-free stocks of *P.*

vannamei may help in preventing its recurrence. No effective therapeutics have been reported for IMN.

5. 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*.

5.1. Causative agent of TS

Initial work suggested that TS was caused by a toxic pesticide. However, it is now known that a single or perhaps several very closely related strains (mutants) of the Taura syndrome virus (TSV) are responsible for the TS. TSV is a single stranded RNA virus of 32 nm size, non-enveloped icosahedrons and more prone to mutations causing more concern.

5.2. Clinical symptoms of TS

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

5.2.1. Acute phase

This is characterized by empty stomach, soft shell pale red body surface and appendages, red tail fan and pleopods due to the expansion of red chromatophores. Moribund shrimp will appear pale reddish in colour with tail and pleopods appearing distinctly red commonly referred as ‘Red tail disease’. In such shrimp, close inspection of the cuticular epithelium in thin appendages (such as the edges of the uropods or pleopods) with hand lens reveals signs of focal epithelial necrosis. The affected shrimp also show signs of soft shells and empty guts and generally die during ecdysis.

5.2.2. Transition (recovery) phase

This phase is seen only for few days and is characterized by multiple, irregularly shaped, and randomly distributed melanized (dark) cuticular lesions death, usually at moulting. These melanised spots are haemocyte accumulations indicating the sites resolving TS lesions in the cuticular epithelium. Such shrimp may or may not have soft cuticles and red-chromatophore expansion and may be behaving and feeding normally. If

shrimp with these black lesions survive the next moult, the lesions disappear and they appear grossly normal, despite the continuing presence of the virus, especially in the lymphoid organ.

5.2.3. Chronic phase

Shrimp in the transition phase move into the chronic phase as there are no obvious gross pathological signs of disease in this phase.

5.3. Diagnosis of TS

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. Acute and chronic phases can be diagnosed consistently by using histological methods. In acute phase, the pathognomonic lesions are seen in the cuticular epithelium, while in the transition and chronic phases there are no pathognomonic lesions. Acute phase of the disease is characterized by multifocal areas of necrosis in the cuticular epithelium of the general body surface, appendages, gills, hindgut, and foregut (the oesophagus, anterior and posterior chambers of the stomach). Cells of the subcuticular connective tissues and adjacent striated muscle fibres are occasionally affected. In some severe cases the antennal gland tubule epithelium is also destroyed. Cytoplasmic remnants of necrotic cells are often extremely abundant, and these are seen as spherical bodies (1–20µm in diameter) which are eosinophilic to pale basophilic. These structures, along with pyknotic and karyorrhectic nuclei, give a characteristic ‘peppered’ or ‘buckshot-riddled’ appearance, which is pathognomonic for TS disease when there is no concurrent necrosis of the parenchymal cells of the lymphoid organ (LO) tubules. The absence of necrosis of the LO in acute-phase TSV infections distinguishes TS disease from acute phase yellow head disease in which similar lesion is seen. In this phase there is absence of haemocytic infiltration or other host-inflammatory response which distinguishes it from the transitional phase of the disease. In transitional phase typical acute phase cuticular lesions decline in abundance and severity are replaced by conspicuous infiltration and accumulation of haemocytes at the sites of necrosis. The masses of haemocytes may become melanised giving rise to the irregular black spots that characterize the transition phase of the disease. In chronic phase there is no gross signs of infection, but the prominent histopathological lesion is the presence of an enlarged LO with numerous Lymphoid Organ spheroids (LOS), which may remain associated with the main body of the paired LO or seen detached and become ectopic

LOS bodies that lodge in constricted areas of the haemocoel (i.e., the heart, gills, in the subcuticular connective tissues, etc.).

5.4. Transmission of TS

The mechanism of transmission of TSV can be through contaminated PL and broodstock. Recently it has been shown that mechanical transfer through insect and avian vectors may be a more likely route of infection. Shrimp-eating seagulls can transmit TSV through their faeces. Hence birds are likely to transmit TSV.

5.5. Prevention / control of TS

Infected stocks must be totally destroyed and the culture facility must be disinfected. The disease can be prevented by avoidance of reintroduction of the virus from nearby facilities, wild shrimp and carriers and stocking with TSV-free PL produced from TSV-free broodstock. Switching culture to refractory species such as *P. stylirostris* has been suggested. Other methods suggested for controlling the virus include BMPs and maintenance of optimal environmental conditions, weekly applications of hydrated lime (CaOH) at 50 kg/ha, polyculture with fish (to consume dying and dead carriers).

6. Yellow head disease (YHD)

Yellow head disease is infection with the pathogenic agent yellow head virus genotype 1 (YHV1). YHV1 has been reported in Chinese Taipei, Indonesia, Malaysia, the Philippines, Sri Lanka, Thailand and Vietnam. YHV1 has been detected in *P. vannamei* in Thailand and Mexico. YHV disease caused 60–70% mortality in farmed *P. vannamei* in Thailand with estimated economic loss of approximately US$3 million in 2008. The susceptible species to infection with YHV1 are *Penaeus vannamei, Penaeus stylirostris*, *Palaemonetes pugio*, *Penaeus monodon and Metapenaeus affinis.* When YHD emerged in Thailand in 1990, losses were severe and the disease listed by the OIE.

6.1. Causative agent of YHD

Yellow head virus genotype 1 (YHV1) is one of eight genotypes belonging to the yellow head complex of viruses known agent causing yellow head disease. YHV1 and other genotypes in the yellow head complex are classified as a single species (*Gill-associated virus*) in the genus *Okavirus*, under the family *Roniviridae* by the International Committee on Taxonomy of Viruses (ICTV). YHV1 is an enveloped, rod-shaped virus of 40–50 nm × 150–180 nm in size with ~26 kb (+) ssRNA genome of helical symmetry. Since the first

report of YHV in Thailand in 1990, eight geographical types (YHV-1 to YHV-8) have been described.

6.2. Clinical symptoms of YHV

YHV affects tissues of ectodermal and mesodermal origin including lymphoid organ, haemocytes, haematopoietic tissue, gill lamellae and spongy connective tissue of the subcutis, gut, antennal gland, gonads, nerve tracts and ganglia. 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 (Fig 3) 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.

6.3. Diagnosis of YHV

Yellow head virus can be detected by RT-PCR or with a probe designed for dot-blot and *in situ* hybridisation 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.

6.4. Transmission of YHV

The primary mechanism of spread of YHV in pond culture appears to be through water and mechanical means or from infected crustacean carriers. YHV is reported to remains viable in aerated seawater for up to 72 hours. However, the most serious threat is latent or asymptomatic carriers, from which the virus can spread either by ingestion or cohabitation. Other shrimp such as *P. merguiensis*, *P. indicus*, *Metapenaeus ensis*, *Palaemon styliferus* and *Acetes spp*. may become infected and act as carriers having latent infections, while others such as *Euphausia superba* may die upon infection. Other crustaceans, such as *Macrobrachium rosenbergii* and many crab species and *Artemia* appear to be refractive to YHV. Infected broodstock can pass on the virus to larvae in the maturation/hatchery facilities if thorough disinfection protocols are not strictly adhered to.

6.5. Prevention / control of YHD

Although YHD is not causing much loss at present, 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, chlorination (30 ppm active ingredient) of reservoir water, filtering inlet water with fine screens, avoidance of live feeds, maintenance of stable environmental conditions, disinfection of YHV infected ponds before discharge, and monitoring (by PCR) and production of virus free broodstock and PL for pond stocking.

6. Infection with decapod iridescent virus 1 (DIV1)

Infection with Decapod Iridescent Virus 1 (DIV1**)** was first isolated and identified in 2017 from Zhejiang Province in China. There have been reports of DIV1 from Thailand at a very low prevalence. Synonyms of infection of DIV1 is infection with shrimp hemocyte iridescent virus (SHIV). Infection with DIV1 has been proposed for listing in the OIE Aquatic Animal Health Code (OIE, 2016). The disease meets the OIE definition of an 'emerging disease' and, members must report it in accordance with Article 1.1.4 of the Aquatic Code. Infection with DIV1 is listed in the OIE/NACA quarterly aquatic animal disease reporting programme (https://enaca.org).

6.1. Causative agent of DIV1 infection

There are two original isolations of Decapod iridescent virus 1 (DIV1): Shrimp haemocyte iridescent virus and Cherax quadricarinatus iridovirus. DIV1 assigned as the only member of the genus Decapodiridovirus within the Iridoviridae family by the International Committee on Taxonomy of Viruses (ICTV).

6.2. Clinical symptoms of DIV1 infection

DIV1 infected shrimp display slightly reddish body discolouration, hepatopancreatic atrophy with colour fading, empty stomach and guts and soft shell in partially infected shrimp. The moribund shrimp loose their swimming ability and sank to the pond bottom. The symptoms and mortality are observed in the infected P. vannamei from post larvae to sub-adult shrimp stages.

6.3. Diagnosis of DIV1 infection

The sensitive nested PCR and real-time PCR methods have been established In situ hybridization (ISH) and in situ DIG-labelling-loop-mediated DNA Amplification (ISDL) have been validated.

6.5. Transmission of DIV1 infection

Challenge tests with P. vannamei via per os and reverse gavage have demonstrated that direct horizontal transmission as an important route of

transmission. There is no evidence of vertical transmission; however, samples from hatcheries have been found to be DIV1 positive.

6.6. Prevention / control of DIV1 infection

Enhanced biosecurity is the key strategy for control of infection with DIV1, including surveillance of farms, quarantine, and testing for DIV1 in broodstock and postlarvae. Biosecurity measures to minimise fomite spread via equipment, vehicles (i.e. cleaning and disinfection) should also be implemented. Restrictions on the movement of live crustaceans and removal of moribund or dead individuals from affected farms will limit the spread of the disease.

7. Viral covert mortality disease (VCMD)

Viral covert mortality disease was first reported in 2009 from China as a serious disease outbreak due to heavy economic loss shrimp aquaculture sector. The disease was commonly named as covert mortality disease (CMD) due to the moribund shrimp which died at the bottom of the pond. Later CMD was renamed as viral covert mortality disease (VCMD). The cumulative mortality of VCMD was variable, but it reaches 80 to 90% in some P. vannamei culture ponds. After the first appearance in China, CMNV was also reported in Thailand, Vietnam and Ecuador.

7.1. Causative agent of VCMD

A new emerging virus, covert mortality nodavirus (CMNV), was identified to be the aetiological agent of covert mortality disease. CMNV is a non-enveloped, spherical-shaped, single-strand RNA virus, a new member of *alphanodavirus*. The virus consists of an icosahedral capsid size ranging from 29 to 35 nm in diameter.

7.2. Clinical symptoms of VCMD

CMNV-infected shrimp shows hepatopancreatic atrophy and necrosis, empty stomach and gut, soft shell, slow growth of infected shrimp, abdominal muscle whitening and necrosis. The histopathology analysis of CMNV-infected shrimp showed coagulative necrosis of striated muscle, eosinophilic inclusions in the epithelium of the tubules in the hepatopancreas and lymphoid organ, mass karyopyknotic nuclei which existed in the muscle and lymphoid organ and the tubular epithelium of the hepatopancreas with significant atrophy.

7.3. Diagnosis of VCMD

Currently, the detection of CMNV include a reverse transcription nested PCR (RT-nPCR) and real-time reverse transcription PCR (qRT-PCR),

quantitative reverse transcription loop-mediated isothermal amplification (qRT-LAMP) assay and fluorescence in situ hybridization (FISH) methods.

7.4. Transmission of VCMD

Experimental challenge by injection mode showed reproduction of VCMD confirmation of the causative pathogen

7.5. Prevention / control of VCMD

Farmers in the shrimp aquaculture need to pay close attention and take measures to prevent disease outbreaks by practicing BMPs.

8. Penaeus vannamei nodavirus (*Pv*NV) infection

The disease was first reported in cultured *Penaeus vannamei* in Belize in 2004 and in Guayas Province, Ecuador in 2006.

8.1. Causative agent of PvNV infection

The causative agent is a positive single stranded RNA virus, named as *Penaeus vannamei* nodavirus (*Pv*NV) and is related to *Mr*NV belong to Nodiviridae.

8.3. Clinical symptoms of PvNV infection

The virus in has been detected in adult shrimps. Affected shrimp exhibit clinical signs, white, opaque lesions in the tail muscle that causes muscle necrosis. Histological examination reveals multifocal necrosis and hemocytic fibrosis in the skeletal muscle and basophilic, intra cytoplasmic inclusions in striated muscle, lymphoid organ and connective tissues

8.4. Transmission of PvNV infection

It appears to affect survival in grow out ponds. The disease is associated with environmental stress, such as crowding and high temperature. When the shrimp are stocked at a high density (>50 m–2), or when the temperature is >32°C, survival decreases by 40% in *Pv*NV-infected ponds. It was estimated 50% production loss due to of *Pv*NV in 2004 for the infected farm in Belize.

8.5. Diagnosis of PvNV infection

In situ hybridization method and a nested RT-PCR assay specific for *P. vannamei* nodavirus (*Pv*NV) have been developed. Also commercial IQ2000 RT –PCR diagnostic kit is available for routine screening.

8.6. Prevention / control of PvNV infection

The infection is need to be monitored, otherwise it may lead to spread of *Pv*NV to other farms and could result in significant production losses in infected areas.

9. General health management

SPF shrimp are expected to be free from the viral pathogens which are known to cause major losses to the shrimp aquaculture, including WSSV, YHV, TSV, IHHNV and IMNV. SPF refers only to the present pathogen status for specific pathogens and not to pathogen resistance or future pathogen status. SPF means that these animals will not suffer from diseases caused by specified diseases for which the animal is declared 'free' when cultured under 'strict' biosecurity. However, it does not guarantee against these shrimp getting infected with unknown pathogens or known pathogens which are not screened. Further, the SPF shrimp are not resistant to pathogens and these shrimp can become infected by any pathogen that they encounter during culture. Focusing efforts are needed on producing high quality seed, following farm biosecurity, better pond management to reduce stress and risk of infection.

10. Conclusion

Aquaculture is vital to the economies of several countries. Growing demand for seafood and limitations on production from capture fisheries will inevitably lead to the increased intensification and commercialization of shrimp aquaculture. This consequently leads to emergence of both known and novel diseases. The occurrence of infectious disease is usually related to a series of happenings involving the interactions between the host, the environment and the presence of pathogens. Enhanced better management practices aid in preventing the diseases epidemics.

11. Further readings

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Bacterial Diseases of Farmed Shrimp and their Management

Sanjoy Das, Leesa Priyadarshani, *M. Poornima, *Sujeet Kumar and *K.P. Jithendran

Kakdwip Research Centre of ICAR-CIBA, Kakdwip, West Bengal, India

** ICAR- Central Institute of Brackishwater Aquaculture, Chennai, India*

E-mail of corresponding author: sanjoy125@yahoo.co.in

1. Introduction

Brackishwater aquaculture is one of the fastest growing sectors of India with high growth rate and export potential. At present, India is the largest shrimp exporter in the world. During 2020-21, India earned INR 437210 million through export of fish and fishery products, and the contribution of frozen shrimp alone was 74.4% (INR 325203 million). Thus, brackishwater shrimp culture can be considered as a highly profitable and export-oriented enterprise. Due to intensification, both production and area under culture are increasing over a period of time, but at the same time it is resulting in spreading of different diseases leading to severe economic loss. Despite the spectacular growth in brackishwater aquaculture, the economic viability of the sector had been threatened by frequent occurrence of various diseases, which are the major constraints in production. The brackishwater aquaculture sector is mostly dominated by shrimp. Presently, Indian shrimp industry is mostly dependent of an exotic species called *Penaeus vannamei*, for which we are dependent on import of brood stock from foreign country. Moreover, the introduction of vannamei has increased the possibility of introduction of many new diseases, which were hitherto not present in India. Although most devastating diseases of shrimp are mostly viral in origin, very often different bacterial pathogens have also been found to affect cultured shrimp with high degree of mortality leading to crop loss. During 2013-14, the shrimp industry of different South-east Asian nations was ravaged by a bacterial disease called Acute hepatopancreatic necrotic disease (AHPND) initially known as Early mortality syndrome (EMS). Due to this disease, approximately there was 60% drop in shrimp production in the affected region (mostly South-east Asian countries) in 2013 compared with 2012. Although there is no confirmed report of AHPND in India till date, but our country needs to be vigilant for this disease as the incidence of this disease was found in Bangladesh, the immediate neighbouring country of India. Apart from AHPND, several other diseases including vibriosis, white faecal disease, black gill disease, black spot disease, netrotizing

hepatopancreatitis, etc. affected shrimp farms of different areas of different parts of the world leading to heavy economic loss.

2. Important bacterial diseases in shrimp culture

2.1. Acute hepatopancreatic necrotic disease (AHPND)

This is the most dangerous bacterial disease of *P. vannamei* and often causes 100% mortality. The disease also affects *P. monodon*. Earlier called EMS is termed as acute hepatopancreatic necrotic disease (AHPND) as the etiology of the disease has been confirmed as a particular strain of *Vibrio parahaemolyticus*, which bears a specific plasmid. This disease was first reported in Taiwan province of China and mainland China in 2009 and later on reported from all shrimp growing countries of South-east Asian region including Thailand, Vietnam, Malaysia etc. The early stages of culture period (20-30 days after culture period) are generally affected and cause upto 100% mortality. In many cases, farmers are unable to detect any shrimp in the pond after a month of stocking. In the infected shrimp, hepatopancreas becomes pale with significant atrophy. The moribund shrimps usually shrink to bottom and die. Temperature fluctuation, high salinity and high stocking density are the predisposing factors for AHPND. Diagnosis is generally done by PCR and histopathological examination of hepatopancreas. Histopathology of hepatopancreas is considered is the most reliable method of diagnosis of AHPND.

2.2. Vibriosis

Apart from AHPND-causing *V. parahaemolyticus*, there are several other species of *Vibrio*, which can affect shrimp. These include *V. parahaemolyticus*, *V. alginolyticus*, *V. mimicus*, *V. harveyi*, *V. fischeri*, *V. litoralis*, *V. metschnikovii* etc. Water bodies of brackishwater aquaculture systems are generally normal habitats of different species of *Vibrio*. However, they also act as opportunistic pathogens of cultured shrimp and affect shrimps during different environmental stress factors including mechanical injury, higher salinity, increased level of ammonia, nitrite and nitrate, low dissolved oxygen, higher stocking density, sudden change of pH, etc. Vibriosis is considered as major problem in hatchery level. In grow-out culture, this disease is characterized by melanised nodules in the gills, opacity of muscle, red discolouration of the appendages etc. Gathering at edge of the ponds is also commonly observed. Haemolymph of the affected shrimp does not clot or clot at very slow rate. Different species of *Vibrio* can be isolated from haemolymph and hepatopancreas by plating on TCBS agar and Zobell marine agar. The luminescence can be demonstrated by preparing a smear from haemolymph and observation in dark. In case of

heavy infection, the luminescence can be seen if the haemolymph is observed in dark. Some species of Vibrio also cause septic hepatopancreatic necrosis causing necrosis of hepatopancreas.

Another manifestation of Vibriosis in shrimp is White gut disease. This disease of shrimp very much prevalent in Andhra Pradesh and Tamilnadu states of India. 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. Six species of *Vibrio* viz. *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus* and *V. splendidus* are generally associated with this disease. 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 gene sequencing.

In grow-out pond, vibriosis can be controlled by application of different probiotics, application of neem leaves at 20 kg/ ha, fertilization of pond with sucrose (20 kg/ ha), etc. Addition of garlic paste (5–10 g of garlic/ kg of feed) and leaves of *Cantella asiatica* (Indian penny wort) (10 g/ kg of feed) in feed have recently been found to be very effective in controlling vibriosis in grow-out pond.

2.3. Luminescent bacterial diseases

Luminescent bacteria (LB) that cause luminescent disease are ubiquitous to the marine environment and mostly include vibrios. Some species of *Vibrio* like *V. harveyi*, *V. fischeri* are considered as luminescent bacteria and they emit luminescence in dark. In case of shrimp, this disease is more problematic to hatcheries than the grow-out systems. As vibrios considered as opportunistic pathogens, most of the times the mortality is mainly due to different stress factors caused by poor water quality, crowding, high water temperature, low dissolved oxygen and low water exchange. Different bacterial species recorded to be the cause for luminescent diseases includes mostly of *Vibrio harveyi*, and the rest belong to *V. splendidus, V. logei, V. fischeri* and *Photobacterium spp.* The main clinical symptoms for this disease are cloudy hepatopancreas, brown gill and body necrosis. Luminescent vibriosis may be controlled in the hatchery by washing eggs with iodine and formaldehyde and avoiding of contamination by spawner faeces. *Vibrio harveyi* in the water column can be inactivated by chlorine dioxide. Probiotics are administered directly into the water or via feeds and the immunostimulants successfully used for reducing shrimp mortalities associated with vibriosis. Though antibiotics are efficient to bring good

control over the disease, the use of it is not recommended in shrimp aquaculture due to development of drug resistance strains of different bacteria. Biocontrol by bacteriophages can also be used as an alternative to antibiotics. Better Management Practices (BMP), associated with the use of probiotics, immunostimulants and biocontrol agents may be effective ways to control luminescence bacterial disease in shrimp hatchery.

2.4. Necrotizing hepatopancreatitis (NHP)

NHP is caused by a rickettsia like bacterium called as *Hepatobacter penaei*, which infects the hepatopancreas of shrimp. The clinical signs of this disease include lethargy, emaciation, soft shells, heavy fouling from external parasites, black gills and reduced growth. The hepatopancreas appears pale to white. NHP requires lengthy periods of high air temperature (29°–31°C) and elevated salinity (20–40 ppt). Mortality can be 90–95% within 30 days of an outbreak. Mortalities usually occur midway through the grow-out phase. NHP appears to be transmitted by direct ingestion of an unidentified carrier (a reservoir host). Diagnosis is generally done by PCR. Histopathology of hepatopancreas shows empty hepatopancreatic tubule with capsule formation. Cell sloughing, tubular atrophy, melanization and formation of multifocal haemocytic capsules are also observed. In some cases, the cluster of intra-cytoplasmic bacteria is also observed.

2.5. Streptococcosis

This is an emerging disease of shrimp caused by a Gram positive chain-forming cocci group of bacteria called *Streptococcus* spp. and generally two species of *Streptococcus* are responsible for this disease viz. *Streptococcus uberis* and *Str. parauberis*. This disease was first reported in the South American country Guatemala during 2009. This organism affects hepatopancreas of shrimp and causes necrosis. On histopathological analysis of hepatopancreas, the characteristic observations include haemocyte infiltration, liquefying necrosis and presence of bacterial mass in the musculature. The presence of high streptococcal load is observed in haemolymph and hepatopancreas. In acute cases, the mortality reached up to 80%.

2.6. Spiroplasmosis

This is also an emerging bacterial disease of cultured shrimp caused by *Spiroplasma penaei*. This disease was first reported in Colombia in 2012. In this disease, the affected shrimp doesn't exhibit no external symptoms of bacterial infections, but external chromatophores are sometimes observed. Organs, which are affected by this disease, are ventral nerve cord, skeletal muscle, heart, antennal gland, lymphoid organs, and fibrous connective

tissues within the hepatopancreas, gill lamellae and sub-cuticular epithelium. However on histopathology, the typical lesion of systemic bacterial infection is observed. This includes haemocytic infiltration, haemocytic nodules, and evidence of phagocytosis, melanization and fibrosis. For confirmatory diagnosis, the combination of histopathology and PCR is required.

2.7. Filamentous bacterial disease

This disease is caused by bacteria called *Leucothrix* spp., which can affect all stages of shrimp starting from larval stages to adulthood. When shrimp eggs are affected, it will cause problems with respiration and hatching. In adult stages, this bacterium mostly affects the gill causing respiratory problems. This disease also causes moulting problem in shrimp. However, mortality rate is very low in this disease, but the growth of culture shrimp is usually affected.

3. Disease syndromes in shrimp culture

3.1. White faeces syndrome (WFS)

Although in many occasion, White faeces syndrome is caused by a microsporidian called *Enterocytozoon hepatopenaei*, but several bacterial species also play a major role. WFS has recently been considered as serious problem for *P. vannamei* throughout the world. However, this disease has been reported from both cultured black tiger shrimp and pacific white shrimp. WFS usually occurs after 50-60 days of culture (DOC). Ponds affected with this disease show white faecal strings floating on the pond surface while the shrimps show white/golden brown intestine, reduced feed consumption, growth retardation and often associated with loose shell. The disease can cause moderate to severe economic loss by reducing the shrimp survival by 20–30 percent when compared to normal ponds. There is multiple causes for WFS, which may be associated with presence of vermiform like gregarine bodies, vibriosis, *Enterocytozoan hepatopenaei*, blue green algae and loose shell syndrome. On bacteriological examination of haemolymph, a very high load of *Vibrio* is generally observed in WFS infected shrimp. Six species of fungi (*Aspergillus flavus, A. ochraceus, A. japonicus, Penicillium* spp., *Fusarium* spp., and *Cladosporium cladosporioides*) were isolated from shrimp naturally infected with WFS. Reduced stocking density, proper water exchange together with better management practices will be helpful in evading WFS. Application of gut probiotics at regular interval can also prevent the onset of this disease.

3.2. Black gill disease

This disease generally occurs when plankton content of water is too high. Poor pond bottom quality, low dissolved oxygen content, poor water quality parameters and high stocking density are also the causal factors of this disease. This is also known fouling disease. Gill becomes black in colour and is generally colonized with different saprophytic bacteria (*Flavobacterium*, *Cytophaga*, etc.) and parasite (e.g., *Zoothamnium* spp.). Addition of lime (quantity depends on pH), water exchange and increase of duration of aeration may help in controlling this disease. This disease is very often associated with deficiency of Vitamin C. To avoid this disease condition, the shrimp must not be overfed.

Black gill disease in shrimp

3.3. Loose shell syndrome (LSS)

This disease also causes a heavy economic loss among the shrimp farmers and was first reported in India in 1998. The incidence is more in summer than in winter. In India, the disease is more prevalent in some districts of Andhra Pradesh (East Godavari, West Godavari and Nellore) and Tamil Nadu. The disease is characterized by spongy abdomen due to muscular dystrophy, shrunken hepatopancreas and poor meat quality, which generally fetch reduced market price. The affected shrimps cannot moult and the gap between muscle and shell is generally increased with accumulation of water. The etiology of this disease is still not confirmed. Different species of *Vibrio* has been isolated from affected shrimp. The involvement of a filterable infectious viral agent has also been suspected for the disease. Maintenance of good aquaculture practices including water quality parameters and adaption of strict biosecurity measures may be of help in controlling this disease. Recently, it is also observed that sometimes high nitrite content of pond water is associated with loose and weak shell.

Shrimp with LSS condition

3.4. Brown spot disease of shrimp

Brown spot occurs when the pond bottom condition deteriorates. This disease occurs due to action of chitinolytic bacteria on shrimp shell. The shell becomes weak with brown or black colouured spots over the shell.

4. Common treatment measures of bacterial diseases of shrimp

In case of treatment of any shrimp disease, it is better to apply any chemical or medicine only after consultation with aquaculture experts or fish heal professional. Indiscriminate use of any chemical or medicine may adversely affect the diseased condition. Moreover, accidental use of any banned chemical or substances may result in export rejection leading to heavy economic loss. However, the common treatment methods are depicted below:

4.1. Application of potassium permanganate

Potassium permanganate may be applied at the rate of 10 kg per hectare in pond.

4.2. Application of lime

Quick lime may be applied at the rate of 10 kg per 1000 m^2. pH of the pond water may be checked before application of quick lime.

4.3. Benzalkonium chloride

Benzalkonium chloride (50%) can be applied @ 5 Lit per hectare to prevent harmful bacterial load in the pond.

4.4. Application of suitable probiotics

Probiotics are the live beneficial bacteria, which can reduce the harmful and pathogenic bacterial load. For control of harmful bacteria in pond water, different water probiotics and soil probiotics may be applied. On the other hand, for controlling pathogenic bacterial infection in shrimp or finfish, gut probiotics can be given along with feed. Different commercial probiotics marketed by different aquaculture companies are available. Manufacturer's instruction should be strictly followed before application.

4.5. Application of herbal formulations

- Thankuni leaves: Application of Thankuni leave (Indian Penny wort) paste @ 5 g per Kg of feed is sometimes very effective in controlling bacterial infection.
- Guava leave: For controlling different bacterial infection, especially Vibriosis, feed can be supplemented with guava leave at the rate of 5 g per kg of feed.
- CIFAX: Application of CIFAX at the rate of 1 kg per hectare of pond water can cure many bacterial infections.
- Application of Garlic-turmeric paste: Garlic-turmeric paste can be applied at the rate of 5 g per kg of feed.
- Application of immunostimulants: Immunostimulants are certain chemical compounds that stimulate immune system. e.g. Glucans, mannans, lipopolysaccharide, etc.

4.6. Application of hydrogen peroxide

Application hydrogen peroxide at the dose of 5 Lit (30%) per 1000 square meter water area (For 1 meter water depth) helps to reduce pathogenic bacterial load in the pond water considerably.

4.7. Commercial products

There are various commercial anti-bacterial preparation sold by different manufacturer by different trade names. They can be used only under proper guidance of aquaculture specialist by following manufacturer's instruction.

5. Measures to be followed at field level for control of bacterial diseases

For shrimp aquaculture, two most important factors for disease management is strict adherence to Better Management Practices (BMPs) and guidelines of Coastal Aquaculture Authority (CAA). The guidelines of CAA is available to their website www.caa.gov.in

- Optimum pond preparation: Optimum pond preparation is the first step for the shrimp culture. The pond should be dried for at least three weeks and the top black soil should be excavated. The pond bottom should be limed with proper dose, which depends upon pH of the soil. For the quantity of lime required, please refer to the CIBA guidelines. However if the pH is not below 7.5, apply a basal dose of 300-500 Kg. / ha.
- Biosecurity measures: Strict biosecurity measures should be adopted as per CAA guidelines
- Disinfect the pond water with optimum dose of bleaching at least 14 days before stocking. There should be at least 20 ppm of effective chlorine.
- Stocking density: The chances of spreading infectious diseases including WSD increases with increasing of stocking density. As per CAA guidelines, the maximum permissible stocking density is 30 and 60 per sq.m. in case of *P. monodon* and *P. vannamei*, respectively. This should be followed strictly.
- Never discharge pond water immediately after harvesting. After harvesting, bleach the water properly, hold for at least 7 days before discharging.
- Monitor the cultured shrimp regularly for any abnormal symptoms or behavior. If observed, report immediately to any aquaculture health expert.
- Monitor the water and soil-water-interface properly throughout the culture period. Test the water and soil-water-interface samples, periodically.
- Don't use antibiotics. Use of almost all antibiotics is banned. Use of antibiotics may give rise to antibiotic-resistant bacteria.
- As much as possible avoid exchange of water. Exchange of water will increase the probability of introduction of pathogen in the cultured ponds. During water exchange special care should be taken to avoid high fluctuation of water quality.
- Depth of water: The optimum depth of water for shrimp culture is 1.25 meter. For vannamei, it is 1.5 meter. Reduced depth of water causes stress in the cultured shrimp.
- Filter the intake water through net filter to prevent the entry of vectors of different pathogens.

- Monitor different water quality parameters through testing of water in any competent laboratory. Different crucial water quality parameters include pH, salinity, dissolved oxygen, total ammonia nitrogen, nitrate nitrogen, nitrite nitrogen, total alkalinity, dissolved inorganic phoshphate, etc. If really, necessary, perform partial water exchange (5-30 %).

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Diseases in Mud Crab Culture and their Management

Sanjoy Das, Leesa Priyadarsani, *M. Poornima, *R. Ananda Raja and *K.P. Jithendran

Kakdwip Research Centre of ICAR-CIBA, Kakdwip, West Bengal, India

** ICAR- Central Institute of Brackishwater Aquaculture, Chennai, India*

E-mail of corresponding author: sanjoy125@yahoo.co.in

1. Introduction

Seafood including crabs are one of the most important traded commodities worldwide. The most important farmed mud crab species is genus *Scylla.* Often culture of mud crab is affected with diseases causing heavy economic loss to the farmers. It may be caused by pathogens like bacteria, viruses, parasites and fungi. Combination of several pathogens also causes few diseases. Various disease conditions of the mud crab and their control measures are described in this chapter.

2. Bacterial diseases

Bacterial diseases (Bacteraemia) of crabs are caused by Vibrio spp., *Aeromonas* spp., *Rhodobacter*-like organism, etc.

2.1. Vibriosis

Vibriosis is caused by several species under Genus *Vibrio* and they are mostly ubiquitous in brackishwater environment. However in case of mud crabs, the *Vibrio* spp. infections are characterized mostly by bacteraemia and shell disease. Bacteraemia caused by *Vibrio* spp. has been reported in mud crabs (*Scylla serrata*). The major species involved are *V.alginolyticus, V. harveyi*, *V. parahaemolyticus, V. cholerae* , *V. vulnificus* and *V. fischeri.* Among different target organs, gills, gut, hepatopancreas, haemolymph and exoskeleton are primarily affected. Clinically, it is characterised by a marked reduction in haemocyte numbers, and intravascular clotting because of an endotoxin in the cell wall of the bacterium.

2.2. Shell disease

Shell disease of crabs is caused by a group of chitinolytic or chitinoclastic bacteria such as *Vibrio* spp., *Aeromonas* spp., *Pseudomonas* spp., *Alteromonas* spp., *Flavobacterium* spp., *Spirillum* spp., *Moraxella* spp., *Pasteurella* spp. and *Photobacterium* spp. These chitinolytic bacteria produce chitinase enzyme, which degrades the chitin present in the carapace of the crab. These result in difficulty in moulting with typical erosions and

pigmentations on the exoskeleton which are often viewed as box burns and black-spots. It can be led to septicaemic condition due to various pathogens.

Shell disease in mud crab

2.3. Red sternum syndrome

The Red sternum syndrome disease was reported in from Thailand in 2010. The affected animals found to be abnormal, with red sternum and orange to white haemolymph and decreased productivity of mud crab. These crabs had hard carapaces, red chelipeds and joints, pale hepatopancreas, gills, soft muscles, almost immobile and eventually dead. The haemolymph revealed three stages of the syndrome, namely orange, orange-white, and milky-white in color. These symptoms are similar to those of infected bitter crab disease (BCD) or Pink crab disease (PCD) reported in other crab species.While the cause of the syndrome is still unknown, in bacteria like *V. alginolyticus, V. Parahaemolyticus* and *Shewanella putrefacien* found to be associated with the disease.

3. Viral diseases

In recent years, viral diseases have become important limiting factors for shrimp production throughout the world. Many viruses affecting the shrimp also affect crabs as well. Some of the viruses affecting the crabs are briefly presented here.

3.1. White Spot Syndrome Virus (WSSV)

WSSV, which causes devastating disease in cultured shrimp, may also attack cultured crab species. However, mostly both wild and cultured crabs

act as a carrier of this virus. The occurrence of WSSV in *Scylla serrata* has been reported. Symptoms are inapparent and the crab can maintain the experimental WSSV infection for many months, though moulting frequency can be reduced. Natural WSSV infections are very often observed in wild crab species. Usually WSSV alone doesn't cause high degree of mortality in crabs. However, high degree of mortality due to WSSV is observed in case of co-infection with other diseases metazoan crustacean parasite infection, stalked barnacle infection, etc. In this case, the available symptoms are presence of white spots on the exoskeleton, chitinolytic spots on the abdominal flaps, broken claw and yellowish to pinkish discolouration, etc

3.2. Mud crab reovirus (MCRV)

Mud Crab Reo Virus (MRCV), which is a RNA virus causes 'sleeping disease' in Mud crab (*S. serrata*) with high mortality rate and economic loss was reported from China in 2007. The virus is icosahedral non-eneveloped virus of 70 nm diameter. It infects the connective tissue cells of hepatopancreas, gill and intestine in mud crab. An RT-PCR detection method for the diagnosis of MCRV has been developed and could be detected in all tissues in advanced stage of the disease. MCRV is reported from India recently

3.3. Baculovirus

Baculovirus infection in crab produces intra-nuclear inclusion bodies in the hepatopancreas epithelium. However, this virus doesn't cause any severe disease in crab.

3.4. Muscle necrosis virus

This disease is caused by an icosahedral virus with around 150 nm in diameter.

4. Parasitic diseases

4.1. Cirripeds (Barnacles)

Usually the barnacles have symbiotic relationship with crab. However in case of environmental stress condition, very often they act as opportunistic pathogen. Recently during 2019-21, the infestation by stalked barnacle *Octolasmis* spp. was found to cause diseases condition of crab with high degree of mortality in cultured mud crab (*Scylla serrata*) at different regions of China and India. This barnacle was also found to infect Sundarban orange crab (*Scylla olivacea*) in Sundarban area of West Bengal. Other important barnacle species, which affect cultured crabs, are *Loxothylacus texanus, Sacculina lata* Boschma, *Chelonibia patula, Balanus venustus, Balanus eburneus,* etc.

Mud crab infested with stalked barnacle *Octolasmis* spp

4.2. Microsporidiasis

These group of parasites are intracellular spore-forming parasites. The important genus of Microsporidia, which infect crab, are *Pleistophora*, *Thelohania, Ameson* and *Nosema.* The spores of the microsporidia are very rigid structure and can withstand the extreme and adverse environmental condition. Infected crabs become inactive, muscles appear chalky white through joints of the appendages, and the abdomen may appear greyish.

4.3. Milky disease

This is an emerging disease of cultured crab and is caused by the dinoflagellate parasite of the genus *Hematodinium.* A large number of crab and lobster species have been reported to be hosts for this parasite. This disease is mostly prevalent in temperate northern hemisphere. The crabs appear to be most vulnerable to infection immediately following moulting. These parasites proliferate rapidly in different tissues and haemolymph. It causes reduction of number of blood cells in infected crab and causes 'milky' appearance of blood. Haemolymph becomes chalky yellow in colour with markedly reduced clotting ability. Severe and heavy infection cause lethargy, inappetence in crab whereas, mild infection is mostly unnoticed without any symptoms. Lightly infected crabs exhibit no overt sign of infection.

5. Fungal diseases

The commonly occurring fungal species, which cause diseases in crabs, are *Lagenidium, Atkinsiella, Haliphthoros* and *Fusarium*. Morbidity and mortality

vary depending upon environmental conditions. Environmental factors such as rainy season, humid condition and low salinity are found to increase the fungal growth. In case of larval infection, it starts with egg stage. As the hatchling grow up, fungus ramifies throughout the body and tissues of the crablet. Ultimately, the entire body becomes covered with mycelia of the fungi. Zoeal stages may become infected during the moulting. Growth of fungus *Fusarium* exhibits cotton wool like growth. The fungus infection can be prevented by filtering the water thoroughly, treating the water chemically and/or irradiation. Adding malachite green at 0.001 to 0.006 mg per litre of water and treflan at 0.01 mg per litre are also found to be effective in controlling the fungal infections.

6. Non-infectious diseases

6.1. Shell discoloration

Crab shells are covered by yellowish-brown to reddish-brown deposits, which are more obvious on the whitish ventral side but easily come off after scraping. It is caused by low soil and water pH. Acidic soil and water cause formation of iron precipitates on crab shells. Etiological factor leading to discoloration may adversely affect other organs like the gills and eyes. Exposure of crabs to acidic soil and water causes impairment of normal metabolism, leading to growth retardation and death.

6.2. Deformities

Deformities are mostly due to failure or delay in moulting from zoea to megalopa and it leads to abnormal swimming behaviour in hatchery phase. Environmental parameters and nutrient inadequacy can be considered as major causes for the delay or failure of moulting.

Limb deformities in mud crab

6.3. Blackened ovaries

This condition is also known as ovarian discolouration, which is usually caused by the deposition of the pigment melanin as a result of mechanical or microbial injury. This ovarian discolouration ranges from orange with black spots to black with orange spots. It leads to spawning failure or incomplete spawning effecting reduced fecundity. This problem can be prevented by supplementation of Vitamin C along with feed.

6.4. Incomplete moulting

Moulting is also known as Ecdysis, which is procedure of shading exoskeleton in case of Crustaceans animals including crabs under influences of certain hormones. The effective moulting is very much important for proper growth of crustaceans. Incomplete moulting is caused by low temperature, nutritional deficiencies, mineral imbalance and hormonal problem. If the conditions are not suitable for complete moulting, the affected animals fail to shed off their old shells. In this case. the parts of the old shell remain attached to the new shell. Incomplete moulting not only affects the growth, but also results in abnormal movement and swimming behaviour rendering the crabs easy prey to healthy individuals.

6.5. Limb loss

Crabs shed their limbs through the process of autotomy to escape from the predators. There are some other factors, which causes loss of limbs. These include over-exposure to dry environment, physical stress due to traumatic moult, cannibalism, etc. Often, the lost limb regenerates. But regenerated limb usually doesn't restore to its original function and it remains relatively smaller than other limbs. If the major appendage like cheliped is lost, then it may lead to impaired feeding, mating and capacity of defence. This condition can be prevented by providing shelters to prevent aggressive behaviours, proper feed management and avoidance to exposure to dry environment.

7. Prevention and control strategy

Like other cultured aquatic species, the famous age-old proverb 'prevention is better than cure' is also applicable in case of crabs. Some of the suitable control measures for prevention of occurrence of diseases in crabs are as follows:

- Grow-out crab culture should always be stalked with disease-free healthy crablets.
- Use of wild broodstock or crablets should be strictly prohibited.

- Optimal stocking density should be maintained. Over-stocking should strictly be avoided.
- Potential vectors (both living and non-living) should be treated appropriately or eliminated from the farm. Appropriate disinfection of non-living vectors such as vehicles, nets, equipment and wastewater can control entry and spread of any infectious diseases.
- Water exchange should be avoided during culture period to reduce the risk of introduction of infectious agents. Water exchange should be done if it is really required and in such cases, only properly disinfected clean water should be used for exchange. There should not be much difference of pH and salinity between existing water and water to be added.
- Movements of people and equipment should be restricted between ponds (or other farming units) to reduce the risk of spreading infection.
- Farm-specific overall health management programme should be in operation to minimize stress to crabs by optimizing the pond environment
- Strict biosecurity measures should be adopted to prevent the entry of wild crabs, birds and other vectors.
- Cultured crabs should be regularly monitored for any abnormal symptoms, disease condition, etc. throughout the culture period.
- Judicious use of probiotics, phage therapy, and immunostimulants can be done with consultation with aquaculture specialists.

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Diseases of Brackishwater Finfishes in India

T. Bhuvaneswari., M. Poornima, P. Ezhil Praveena and S.K. Otta

ICAR- Central Institute of Brackishwater Aquaculture, Chennai, India

E-mail of corresponding author: t.bhuvaneswari@icar.gov.in

1. Introduction

Brackishwater finfish aquaculture is a successful fish farming gaining importance in India. Among the domesticated brackishwater finfishes, high valued species like Asian Seabass (*Lates calcarifer*), Milk Fish (*Chanos chanos*), Pearl Spot (*Etroplus suratensis*), Cobia (*Rachycentron canadumcanadum*) and GIFT tilapia are alternative for shrimp farming in fetching profit to the aquafarmers. Intensification of domesticated finfish are vulnerable to viral and bacterial diseases. Viral diseases like *Viral Nervous Necrosis* (VNN), *Red sea bream Irido viral* disease (RSIVD), *Tilapia lake virus* disease (TiLVD) are three important viral diseases reported from India. Vibriosis is the bacterial disease reported as secondary pathogens in cultured brackishwater finfishes.

2. Viral Nervous Necrosis (VNN)

VNN also called as *Viral Encephalopathy and Retinopathy* (VER) is economically significant viral disease causing loss to fish culture to major extent. The virus is distributed across the world infecting freshwater, brackishwater and marine fishes belonging to 120 species from 30 families of fishes. . The virus is classified under the family Nodaviridae and genus betanodavirus. The virus is non-enveloped with icosahedral symmetry. VNN virus is about 25 nm in diameter. The genome of the virus consists of two single stranded positive sense RNA molecules, RNA1 and RNA2 of 3.1 kb and 1.4 kb in size respectively. Betanodavirus is classified into four genotypes viz. tiger puffer nervous necrosis virus (TPNNV), striped jack nervous necrosis virus (SJNNV) (type species), barfin flounder nervous necrosis virus (BFNNV), and red-spotted grouper nervous necrosis virus (RGNNV) . RGNNV is the only genotype reported in India.

Clinical signs depend on the age and size of the fish. Erratic swimming behaviour such as whirling movements, swimming with belly-up due to hyperinflation of swim bladder, skin darkening and loss of appetite are some of the common clinical signs observed in most of the species. Adult and sub-adult fish do not exhibit clinical signs and remain as asymptomatic carriers. The virus is transmitted both horizontally and vertically. Virus is shed through gills, skin and intestine aiding in horizontal transmission and

spread by transport of infected larvae and juveniles for culture. Prevalence of virus in wild fish also serves as a source of infection to the farmed fish. Contaminated inanimate objects can serve as a source of the contaminated virus. Invertebrates acts as carriers of the virus and can be a potential source of infection to susceptible population. Live feed organisms such as artemia and rotifer can also harbour the virus and aid in its transmission. The virus also reaches gonads and shed through eggs and milt aiding in vertical transmission of the virus. VNN affects mostly larval stages and early juveniles. However, adult and market size fishes are also reported to be affected. Fish surviving VNN remain positive for the virus for more than a year indicating that these fish remain as subclinical carriers of the virus transmitting the virus to susceptible population. NNV infection in fish results in initial viremia during which the virus spread through the circulatory system followed by neuronal spread. NNV replication is fast and the virus genome can be detected as early as 4 hours post infection in brain of infected pompano. The clinical signs appear between 3-10 days post infection in experimentally infected fish. In early life stages the virus causes hyperplasia and epithelial degeneration in gill operculum, skin and oral cavity.

Diagnoses of VNN are done with whole larvae or the brain, spinal cord and retina of fingerlings and adult fish, which are the organs of predilection. The only practical way of preventing disease outbreaks in hatcheries and nursery rearing facility is to screen and use virus free brooder and seeds and to vaccinate the brooders and juveniles. Vaccination of Asian seabass (*L.calcarifer*) and grouper (*Epinephelus tukula*) brood stock have been reported to prevent or reduce the risk of vertical transmission of the virus. Two inactivated vaccines are commercially available in European market viz., ALPHA JECT micro® 1 Noda and Icthiovac® VNN.

3. Red sea bream iridovirus disease (RSIVD)

Emerging viral pathogen causing systemic fatal infection in many wild and cultured fishes. In 1990 the first outbreak of megalocytivirus disease was recorded in cultured red sea bream (*Pagrus major*) from Japan. The causative agent was designated as red sea bream iridovirus (RSIV). Subsequently, different megalocytivirus isolates were identified and reported. The type species infectious spleen and kidney necrosis virus (ISKNV) is closely related to red sea bream iridovirus. OIE Reference Laboratory designates both the genotypes RSIV and ISKNV as causative agents of RSIVD and both are notifiable pathogens. Iridovirids are enveloped icosahedral DNA viruses of 120–200 nm in size, with genome ranging from 103 to 220 kbp. The host range of Megalocytiviruses is diverse with

widespread geographical distribution and freshwater and brackish water fish species are the predominant species affected. RSIV has been reported from several Asian countries. While RSIV infection is reported in 40 fish species, outbreaks of *Megalocytivirus* in *L. calcarifer* and tilapia farms have caused considerable mortalities with huge economic losses in Vietnam, USA and Thailand. In India, a disease outbreak due to RSIV in cultured Asian seabass (*L. calcarifer*) has been reported.

High mortality rates of infected fish were reported with the clinical signs including lethargy, anorexia, unusual swimming behaviour, exophthalmos, skin lesions, dark discolouration, haemorrhage and fin erosion. Big belly, red eye including skin nodules, frayed fins, opaque eyes, loss of scales, exophthalmia, anorexia, decolouration of skin, skin haemorrhages, hypersecretion of mucus, gill pallor ascites and distended abdomen are some of clinical signs observed in different fish species. Experimental studies showed the virus spread by both vertical and horizontal modes. Movement of infected carriers and the ornamental fish trade are the most common means of spread of RSIVD to new host species in geographically distant regions. Farmed seabass fish had massive mortality (~50%) due to dual infections of *Megalocytivirus* and piscine *Nodavirus* (NNV). Diagnoses by various rapid and sensitive confirmatory PCR based methods are available as diagnostic for RSIVD. LAMP assay and LAMP combined with a lateral flow dipstick (LFD) are developed for rapid and visual detection of RSIV. To control and prevent megalocytivirus infections quite a few vaccines have been developed and evaluated experimentally. The implementation of stringent biosecurity practices such as introducing pathogen-free fish; implementing on farms hygiene practices; and avoiding stress inducing practices like poor water quality overcrowding and overfeeding would be practical to avoid a major impact on susceptible cultured food fishes and likely negative effects on wild fish populations as well.

4. Tilapia lake virus disease (TiLVD)

TiLVD is a highly contagious emerging disease caused by a relatively novel pathogen termed as *Tilapia tilapinevirus*, which is commonly known as tilapia lake virus. The disease was first reported from Israel in 2009, and the causal agent was identified as tilapia lake virus (TiLV) provisionally positioned as a novel virus in the Family *Orthomyxoviridae*. International Committee on Taxonomy of viruses (ICTV), TiLV called as Tilapia tilapine virus is a single novel type species in the new genus Tilapine virus, currently placed under the new family, Amnoonviridae, belonging to the order Orthomyxoviridae (Articulavirales). TiLV is an enveloped icosahedral virus

measuring about 55 to 75 nm in diameter with a 10–15-nm wide surface projections. The clinical features of TiLV in infected tilapines from various geographical locations include skin erosions, lethargy, abdominal distension, scale protrusion, gill pallor, open wounds, dark discoloration , fin rot and ocular lesions such as opacity of the lens (cataract) uveitis, buphthalmia and phthisis bulbi. TiLV has been identified from wild tilapines, experimentally TiLV caused infection in finfishes. TiLV genomic RNA was detected in wild river barb (*Barbonymus schwanenfeldii*) without clinical signs. TiLV virus has been reported from different countries from various geographical locations across. The modes of transmission includes both horizontal and vertical. Mortality rates ranged from 5% to 90% in different outbreaks, whereas >70% mortalities were observed in cage cultured red tilapia in Thailand and GIFT tilapia in open floating cages in India. The pathogenesis of TiLV disease remains unknown. Based on research studies TiLV spread through direct contact of skin, mucus of infected fish, direct gills exposure or oral route probably enters into susceptible fish and systemically spread to internal organs like spleen, liver, kidney and gonads. Besides, during a TiLV infection severe liver damage and gastrointestinal tract disruption, enteropathy, coinfections, multiple organ failure and secondary bacterial infection in affected fish may be contributing to the mortality of fish. Fish mucus and blood and liver biopsy could be used as non-destructive methods for screening of TiLV. Prevention and control strategies should include improving biosecurity and stocking practices. Quarantining period for imported stocks held at 25°C should be implemented with daily monitoring for development of clinical signs of TiLV disease, including, screening of sick fish to rule out TiLV and other diseases. Strict biosecurity measures of farming practices will ensure the prevention of TiLV.

5. Vibriosis

The disease is caused by Gram negative bacteria in the family Vibrionaceae. This group of bacteria includes two important genera which can be significant fish pathogens. Bacteria in the genus *Vibrio* are significant pathogens of marine and brackish water fish, although they occasionally are reported in freshwater species. *V. anguillarum, V.alginolyticus, V. harveyi, V. parahaemolyticus, V.ordalli, V. vulnificus, V.damsela, V. carchariae and V.salmonicida* of *Vibrio* have been associated with disease in fish. The disease can cause significant mortality (50%) in fish culture facilities once an outbreak is in progress. Common names for *Vibrio* infections of fish include salt-water furunculosis, red boil, and pike pest. *Vibrio* infections can spread rapidly when fish are confined in heavily stocked, commercial

systems and morbidity may reach 100% in affected facilities. Clinical signs usually start with lethargy and a loss of appetite. As the disease progresses, the skin may become discolored, red and necrotic (dead). Boil-like sores may appear on the body, occasionally breaking through the skin surface resulting in large, open sores. Bloody blotches (large discoloured mark) or erythema are common around the fins and mouth. When the disease becomes systemic, it can cause corneal lesions with ulceration and evulsions of the orbital contents called exopthalmia ("pop-eye"), and the gut and rectum may be bloody and filled with fluid. Extremely pathogenic *Vibrio* infection of cold-water marine fish (i.e., salmon) is caused by *V. salmonicida* and is referred to as "cold-water vibrio" or "hitra" disease. Cold-water vibrio has not been reported in warm-water fish. Formalin-inactivated vaccines are commercially available against *V.anguillarum*. Bacterial diseases are treated best by injection or use as food additives or antibiotics. Oxytetracycline, Sulfamerazine, Nitrofurans, Furazolidin and Erythromycin are commonly used antibiotics and proper withdrawal period should be given to get rid of antibiotic residues before harvesting.

6. Sampling of fish for disease diagnosis

If there is a disease outbreak, number of similarly affected animals should be examined. The commonly recommended number of fish to examine is 5-10 fish. Fish that are alive, suspected to be infected showing the symptoms are the most useful specimen to sample. The sample should be taken from all damaged or affected tissues. Moribund samples can also be used for sampling. The number of fish being screened must be representative of the entire population and information on the fish species, age or growth should be collected. More fish specimens required for examining rare infection than those are need to detect a high prevalent infection. Important components are necessary to implement an effective detection and control programme.

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Molecular Tools for Rapid Diagnosis of Shrimp and Brackishwater Finfish Diseases

P. Ezhil Praveena, T. Bhuvaneswari, S.K. Otta and M. Poornima

ICAR- Central Institute of Brackishwater Aquaculture, Chennai, India

E-mail of corresponding author: ezhilpraveena@gmail.com

1. Introduction

"Diagnosis is not the end; it is the beginning for accurate treatment"

Shrimp aquaculture is a promising sector giving livelihoods to many people of our country. Shrimp aquaculture industry has experienced rapid changes over the last few decades due to intensification of culture it is facing many impediments. One major glitch for its growth is infectious diseases in culture ponds. Infectious diseases are becoming increasingly difficult to manage and they have to be diagnosed in right time in laboratories. 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. It is critically important that laboratories should be equipped to identify disease accurately. Diagnosis is the foundation for decision-making in treatment. A diagnostic error may result in treatment fault or incorrect medications. Nowadays molecular method of disease diagnosis is more popular and it helps diagnosing the disease very rapidly.

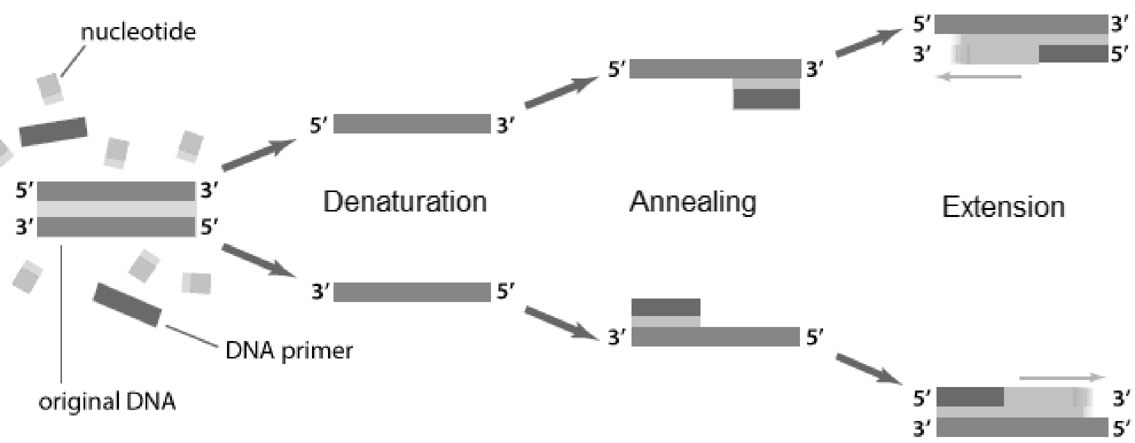
2. Importance of molecular diagnosis

Conventional diagnostic methods are used in case of identifying the infectious cause, it is more time consuming and lack sensitivities to detect latent pathogens. Efforts to overcome these problems have led to the development of molecular diagnostic methods. These molecular techniques facilitate the specific detection with high sensitivity, thus allowing rapid screening of viral pathogens.

3. Polymerase Chain Reaction (PCR)

This technique has become an indispensable tool in modern molecular biology and has completely transformed scientific research. It has also opened up the investigation of cellular and molecular processes to those outside the field of molecular biology and consequently also finds utility by scientists in many disciplines. Whilst PCR is itself a powerful standalone technique, it has also been incorporated into wider techniques, such as cloning and sequencing, as one small but important part of these workflows.

It is a highly sensitive and robust technique for detection of shrimp pathogens. PCR is a nucleic acid amplification technique wherein a specific portion of nucleic acid from a target organism is amplified using oligonucleotide primers that are specific for the portion of the DNA to be amplified. The components of PCR reaction are DNA template (the sample DNA that contains the target sequence to amplify), Deoxyribonucleoside triphosphates (dNTPs), PCR buffer, Primers (forward and reverse), Taq polymerase. The reaction is performed in several cycles, each cycle consisting of three steps (a) *DNA denaturation*: In this step, the target DNA strands are separated by heating to about 95°C, (b) *Primer annealing*: In this the primer binds specifically to the target region. This step is carried out at 55-65°C, (c) Primer extension: where in 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 thermo stable.



Steps in DNA molecule amplification by polymerase chain reaction

3.1. Nested PCR

Nested PCR technique has higher sensitivity; hence even if the sample contains lower DNA, it can be amplified which is not feasible in the conventional PCR technique. In this technique, two sets of PCR primers are sequentially used. Nested PCR is a useful modification of PCR technology wherein the specificity of the reaction is enhanced by preventing the non-specific binding with the help of the two sets of primer. The first set of primer binds outside of our target DNA and amplifies larger fragment while another set of primer binds specifically at the target site. In the second round of amplification, second set of primer amplifies only the target DNA. Nested PCR is a helpful method for detection of different pathogens.

3.2. Multiplex PCR

Multiplex PCR is a common molecular biology technique used for the amplification of multiple targets in a single PCR test run. In Multiplex PCR, multiple primers and a temperature-mediated DNA polymerase are used for the amplification of DNA in a thermal cycler. 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. 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 the primers pairs designed for Multiplex PCR has to be optimized so that the same annealing temperature is optimal for all the pairs during PCR. When multiple sequences are targeted at once, additional information can be generated from a single test run which otherwise would require a larger amount of the reagents and extensive time and effort to perform. 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. In diagnostic laboratories, multiplex PCR is useful to detect different microorganisms that cause the same types of diseases.

3.3. Hot start PCR

This is a novel form of conventional PCR where in it reduces the occurrence of undesired products and formation of primer-dimers due to non-specific DNA amplification at room temperatures. The basic principle of hot-start PCR is the separation of one or more reagents from the reaction mix until the mixture reaches the denaturation temperature upon heating. Hot start PCR significantly reduces non-specific binding, the formation of primer-dimers, and often increases product yields. It also requires less effort and reduces the risk of contamination.

3.4. Reverse-transcription PCR (RT – PCR)

In reverse-transcription PCR, the RNA target 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.

4. Real-time PCR

Conventional PCR is a time-consuming process where the PCR products are analysed through gel electrophoresis whereas real time PCR facilitates the analysis by providing real time detection of products during the exponential phase. It eliminates post-PCR analysis process. The principle of real-time PCR depends on the use of fluorescent dye. The concentration of the nucleic acid present into the sample is quantified using the fluorescent dye or using the fluorescent labelled oligonucleotides. Real-time PCR is used to amplify and simultaneously quantify a targeted DNA molecule and enables detection and quantification of the viral pathogen in the infected shrimp tissues. 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. Real-time PCR uses specialized thermal cyclers equipped with fluorescent detection systems that monitor the fluorescent signal as the amplification occurs. Quantitative PCR (qPCR), also called real-time PCR or quantitative real-time PCR, is a PCR-based technique that couples amplification of a target DNA sequence with quantification of the concentration of that DNA species in the reaction. Probes bind specifically to DNA target sequences within the amplicon and use the principle of Förster Resonance Energy Transfer (FRET) to generate fluorescence via the coupling of a fluorescent molecule on one end and a quencher at the other end. 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 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.

4.1. Reverse transcription-quantitative PCR (RT-qPCR)

Reverse transcription (RT) -PCR and RT-qPCR are two commonly used PCR variants enabling gene transcription analysis and quantification of viral RNA, both in clinical and research settings. Reverse transcription is the

process of making cDNA from single-stranded template RNA and is also called first-strand cDNA synthesis. The first step of RT-PCR is to synthesize a DNA/RNA hybrid between the RNA template and a DNA oligonucleotide primer. The reverse transcriptase enzyme that catalyzes this reaction has RNase activity that then degrades the RNA portion of the hybrid. Subsequently, a single-stranded DNA molecule is synthesized by the DNA polymerase activity of the reverse transcriptase. High purity and quality RNA are essential for a successful RT-PCR and can be performed following two approaches: one-step RT-PCR and two-step RT-PCR. In the one step RT-PCR, the RT reaction and the PCR reaction occur in the same tube, while in the two-step RT-PCR, the two reactions are separate and performed sequentially.

4.2. 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 109 copies at 60–65°C within 1 h. 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 amplicon 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, Thermocycler 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 WSSV, IMNV, IHHNV, MBV, TSV, YHV, and HPV.

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

The PCR–ELISA is an alternative method for the detection of nucleic acids which mimic enzyme linked immunosorbent 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 are hybridized to an immobilized probe. This can be carried out with a simple and inexpensive water bath so that a thermal with sequences internal to the PCR

product. Thus, it is an alternative and less expensive technique than real-time PCR. It is 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 can be used for field level applications where large numbers of samples can be analyzed simultaneously.

5. CRISPAR based diagnosis

Diagnostics based on the detection of nucleic acids are among the most sensitive and specific, yet most such assays require costly equipment and trained personnel. Recent developments in diagnostic technologies, is Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), aim to enable accurate testing. CRISPR-based diagnostic methods rely primarily on identifying a certain sequence associated with a disease and then cleaving it in order to produce a readable signal. Examples of target sequences include oncogenic mutation sequences or viral and bacterial sequences derived from the infectious agent. The goal of CRISPR systems is to identify the specific pathogens, as well as to repair alleles that cause disease through specific DNA sequence editing at exact locations on the chromosome. They have the capacity not only for detection of pathogens during an epidemic, but also in genetic disease detection. The highly sensitive nature of CRISPR diagnostic tests is derived from the fact that most are able to utilize fluorescent probes which are highly sensitive. The tests can proceed at a rapid pace since it is not necessary to culture isolates or extract genomic DNA. Further diagnosis of infectious diseases using methods such as PCR requires high expertise and sophisticated equipment, all of which are limited especially in underdeveloped countries. The CRISPR-based diagnostic system makes it possible to diagnose infections with the same accuracy of conventional methods but with the lower cost. CRISPR-Cas technology is highly flexible RNA-guided endonuclease (RGEN)-based nucleic acid editing tool. The immune response provided by the CRISPR-Cas system includes three stages, viz. adaptation, pre-CRISPR RNA (crRNA) expression/processing, and interference. The adaptation stage begins with the expression of a complex of Cas proteins by the CRISPR-Cas loci and binding of these Cas proteins to the target DNA sequence, followed by two double-strand breaks in the target DNA based on the Protospacer Adjacent Motif (PAM) which is a distinct short motif of 2-4 bases. The released segment of the target DNA, called as protospacer, is inserted between two repeats of the CRISPR array and then acts as a new spacer. The transcription of CRISPR array occurs in expression processing stage to produce a single long pre-crRNA which is processed by a distinct set of Cas proteins to generate mature crRNA. This is

followed by the interference stage wherein the mature crRNA, bound to the processing complex, acts as guide RNA to recognize similar sequences in the invading viral RNA that is then cleaved and inactivated by one of the Cas proteins. The CRISPR-based diagnostic techniques are rapid, sensitive, specific, accurate, cheap, and reliable; provide huge potential for applications in a wide range of disease diagnosis in shrimp.

6. Probe Techniques

The development of non-radioactive labeling of nucleic acid fragments 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 *P. vannamei* viz IHHNV, TSV, YHV, WSSV, HPV, MBV etc.

7. Lateral flow immunodiagnostic tests

Recently, lateral flow chromatographic immunodiagnostic strips are available for shrimp disease diagnosis like WSSV, EHP and vibriosis. These tests can be easily performed by unskilled farm personnel and can diagnose shrimp disease outbreaks at the pond side at an earliest. The strips are relatively cheap and diagnosis can be made very rapidly.

8. Conclusion

Molecular diagnostic methods, revolutionised the disease treatment, an important aspect of any disease control program is easy and convenient, rapid and reliable pathogen detection methods, all together with the ability to interpret results and apply them in a proper manner in health management programs. PCR and RT-PCR methods have been very important in contain the spread of major shrimp disease agents, but they have the disadvantage like requirement of sophisticated equipment and highly trained personnel.

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Emerging Microbial Diseases and Issues in Brackishwater Aquaculture

T. Jawahar Abraham

Department of Aquatic Animal Health, Faculty of Fishery Sciences,

West Bengal University of Animal and Fishery Sciences,

Kolkata 700094, India

E-mail of corresponding author: abrahamtj1@gmail.com

1. Introduction

The aquaculture and fishery sector, a source of cheap and nutritious food and a foreign exchange earner plays a major role in the socio-economic development of the country. It has been recognized as a powerful income and employment generator. India is the second-largest aquaculture fish-producing nation in the world after China, contributing about 8.61% of the global fish production. Shrimp aquaculture has witnessed rapid growth in the last couple of decades. Shrimp is the largest single seafood commodity by value, accounting for 17% of all internationally traded fishery products. Approximately 75% of production is from aquaculture, which is now almost entirely dominated by two species namely the black tiger shrimp (*Penaeus monodon*) and the Pacific white shrimp (*Penaeus vannamei*) that represent the two most important invertebrate food animals. Since 2017, India has become the fourth major exporter, boosted by a steep increase in farmed shrimp production. However, after peaking at USD 7.2 billion in 2017, the value of India's exports declined by 3% in 2018 and by a further 1 percent in 2019 (USD 6.8 billion), driven primarily by a decline in shrimp prices. The rapid growth of aquaculture and the intensification of shrimp farming have increased the complexity of the health challenges for the industry. The incidence of diseases is on the surge with the expansion of the shrimp culture sector. The impacts of emerging diseases on aquatic animals have been substantial. The most devastating economic and social impacts have been in shrimp aquaculture for which it was estimated in 1996 that the global direct and indirect costs of emerging diseases had reached $US 3 billion annually or 40% of the total production capacity of the industry. White spot virus (WSV) has been by far the most devastating of the shrimp pathogens. It has been estimated that the impact of WSV in Asia alone during the 10 years after its emergence in 1992 was $US 4–6 billion. Recent FAO and World Bank estimates indicate that, annually, the aquaculture sector suffers losses of over 6.5 billion US Dollars due to diseases. The consequences of disease emergence for some countries have been so severe

that shrimp production has never fully recovered. Shrimp diseases are the shrimp farming industry's biggest concern. In Asia, diseases cost the shrimp industry billions of dollars annually (Shinn, et al., 2018). In India, the total employment loss due to diseases was estimated to be 1.65 M man-days worth US$ 7.07 M. A recent study revealed that the overall probability of infectious disease occurrence in India was at 49% leading to an annual loss of 0.21 M ton shrimp worth US$ 1.02 billion. The impacts of diseases and their severe outcome impinge more on the shrimp farming community. To uphold the sustainability of the shrimp industry a brief account of the management strategies to control the emerging shrimp diseases that can be routinely followed by the farmers is provided below:

2. Hepatopancreatic Microsporidiosis (HPM)

2.1. Signs of disease

- Severe growth retardation/ growth stunting
- Disparate growth, i.e., high size variation
- Floating white faeces (White faeces observed with other diseases also) in ponds
- Low-level mortality associated with a secondary bacterial infection

2.2. Disease agent

The disease is caused by a microsporidian parasite - *Enterocytozoon hepatopenaei* (EHP).

2.3. Susceptible stages of the host

All stages

2.4. Diagnostics

PCR method

- Sample for PCR test – Hepatopancreas (HP) and faecal samples
- Histopathology

2.5. Host range

EHP infects *Penaeus monodon* and *Penaeus vannamei*

2.6. Vector

None identified

2.7. Transmission mode

- Horizontal and vertical

- Primarily through oral route (through faeces and cannibalism of infected shrimp)
- EHP spore present in the pond sediment will cause fresh infection

2.8. Management

- If a pond is affected by EHP then, the following pond soil treatment is suggested for subsequent crop
- The spores of EHP have thick walls and are not easy to inactivate. Even high levels of chlorine alone are not effective
- For pond sediment treatment apply CaO (quick lime, burnt lime, unslaked lime, or hot lime) @ 6 ton/ha
- Plough the CaO into the dry pond sediment (10-12 cm) and then moisten the sediment to activate the lime
- Leave for one week before drying or filling
- After application of CaO, the soil pH should rise to 12 or more for a couple of days and then to the normal range as it absorbs carbon dioxide and forms $CaCO_3$
- Hatcheries should test fresh feeds and artemia by PCR method
- Stock PCR negative seed, healthy and strong seed
- Stock seed in ponds with good plankton/bloom

3. Acute Hepatopancreatic Necrosis Disease (AHPND)

An emerging shrimp disease, i.e., early mortality syndrome (EMS) or acute hepatopancreatic necrosis disease (AHPND) has been reported to cause significant losses among shrimp farmers in China (2009), Vietnam (2010), and Malaysia (2011). It was also reported to affect shrimp in the eastern Gulf of Thailand. The disease affects both *P. monodon* and *L. vannamei* and is characterized by mass mortalities (reaching up to 100% in some cases) during the first 20-30 days of culture (post-stocking in grow-out ponds). Clinical signs observed include slow growth, corkscrew swimming, loose shells, as well as pale coloration. Affected shrimp consistently show an abnormal hepatopancreas (shrunken, small, swollen, or discouloured). Histological examination showed that the effects of AHPND in both *P. monodon* and *L. vannamei* appear to be limited to the hepatopancreas (HP) and show the following pathology:

- Lack of mitotic activity in generative E cells of the HP;
- Dysfunction of central hepatopancreatic B, F, and R cells;
- Prominent karyomegaly and massive sloughing of central HP tubule epithelial cells;

- Terminal stages include massive intertubular hemocytic aggregation followed by secondary bacterial infections.

3.1. Signs of disease

- Often pale to white hepatopancreas (HP)
- Significant atrophy of HP, I e., shrinkage of HP
- Often soft shells and guts with discontinuous contents or no content
- Black spots or streaks sometimes visible within the HP
- HP does not squash easily between thumb and finger
- Onset of clinical signs and mortality from the tenth day of stocking
- Moribund shrimps sink to the bottom or floats sometimes

3.2. Disease agent

The disease is caused by a pathogenic strain of *Vibrio parahaemolyticus* containing a virulence plasmid/toxin gene

3.3. Susceptible stages of the host

Juveniles

3.4. Diagnostics

- PCR method targeting the toxin gene
- Histopathology

3.5. Host range

AHPND affects *Penaeus monodon* and *Litopenaeus vannamei*

3.6. Vector

None identified

3.7. Transmission mode

Horizontal and vertical

3.8. Management

- When signs of AHPND / EMS disease is observed immediately submit samples for analysis immediately to reputed labs and inform appropriate authorities
- Stock PCR negative seeds
- Select healthy and strong seeds
- Nursery rearing of PL
- Stock seed in ponds with good plankton/bloom

- Monitor and maintain *Vibrio* load under control

3.9. Infrastructure improvements

- Provide central drain in ponds to flush out organic wastes
- Position aerators in the pond to concentrate and help to remove organic wastes towards a central drain
- Provide nursery in the farm to rear PL for 25 - 35 days
- Remove sludge after every crop
- Stock salinity resistant strain of tilapia in cages @500 - 800 nos/ha in nursery
- Use tilapia culture water to manage AHPND

The following products/technology are reported to help manage AHPND: Probiotics, tilapia culture water, use of bacterial biofloc, bioremediators, toxin binders, immunostimulants, vitamins, phage therapy, etc.

4. Covert Mortality Disease (CMD)

4.1. Signs of disease

- Atrophic or pale HP
- Whitish abdominal muscle
- Partial/empty stomach and guts
- Softshell, slow growth

4.2. Mortality pattern

- Shrimp dies at the pond bottom
- Mortality during 30 - 80 Days of culture (DOC)
- Moribund and dead shrimp can be found daily
- Disease worsens after 60 - 80 DOC accompanied by high NO_2-N and temperature > 28°C
- Cumulative mortality up to 80 %

4.3. Disease agent

Covert Mortality Noda Virus

4.4. Diagnostics

- Reverse transcription (RT)-PCR method
- Sample for PCR test - Haemolymph, Hepatopancreas (HP), muscle
- Histopathology

4.5. Host range

CMNV infects *Penaeus vannamei*

4.6. Vector

None identified

4.7. Transmission mode

Horizontal

4.8. Management

- Stock PCR negative seed
- Stock healthy and strong seeds

5. White gut/faeces syndrome

- Vermiform, gregarine-like bodies within HP and midgut
- Show no cellular or subcellular organelles
- No gregarine, other protozoan or metazoan involved.
- Microvilli found peeled away from HP tubule cells and aggregated in HP tubule lumen
- Stripped of microvilli, the originating cells undergo lysis.
- Cause is unknown, but the loss of microvilli and subsequent cell lysis indicates a pathological process.
- May retard shrimp growth and may predispose them to opportunistic pathogens.

6. Shrimp health management

The production cycle of shrimp farming consists of four components: broodstock, hatchery, nursery, and grow-out. According to the FAO, each of these components would require clean water, clean rearing facilities, clean feed, hygienic protocols, and dry-out and break-cycle practice. Shrimp disease treatment is not easy; often, it is more complex than disease prevention. No single solution fixes all problems, but there are preventive measures that keep pathogens from filtering through shrimp ponds and that keep shrimp healthy. The FAO recommends the following best practices in shrimp culture:

6.1. Choose the right shrimp genetics

Specific pathogen-free (SPF) and high performers for high biosecurity systems. Better biosecurity in shrimp aquaculture begins with clean broodstock that supplies eggs and nauplii for hatcheries. The broodstock, whether SPF or non-SPF, should be carefully sourced and certified. This is

the first and most important step to take, as it could prevent the disease carriers from entering the culture system.

6.2. Keep pathogens out

Dry out facilities regularly; disinfect fill and exchange water; control feed, and prevent air and droplet contamination.

6.3. Biosecurity implementation

Limit the number of visitors (e.g., vehicles, humans, birds, other carriers) to a minimum.

6.4. Hygiene

Install hand-wash stations, foot baths, and wheel washes or tire baths; put up warning signs; disinfect footwear before entering the facilities; and wear boots.

6.5. Enhancing the immune system of shrimp

It is important to take good care of shrimp's gut health and work to bolster their immune systems. This can be achieved by using the right feed additives. One example is supplementation with, probiotics, immunostimulants, zinc, and selenium. Zinc plays a key role in modulating immune function, resulting in shrimp with increased immune capabilities. Zinc also helps maintain skin integrity and speeds up wound healing. Selenium supplementation has also been proven to promote shrimp growth and immune health.

7. Further readings

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Parasitic Diseases of Brackishwater Fish, Shrimp and Crab and their Management

R. Ananda Raja and K.P.Jithendran

ICAR- Central Institite of Brackishwater Aquaculture, Chennai, India

E-mail of Corresponding Author: ananda.raja@icar.gov.in

1. Introduction

The brackishwater aquaculture assumes importance by effectively utilizing the non-potable water with tremendous scope for the expansion world-wide. The ultimate goal should be in achieving sustainable brackishwater aquaculture for food with diversification of species, different farming systems, popularisation of the ornamental fish industry, and employment generation. But, any disease outbreaks pose the biggest challenge for this growth. History has shown that diseases play a significantly detrimental role in aquaculture. Among which the parasites could be viewed as spanning the gamut of diseases in the way of either parasitic, hyperparasitic, commensalistic, mutualistic, or phoretic, but not predatory, relationships. There are many commercially important brackishwater fish and shrimp species with aquaculture significance such as Asian seabass (*Lates calcarifer*), Grey mullet (*Mugil cephalus*), Milkfish (*Chanos chanos*), Pearlspot (*Etroplus suratensis*), Spotted scat (*Scatophagus argus*), Mangrove red snapper (*Lutjanus argentimaculatus*), Silver moony (*Monodactylus argenteus*), Tiger shrimp (*Penaeus monodon*), Pacific white shrimp (*Litopenaeus vannamei*), Indian white shrimp (*Fenneropenaeus indicus* or *Penaeus indicus*), Banana shrimp (*Fenneropenaeus merguiensis*), and Mud crabs (*Scylla serrata* and *Scylla olivacea*). This chapter describes the major diseases attributed to parasites of finfish and shellfish and their diagnosis, treatment and control.

2. Parasitic diseases of finfish

2.1 Protozoa

Protozoans can be either ectoparasites or endoparasites depending on their host species and the predilection site of infection. Ciliates and flagellates feed on the most superficial skin layers leading to a reactive hyperplasia of the epithelium and increased mucus production. Hyperplasia appears as cloudiness on the skin and leads to hypoxia when gills are affected. Other groups of parasitic protozoa such as intracellular Microspora and intercellular Myxosporea are affecting a wide variety of vertebrates and invertebrates. The affected animals shows the most common clinical signs

such as changes in swimming behaviour due to loss of equilibrium, flushing or scraping, inappetence, discoloration, tissue erosion, excess mucus production, haemorrhage, swollen body and distended eyes.

2.1 Flagellates (Mastigophora)

2.1.1 Dinoflagellates (Phytomastigophora)

Phytomastigophora contains chloroplasts in their cytoplasm and the dinoflagellate parasites such as *Amyloodinium* spp., *Piscinoodinium* spp. and *Hematodinium* spp. are common under this group. *Amyloodinium* spp. and *Piscinoodinium* spp. cause necrotic dermatitis to their host. Amyloodiniosis also known as velvet disease, is caused by a dinoflagellate, *Amyloodinium ocellatum*. It is one of the common microscopic ectoparasites with flagella for movement, found on the skin and gills of different fish species. Other dinoflagellate (*Piscinoodinium* spp.) parasitizes many freshwater fish species. High level of organic matter in water and higher stocking density of fish are considered as predisposing factors. Clinical signs consist of velvet appearance, anorexia, scratching, darkening of the body surface and gathering on the surface or near the source of aeration. Histopathological lesions include gill and skin inflammation, haemorrhages, hyperplasia and necrosis.

2.2.2. Flagellates (Zoomastigophora)

Flagellates do not contain chloroplasts in their cytoplasm, comprises three orders affecting the fish namely Kinetoplastida, Retortamonodida and Diplomonadida. Kinetoplastida have one or two flagella as in *Cryptobia branchialis* and *C. eilatica*. Cryptobiasis in marine fish species is caused by ectoparasitic flagellates with a direct life cycle. Some species of these genera like *Cryptobia iubilans* also parasitize internal organs of fish. It causes gastric dilation, submucosal granuloma, gastric perforation, peritonitis and full thickness necrosis of the body wall musculature. The infection can produce trickling but persistent mortalities, leading to cumulative mortality rates up to 10% after several weeks. Costiasis or ichthyobodiasis is the infection caused by ectoparasitic flagellate, *Ichthyobodo* spp. from the Order Retortamonodida, which possesses two to four flagella. It causes a disease in both freshwater and marine fish and affects the gills and skin. Affected fish show clinical signs such as lethargy, emaciation and flashing behaviour. Clinical lesions such as grey-whitish pellicle on skin, severe epidermal erosion, increased mucus production, ulcers, ulcerative dermatitis, haemorrhages, gill hyperplasia, lamellar fusion (clubbing), oedema, and mortality are observed. Diplomonadida have one to four flagella and two-fold rotational or bilateral symmetry such as *Hexamita* spp. and *Spironucleus*

spp. *Hexamita* spp. are endoparasitic flagellates of the intestine and gall bladder in freshwater fish, mainly salmonids but also cyprinids, mullets and ornamental fish. Hexamitiasis is observed typically in weak fish frequently as a secondary infection. Affected fish can show nervous behaviour, and internally the intestine may appear pale. Mortalities can occur in fry and ornamental fish. *Spironucleus* spp. and *Hexamita* spp. are the cause of hole-in-the-head disease in aquarium cichlids, associated with large erosions in the cranial cartilages.

2.2. Ciliates (Ciliophora)

2.2.1. Kinetofragminophorea

This belonging to the genus *Chilodonella*, free living organism, have oral ciliature slightly distinct from body ciliature. Only two species *C. piscicola* and *C. hexasticha* are pathogenic for freshwater and marine fish. *Chilodonella* spp. attaches on skin and gills, especially in ornamental fish (Koi and goldfish). *Chilodonella* spp. feeds directly on epithelium by penetrating the host cells with its cytostome and sucking their contents. After infection, fish secrete excessive mucus, with acute to subacute dermatitis, rotting or fraying of fins, hyperplasia, degeneration, necrosis and respiratory distress. Chilodonellosis is a serious ectoparasitic infestation causing heavy losses in aquaria and in farming systems.

2.2.2. Oligohymenophorea

Class Oligohymenophorea has well-defined oral apparatus and oral ciliature, is distinct from somatic ciliature. The major parasites are *Ichthyophthirius multifilis*, *Trichodina* spp. *Tetrahymena* spp., *Epistylis* spp., *Vorticella* spp. and *Scuticociliatida* (*Uronema* spp., *Phylasterides* spp. and *Miamiensis* spp.). *I. multifilis* parasitizes on many species of freshwater fish and cause Ich or white spot disease. Fish trichodinids include mainly *Trichodina* spp., *Trichodinella* spp., *Tripartiella* spp. *Paratrichodina*, *Hemitrichodina* and *Vauchomia* spp. from freshwater and marine source. *Trichodina* spp. infects mainly gills, body surface and fins. Clinical signs and lesions of Trichodiniosis / Trichodiniasis include excess mucus production, respiratory distress by clogging of gills by mucus, flashing, debility, pale gills, hyperplasia, irritation on body hence rubs body against objects, subacute dermatitis, necrosis of the epidermis, grey-blue turbid layer on the skin and high mortality.

2.2.3. Prostomatea

Cryptocaryon irritans is the member of class Prostomatea, which cause marine "White Spot Disease". They feed on the host's cells underneath the

epithelium and cause heavy irritation resulting in excessive production of mucus, acute to subacute dermatitis, hyperplasia white spots over the body surface, ulcers, wound, secondary bacterial infection, rubbing the body against submerged objects, and finally completely destroying the gill filaments leading to respiratory distress and mass mortality in untreated cases.

2.3. Microsporidia

Microsporidians are intracellular internal parasites, represented in fish by different genera, mainly *Enterocytozoon*, *Glugea*, *Loma*, *Pleistophora* and *Tetramicra*. Poor water quality and poor nutrition are the predisposing factors. No visible clinical signs of microsporidiosis are observed. Cysts are observed in various internal organs like intestinal wall, ovary, fat tissue etc. These cysts are brown or black in colour and are of various size and shape called xenoma. Pathological concern of microsporidiosis in fish is dependent on location and intensity of infection.

2.4. Amoebozoa

Different species of amoebas have been associated to amebiasis in fish. *Neoparamoeba pemaquidensis* is identified as specific ethology of amoebic gill disease (AGD). Amoebas may appear in small numbers trapped in the gills without causing any damage, but in heavy infections, the parasites elicit epithelial hyperplasia, metaplasia, resulting in complete fusion of secondary lamellae and subsequent gill dysfunction.

2.5. Apicomplexa

Endoparasitic protozoans belonging to Apicomplexa are known from both freshwater and marine fish species, but their pathological significance for aquaculture is very variable. The genera *Eimeria, Goussia* and *Cryptosporidium* are more frequently reported from cultured fish.

2.6. Metazoa / Helminthiases

2.6.1. Myxozoans

Myxozoans are microscopic internal fish parasites cuasing whirling disease (*Myxobolus cerebralis* or *Myxosoma cerebralis*), proliferative kidney disease [PKD] (*Tetracapsuloides bryosalmonae, Tetracapsula bryosalmonae* or *T. renicola*), sphaerosporosis (*Spaherospora renicola*) and ceratomyxosis (*Ceratomyxa shasta*). *Henneguya* spp. is a flagellated myxosporidan parasite found attached mainly to the gills. Poor water quality, high stocking density, feeding with infected trash fish and lack of quarantine measures facilitate infection. Clinical signs are not apparently visible. However, white or black cysts may be seen on body surface, gills, fins and internal organs. The

parasites invade all major organs and forms cysts or freely floating mass called pansporoblast. They destroy gills and all major target organs of the fish. Diseased fish exhibits chronic mortalities with clinical sign of anaemic gills. The major lesions are enlarged bulbus arteriosus and internal haemorrhages in the pericardial cavity. Irregular-shaped plasmodia developed in the bulbus arteriosus which releases mature spores accumulating in the lumen. Massive influx of spores into the gills causes local occlusion and congestion of gill capillaries resulting in proliferative and granulomatous branchitis, lamellar hypertrophy, degeneration of the gill epithelium, and degenerative cardiomyopathy. It is commonly called as "Hamburger Gill Disease" or "proliferative gill disease".

2.6.2. Trematodes (flukes)

2.6.2.1. Monogeneans

Skin flukes: Skin flukes are 2-6 mm long and the most common flukes are *Gyrodactylus* spp., *Benedinea* spp., *Neobenedenia* spp. and *Pseudorhabdosynochus* spp. Gyrodactylosis are mainly typical from freshwater fish (*G. salaris*), however, it has also been reported in marine fish. The infection sets in when there is high stocking density, poor water exchange and polyculture. The parasites mainly affect body surface, fins, eyes, and sometimes gills. Mass mortalities may appear in moderate or heavy infections, mainly in juvenile fish, increasing with water temperature. Clinical signs include lethargy, anoxia, loss of appetite, scratching, excessive mucus production, corneal opacity, blindness and ulcers or haemorrhages with secondary bacterial infection.

Gill flukes: These common ectoparasites in fish are *Diplectanum* spp., *Dactylogyrus* spp. and Ancyrocephalids. High density and poor sanitation serve as predisposing factors. Clinical signs are pale gills, low consumption of feed, erratic swimming behavior and mucus production on gills. Mass mortality with respiratory problems is observed in severely affected fish. Gill histopathological lesions include focal hyperplasia, lamellar fusion, haemorrhages and inflammatory infiltration.

2.6.2.2. Digeneans

Digeneans are generally hermaphroditic platyhelminths that typically have a selectively absorptive tegument, a blindly ending alimentary tract, and two suckers. They are mostly intestinal parasites and involve more than one host for completing their life cycle. Digenetic trematodes such as *Lecithochirium* spp. and *Pseudometadena celebesensis* are found in the intestine especially in wild fish. Digeneans complete their life cycle in a

molluscan host; therefore, elimination of molluscs from the culture facility should stop the transmission cycle of the parasite.

2.6.3. Cestodes (tape worms)

These platyhelminthes may parasitize fish in larval or adult stages, sometimes causing diseases in cultured fish with variable economic impact. The life cycle involves at least one intermediate host. Most species causing disease in fish of economic importance fall within four orders: Caryophyllidea (*Caryophyllaeus* and *Khawia*), Pseudophyllidea (*Bothriocephalus, Diphillobothrium, Ligula* [ligulosis] and *Triaenophorus*), Proteocephalidea (*Proteocephalus*) and Tetraphyllidea (*Acanthobothrium*). Cestodes in fish usually do not cause mortality, though poor condition is frequently observed, mainly in heavy infections. In heavy infections, abdominal swelling and poor condition can be observed, mainly in small fish.

2.6.4. Nematodes (round worms)

Nematodes or roundworms are large, intestinal parasites with un-segmented body and mostly 1-2 cm length. Nematode of the genus *Cucullanus* is found more commonly in the gut of larger fish than in that of young fish. Ascaridoid *Contracaecum robustum* Chandler 1935 or *Contracaecum multipapillatum* is the nematode with potential public health hazard. C. *robustum* larvae (L3's) are encapsulated in the liver, kidneys, and mesentery of Mullets. Related ascaridoid nematodes, *Anisakis* spp. otherwise called as herring worms similarly found in mullets cause human anisakiasis. Infection is prevented by maintaining hygienic conditions. *Philometra barnesi* and *P. lateolabracis* (Philometridae) are described from the skin, muscle and ovary of the marine teleost fish causing reproductive failures.

2.6.5. Acanthocephalan

Acanthocephalid worms with their fearsome-looking proboscis and rows of hooks known as 'thorny-headed' or 'spiny-headed' worms infect the intestines of many fish species. Members of this phylum superficially appear like nematodes, but closer examination of these reveals a protrusible spiny proboscis and no intestine. Mostly reported Acanthocephalan are *Neoechinorhynchus bangoni* Tripathi (in *Mugil tade*), *N. agilis*, *N. chitkaensis* Podder (in *M. cephalus*), *N. elongatus* Tripathi (in *M. subviridis* and *M. dussumieri*), *N. karachiensis* Bilqees (in *M. spegieleri*), *N. agite* (Rudolphi) (in *Mugil* spp.), *N. coiliae* (in *Liza carinata*), *Gracitisentis mugilus* Gupta & Lata (in *Mugil* spp.), *Dispiron mugili* Bilqees (in *M. buchanani*), *Floridosentis mugilis* (Machado Filho) and *F. elongatus* [in *Mugil* spp.], *Paulisentis* spp. (in

Mugil spp.), *Tenuiproboscis* spp. (in *Lutjanus argentimaculatus*) and *Tenuiproboscis keralensis* (in *Scatophagus argus*).

2.6.6. Arthropods

There are several hundreds of parasites considered to be of economic importance in brackishwater aquaculture under phylum Arthropoda and sub-phylum Crustacea. They are of Class or Sub-class such as Copepoda (Order: Cyclopoida and Siphonostomatoida), Branchiura (Order: Arguloida), Pentastomida (Order: Porocephalida) and Malacostraca (Order: Isopoda). Cyclopoida is further divided at genus level in to *Ergasilus* (Family: Ergasilidae), *Lernaea* and *Lamproglena* (Family: Lernaeidae). Siphonostomatoida is divided at genus level in to *Caligus* (Family: Caligidae) and *Lernanthropsis* (Family: Lernanthropidae). Arguloida is identified at genus level as *Argulus* (Family: Argulidae). Similarly, Porocephalida is identified at genus level as *Linguatula* (Family: Linguatulidae) while the Isopoda is recognized at genus level as *Cymothoa* (Family: Cymothoidae). All these parasites are paramount economically significant causing growth retardation and mortality in wide range of hosts in fresh, brackish and marine environments. Hence, prevention and control of these parasites are very much essential to avoid surreptitious economic loss in the aquaculture industry.

2.6.7. Annelids

Leeches are segmented parasitic or predatory worms that belong to the phylum Annelida and comprise the subclass Hirudinea. Leeches act as micropredators rather than parasites, but some leeches exhibit considerable host-specificity. Excessive blood-loss probably occurs only with intense and prolonged infestations. Small hosts, of course, could be more easily harmed and secondary infections can follow any infestation. They probably act as vectors for the blood protozoan parasites. The most common genera are *Myzobdella* and *Zeylanicobdella*.

3. Parasitic diseases of shrimp

3.1. Protozoa

3.1.1. Endocommensal / Invasive protozoa

3.1.1.1. Microsporida

Microsporidia are obligate intracellular parasites known to infect a wide range of eukaryotic hosts. Shrimps are infected by ingestion of spores. There are several genera of microsporidia found to infect crustacean hosts such as *Thelohania, Agmasoma* (8 spores / envelope), *Ameson, Nosema, Pleistophora* (>8 spores / envelope), *Tuzetia, Flabelliforma, Glugoides, Vavraia, Ordospora,*

Nadelspora, Enterospora and *Enterocytozoon*. Microsporidia infects various crustacean tissues for example *Agmasoma penaei* infects muscle and connective tissue, *Tuzetia weidneri* affects muscle, *Enterospora canceri* in hepatopancreas, and *Enterocytozoon hepatopenaei* (EHP) in hepatopancreas (intranuclear). Microsporidia affecting the muscle tissue causes "milk or cotton shrimp disease" or "cotton tail disease" and EHP is affecting the hepatopancreas, which causes "hepatopancreatic microsporidiosis (HPM)". Recently, many studies reported the EHP organism as a microsporidian fungal parasite in shrimp. The affected shrimp show cooked-muscle appearance and the exoskeleton appears bluish black, and white tumor-like swellings may be found on gills and subcuticle. Microsporidia may also localize in shrimp gonads leading to reproductive failure or vertical transmission and granulomatous lesions in hepatopancreas.

3.1.1.2. Haplosporida

Haplosporidia is another spore forming protozoan group affecting the digestive glands (hepatopancreas) of shrimp but the incidence is rare among the well maintained shrimp farms.

3.1.1.3. Gregarina

Gregarina (Protozoa, Apicomplexa) affects the digestive tracts, hepatopancreas and other tissues of shrimp. Worldwide, penaeid shrimps are natural host for a number of gregarine species which are placed into either of three genera, *Nematopsis*, *Cephalolobus* and *Paraophioidina*. Larval stages and cultured shrimps are infected by ingestion of the gregarine spores in the slime of clams or tissues of the polychaete worms. The developed sporozoites attach to the gut wall, grows into trophozoites and form gametocysts. Gametocysts undergo multiple divisions to produce gymnospores which are released outside and are an infective stage for invertebrates such as clams, snails or marine worms. Spores are developed in the intermediate hosts and released in mucous strings which become infective to shrimps. Heavily infected shrimp shows sluggish movement, retarded growth, increased feed conversion ratio (FCR), yellow discoloration of midgut and reduced survival.

3.1.1.4. Body invaders

There are several protozoa which invade the body and feed on tissues of weakened and diseased shrimps. They are *Paraurونema* spp., *Leptomonas* spp. and *Paranophrys* spp. and amoeba (amebiasis).

3.1.1.2. Ectocommensal protozoa

They are found on the gill and body surface of the shrimp. They are peritrich protozoans with body cilia (*Zoothamnium* spp., *Epistylis* spp., *Vorticella* spp., *Lagenophrys* spp. and *Apostome* ciliates.) and suctorians without body cilia (*Acineta* spp., and *Ephelota* spp.). *Zoothamnium* spp. is a frequent inhabitant of the gills of shrimp which are grown in ponds with low dissolved oxygen (DO), leading to suffocation and mass mortality. *Apostome* ciliates interfere with larval moulting in hatchery. Infestations on cultured shrimp are usually a mixture of protozoans, filamentous and non-filamentous bacteria, and algae. They cause "protozoan fouling" and "Fuzzy mat-like appearance" due to ciliate fouling. The affected shrimps show restlessness and difficulty in locomotion and respiration. Another condition called black gill is reported in *L. vannamei*, but not harmful to humans and are safe to eat. Black Gill may not kill shrimp directly but rather compromise individuals, making shrimp increased number of molting events, to remove a damaged gill, more vulnerable to predators and environmental pressures. It is caused by a cryptic parasite, ciliate, a one-celled protozoan animal closely related to *Hyalophysa chattoni*. The ciliate invades and kill the gill tissue. The presence of the invading ciliate stimulates an immune response in the shrimp thereby increase the melanin production. The dark protective layer of melanin forms around the ciliate on the gill tissue, possibly attempting to minimize the amount of tissue damage from the ciliate. It is also surprising to note that some shrimp carrying the ciliate develop black gill and some do not. It is possible that the quantity of ciliates infecting a single shrimp may determine if infection is visually displayed as black gill.

3.2. Metazoa / Helminthiases

3.2.1. Trematodes (flukes)

The cercarial forms of the flukes are infective to the shrimps, which penetrate the shrimp and encysted in the form of metacercarial forms in tissues which are infective to the first intermediate host (fish). The metacercaria develops in to adult and release eggs. The eggs are hatched out and miracidia released which penetrate second invertebrate host, snail and develops into sporocysts. Cercarias develop inside the sporocysts in second intermediate host and released in to water which become infective stage for shrimp. Eg. *Opecoeloides fimbriatus*, *Microphallidae* spp. and *Echinostomatidae* spp.

3.2.2. Cestodes (tape worms)

Shrimp ingest copepods or other crustaceans with larval form of tape worm which develop into advanced larval stage in shrimp. The advanced larval

stage enters the first intermediate host (sting ray) by ingestion of the infested shrimp and develops in to adult and release eggs. The eggs are eaten by copepods, second intermediate host and develop in to larvae which are infective to shrimps. Eg. *Prochristianella penaei*, *Parachristianella* spp., *Renibulbus* spp., Pear shaped worms, and *Cyclophyllidean* group.

3.2.3. Nematodes (round worms)

Round worm larvae also get entry into shrimp through copepods or other small crustaceans. The larvae develop in to an advanced larval stage in shrimp tissues. The advanced larval stage enters the first intermediate host (toad fish) by ingestion of the infested shrimp and develops in to adult worm in the fish gut. Adult worm releases eggs, which are eaten by copepods, second intermediate host and develop in to larvae. The copepods carrying larvae are infective to shrimps. Eg. *Spirocumallanus pereirai*, *Leptolaimus* spp., *Ascaropsis* spp., and *Hysterothylactum reliquens*.

3.3. Other infestations

Single cell plant diatoms (on larval stages), multiple species of algae, barnacle, leeches, colonial hydriod *Obelia bicuspidata*, insects eggs, isopods - *Aega* spp. are occasionally observed among wild and poorly farmed shrimp populations. 'Red rostrum' condition is observed in shrimp grown in ponds with abundant diatom, *Peridinium* spp. Bopyrid isopod parasitic infestation caused by *Epipenaeon* spp. belong to family *Bopyridae.* The parasites lodged in the brachial cavity leads to impaired respiration and reproductive failures.

4. Parasitic diseases of crabs

4.1. Protozoa

Peritrich ciliates (*Zoothamnium* spp., *Vorticella* spp. and *Epistylis* spp.) and suctorian ciliates (*Acineta* spp. and *Ephelota* spp.) are fouling protozoans in crabs. They infest gills, body and appendages. Peritrichs interfere with locomotion, feeding and moulting of crab larvae, while the suctorians feed mainly on other protozoans and in high numbers may interfere with respiration leading to mass mortality. These parasitic disease outbreaks occur with poor water quality and low DO. Additionally, one of the most economically significant diseases of marine decapod Crustacea is caused by the genus *Hematodinium*, is a parasitic dinoflagellate which proliferate internally in the hemolymph and tissues of crustaceans. It is highly pathogenic to the blue crab causing milky disease, bitter crab disease (BCD) or pink crab disease (PCD) with 100% mortality.

Microspora is a phylum containing strictly intracellular parasitic species that produce small (usually <6 μm) unicellular spores with an imperforate wall. The spores lack mitochondria, but contain a sporoplasm and a hatching apparatus, including an extrusible hollow polar tube that injects the sporoplasm into the host cell. Genera such as *Ameson* (single spore), *Pleistophora* (32 to >100 spores) and *Thelohania* (eight spores) are parasitic to crabs. All microsporidians are parasitic, with some infecting vertebrates and others infecting invertebrates; some have a direct life cycle not requiring an intermediate or additional host, but others need another host or life stage. When infected crab tissues or spores are ingested by an uninfected crab, the spore everts its coiled polar tube by means of its lamellar polaroplast and rapidly injects the sporoplasm into an epithelial cell lining the lumen of the mid-gut. The vegetative cell then invades, develops, and multiplies in the hemocytes in the adjacent submucosal connective tissue of the midgut. When the hemocytes reach skeletal muscles, the parasite in these cells undergoes further development in the myofibrils, first forming chains of eight meronts (merogony) that separate into pairs of cells that finally result in isolated mature spores (sporogony). Crabs infected by *A. michaelis* are considered to have "sick crab disease," or "cotton crab disease" with high mortality. The parasite lyses infected muscle and adjacent tissues. The actin and myosin filaments of the host disassemble in the presence of the sporoblasts, and a cell free extract of infected tissue can produce lysis of normal blue crab muscle tissue. Grossly, the infected muscles appear chalky white through joints of the appendages, and the abdomen may appear greyish.

Haplosporidia is a small phylum of spore forming protozoans which have a multinucleated naked plasmodial stage in their life cycle. Members contain uninucleated spores without extrudible polar tubules, but they contain mitochondria, characteristic haplosporosomes, and an anterior orifice or operculum. Haplosporidian, *Urosporidium crescens* causes "pepper crabs," "pepper-spot," or "buckshot," in the skeletal muscles, visceral organs, and gills of the blue crab. It does not infect the actual crab tissue, but rather it hyperparasitizes the encysted metacercaria of the digenean *Microphallus basodactylophallus* that infects the crab. When this parasite infects the fluke and undergoes extensive multiplication, its brownish colored spores in the greatly enlarged worm create a black spot readily visible to the naked eye. Affected crabs exhibited opaque hemolymph with uninucleated cells containing perinuclear haplosporosomes and mitochondria; interstitially, multinuclear plasmodial stages of this parasite occupied much of the vascular spaces. Amoeba, *Paramoeba perniciosa* belonging to Rhizopoda causes "Gray crab" disease, a systemic infection in

crabs. The disease is named so for the darkly discolored sternum and ventral surfaces of heavily infected and dead crabs.

4.2. Metazoa

Trematodes (*Microphallus basodactylophallus*, *Microphallus pygmaeum* and *Levinseniella capitanea*), cestodes (*Prochristianella* spp. in the hepatopancreas of the crab and *Rhynchobothrium* spp. encysted in body cavity), nematodes (ascaridoid *Hysterothylacium reliquens* in hepatopancreatic tubules, *Gammarinema* spp., *Tripylium* spp., *Monhystrium* spp., *Monhystrium* spp., nemertean worm *Carcinonemertes* spp. in gill chambers), annelids (leeches: Hirudinea, *Myzobdella* spp.; branchiobdellid worm: mullet bug, *Cambarincola vitreus* in gills and carapace) and Carripeds (burnacles and *Sacculina* spp.) are reported in different species of crabs. Further studies are required for identification, life cycle and pathogenesis of these parasites in crabs.

5. Diagnosis

The correct diagnosis is obviously a critical step in any disease control program. Major diagnostic methodologies are mentioned below as it would be very exhaustive to elaborate all methodologies under this chapter.

a. Rapid diagnosis based on the anamnesis, clinical signs and post-mortem findings.
b. Wet mount microscopical findings.
c. Isolation and identification of aetiological agents.
d. Bioassay.
e. Histopathology and histochemistry.
f. Electron microscopy.
g. Molecular methods.
h. Serological methods.

6. Prophylactic and therapeutic approach

Application of therapeutic agents directly into the pond water is not an effective and safe method as it requires higher quantities of chemicals and possible toxic effects on target and/or non-target organisms in the pond ecosystem. Toxic accumulation of those compounds and their metabolites in fish tissues and other organisms could pose risk to the safety of consumer and affect the biodiversity leading to ecological imbalance. Hence application of safe and effective chemical agents through feed is commonly practiced in many countries in Europe and South America. But, no such approval is

available in India due to lack of scientific data. The following details are provided from the published reports related to fish culture but not as an approved recommendation for use in aquaculture except anti-parasitic drug, emamectin benzoate.

6.1. External parasites

- Oral in-feed anti-parasitic drug, emamectin benzoate @ 50 µg kg^{-1} body weight day^{-1} for 7-10 consecutive days is recommended via medicated feed, against all crustacean parasites. No recurrence of parasitic infestation for a period of 60 days is reported post medication.
- Formalin 15–25 parts per million (ppm) as a pond treatment or dip treatment of affected fish with 200-250 ppm for one hour at temperature below 15.6 °C and 100 ppm for one hour at higher temperature or 100 ppm hydrogen peroxide for 30 minutes against all species of ectoparasites. Mild infestations can be controlled by simple freshwater bath for 10-15 minutes.
- Though use of organophosphates for treatment in aquaculture has legal restrictions, Dichlorvos @ 1 ppm is used as an effective control against external parasites.
- Ergasilosis can be treated successfully with a combination of 0.5 ppm copper sulphate and 0.2 ppm ferric sulphate for 6 to 9 days.
- Combination of 25 ppm formalin and 0.1 ppm malachite green dip is found to be effective against cryptocaryon.
- Malachite green as a dip treatment at a concentration of 1:15000 effectively controls fungal infection on both fish and eggs.
- Acetic acid at a 1:5 concentration for 1–2 minutes and acriflavin at 3–5 ppm to control external parasites.
- Organo phosphorus compounds, Dylox (Dimethyl phosphonate) at the concentration of 0.25 ppm helps to control anchor worm, crustaceans, gill and body flukes but is not effective against protozoans.
- Cypermethrin 10% w/v– 10-15 mL/1000 sq. m. water spread area.
- Costiasis can be treated with formalin 1:4000 or 1:6000 in baths with a good aeration.
- Formalin baths (150 mL/L) can be effective for treatment of Cryptobiasis.
- Cryptocaryosis can be treated with copper sulphate bath with 25 ppm and/or formalin bath with 0.5 ppm for 5-7 days with aeration and daily replenishment of water.

- Short bath treatment with 200 ppm formalin for 30-60 minutes with strong aeration or extended bath treatment with 25 ppm formalin for 1-2 days with good aeration and daily replacement of water is practiced against Trichodiniasis.
- Flukes are treated by short bath with 100 ppm formalin or freshwater for 10-30 minutes or 150 ppm hydrogen peroxide for 10-30 minutes with strong aeration.

6.2. Internal parasites

- Oral in-feed anti-parasitic drug, emamectin benzoate @ 50 μg kg^{-1} body weight day^{-1} for 7-10 consecutive days is practiced against some nematodes.
- Antihelminthes Di-N-Butyl tin oxide mixed in the food at the rate of 1% and fed at 3% of BW for three days.
- Control of animal coccidians is based on the use of different coccidiostatics or coccidiocides, but information regarding fish coccidia is very scarce. Furazolidone, amprolium chloride and furanace, among others, have been tried to treat different fish coccidia.
- Toltrazuril has apparently given better results than fumagillin and amprolium against Microsporidiosis.

7. Prevention and control

No scientifically effective and legally approved treatments are internationally available at present. Hence prevention and control is followed based on breaking of transmission chain, avoiding the presence of intermediate hosts, and minimising or following standard stocking density. Further, a sustainable integrated pest management (IPM) approach should be based on knowledge of the ecology of the parasite along with adoption of several prevention and control methods.

- Prevention relies on hygienic measures.
- Dry the pond before starting the culture.
- Use sieve at water inlet, bleach before stocking to weed out wild shrimp, fish and intermediate hosts.
- Maintain good water quality throughout the culture.
- Use disease-free genetic strain of broodstock and develop resistant stocks.
- Supply of adequate balanced nutrition.

- Regulate population density, periodical size grading and proper disposal of dead animals.
- Handle the animals with good care and control entry of other animals.
- Proper chemical prophylaxis and vaccine development for immunological protection.
- Regulations to prevent transfer of pathogens from one host population to another, nationally or internationally.
- Proper destruction and disposal of infected animals.
- Sanitation and disinfection of hatchery and equipment.
- Efficient water exchange, good feeding practices (avoiding trash fish as feed) and quarantine measures.

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Biosecurity and Quarantine Measures for Aquaculture Health Management

S.K. Otta, P. Ezhil Praveena, M. Poornima and T. Bhuvaneswari

ICAR- Central Institute of Brackishwater Aquaculture, Chennai, India

E-mail of the corresponding author: subhendu.otta@icar.gov.in

1. Introduction

The nutritional requirement of large world population is compensated by fish and fishery products to a great extent as these are considered as the source for cheap animal protein. As per the current trend, the production from capture fisheries has been continuously declining and therefore aquaculture can be considered as the future source to meet with the increasing demand for fish. Brackishwater ecosystem in coastal zones are potential source as these are considered as the zero foot print because the water is not suitable for drinking, agriculture or construction and therefore very appropriate for aquaculture practice. Among all the aquatic animals, crustaceans contribute significantly to the total fish production. Based on the recent observation, crustaceans alone contribute more than 9 million tons of the total more than 114 million tons of aquaculture production. Among the crustaceans, shrimps forms a major share as shrimps are synonym to crustacean culture which contributes more than 60% of the total production. Shrimp aquaculture has been continuously evolving and considered as a lucrative seafood industry because of its high export value from its short rearing period. Shrimp form almost 22% of the total global international trade of fishery products. India has been in the forefront as far as shrimp aquaculture is concerned. India earns almost $ 4 billion from the shrimp export which is about 16% of global export. During the recent times, farmers are adopting modern culture practices such as biofloc and raceway culture and thereby there has been a continuous increase in production and export value.

In spite of the steady progress in production, shrimp aquaculture has continuously been threatened by disease related crop loss. Disease appears to be the prime obstacle for the smooth progress of shrimp aquaculture industry. Disease continues to remain as the primary issue for farmers for the past several years based on Global Aquaculture Alliance (GAA) survey report. Frequent appearance of several existing and emerging diseases brings huge economic loss to the farmers. Globally more than 1 billion US $ disease related annual loss in shrimp aquaculture has been estimated. Similar to other countries, India also continuously records disease related

crop loss in shrimp aquaculture and as per the recent estimation it can be more than 6000 crores Indian rupees per annum. India has a huge potential for brackishwater aquaculture and still a major chunk of it remains unexploited. With years to come, it is highly expected that there will be increase in effort to explore and utilise this unexploited treasure land. At the same time, there should be proper planning and protocols in hand to counteract all the associated risk factors.

To minimize the introduction and spreading of infectious diseases, a set of well-designed practices, called as Biosecurity need to be followed. Through the adoption of biosecurity, animals can avoid the unnecessary burden of several stress factors and mortality and thereby avoid huge economic loss to the shrimp farmers. In a well-designed biosecurity protocol, it is highly essential to accurately determine the specific points of production system that favours the introduction and spread of pathogens. Once the specific points are determined, it will then be possible to bring corrective measures to check disease occurrence and avoid untimely losses.

Quarantine is a standard protocol to isolate individual/group of organisms, conduct all the necessary tests and ensure existing/new pathogen entry to the point of release. Like biosecurity, this is also an important step in aquaculture practice to avoid disease outbreak and prevent loss. Both biosecurity and quarantine are very much essential for a sustainable aquaculture practice.

2. Biosecurity measures for Brackishwater Aquaculture

In order to design and develop successful biosecurity protocols, initial determination of specific point of entry of pathogens and their spreading possibilities are very essential and these two points are considered as the major steps. These points will vary from case to case which can be as given below.

- **Aquatic organisms considered for culture practice** – Disease tolerance and infection status of animals selected for culture practice varies from species to species or even to strains. Further, this will also vary based on life stages selected for stocking, initial health status during stocking and maturity of immune system of the stocking animals to provide disease resistance.
- **Aquatic environment where animals are cultured** – Depending on the source water selected for culture practice, initial pathogen load and bacterial diversity and water quality, disease appearance and spread will vary. Also how the water is being maintained during the culture practice

and the type of culture practice adopted decides about types of biosecurity need to be practiced.

- **Type, nature and characteristics of pathogen**: Basic biology and life cycle of the pathogen and their survival strategy in the environment (free living state, ability to form spore, ability to survive on inanimate objects, adopting a carrier etc.) determines the criteria to develop biosecurity protocols.
- **People involved with aquaculture practice (Directly or indirectly)** – This includes management staff, workers and visitors. This entirely depends on the nature and education status of all the peoples (how much they understand the principle and how they follow it)

Once the above points are decided, maintenance of biosecurity in aquaculture can mainly aims at managing and maintaining the followings to avoid the occurrence and spread of diseases;

- **Animal Management**: While selecting seeds from a hatchery, their pathogen free status should be accurately determined to ensure no pathogens can enter from the hatchery to the culture ponds. The next criteria is to select healthy larvae for which established stress test should be carried out on a sample of the lot from where larvae will be taken for the stocking. Visual and microscopic observation should also be carried out to determine the health status. Wherever possible, it should be tried to get specific pathogen free (SPF) or specific pathogen resistant (SPR) stocks. Maximum numbers of samples of larvae should be tested for the presence of pathogens before packing through a well-established diagnostic protocol. If SPF/SPR stocks are imported, these should pass through proper quarantine for the detection of all possible pathogens. Care should be taken to avoid importing larvae/broodstock from areas known to be affected with specific diseases. For stocking, the concept of "all-in-all-out" should be followed. This means a single batch or group of animals from a single source should be stocked till harvest and any additional stoking during the culture period should be avoided to the maximum possible extent.
- **Environment management:** Starting from the pond preparation it includes several aspects. Pond design and construction should be appropriately done based on the requirement if new ponds are constructed. Necessary soil test should be carried out. Preparation of ponds during infection to eliminate the pathogens and preparation between the culture periods to further carryover of pathogens is also very important. When chlorine is used to treat the pond water, effective

concentration should be used. Based on the research work carried out in CIBA, it was observed that effective concentration to kill WSSV only with water base is 5 ppm for 2 day, if the water contains infected animals (dead) it is 10 ppm 2 days, in soil based system, with planktonic WSSV, it is 15 ppm for 2 day and in soil based system, with dead infected animals it is 20 ppm for 2 days, Soil based system, WSSV (filtrate)added, water drained, exposed to sunlight for 2 days and then chlorine treatment, it is 10 ppm 2 days. Chlorine concentration also depends on the organic load and therefore should be determined based on the water condition. Accurate chlorine concentration will help to eliminate the pathogen, avoid unnecessary expenditure and will also help to maintain soil health condition. It is necessary to provide sufficient time gap between the culture practices. Again with respect to WSSV, the work carried out at CIBA indicates, the pathogen can remain viable for at least 35 days in non-drainable ponds and in drainable ponds it is 19 days. In addition to this other treatments such as drying the pond, ploughing and application of appropriate amount of lime should also be carried out.

Getting good water is an important aspect to carry out aquaculture practice. Water can be a primary source for the entry of pathogens. Wherever possible, it is advisable to go for recirculatory systems and thereby any pathogen entry can be avoided/minimised. Otherwise, it is necessary to go for adequate amounts of reservoir ponds where water can be stored initially, treated and finally matured before taking into culture ponds. Throughout the culture period, it is required to maintain good water quality and thereby avoid stress. Along with feed and other management practices, the aquatic environment should always be maintained healthy. Animals should be supplied with good quality diets and preferably supplemented with immunostimulants to maintain good immunity during the culture period and thereby avoid infection by opportunistic pathogens.

Some of the commercial feed and live feeds can also serve as a source for pathogens. These feed should be properly tested before use. If necessary proper steps such as pasteurization should be adopted

All the animals and their environment should periodically be monitored for their health status.

Biosecurity measure for prevention of bird and other animals to shrimp ponds

3. Pathogen management

Utmost care should be taken to pathogen management by preventing the entry. However, if due to unovoidable reason, if the pathogens enter the system, then take necessary steps either to eliminate it completely or reduce the number substantially to prevent mortality.

It is necessary to know the nature and virulence status of each pathogen and act accordingly. Many of the pathogens have reservoirs either as living organisms, water or inanimate objects. Therefore, one should be thorough with the nature of the pathogen and accordingly steps should be taken. Suitable environment and conditions for pathogen multiplication should be avoided. Ponds should have proper fencing system to avoid reservoirs or passive carriers for disease spread.

It is always better to have preventive methods such as vaccination or use of immunostimulants than treatment. Similarly biological controls such as phage therapy should be preferred. However, during inevitable infection period, appropriate sanitizers should be used to reduce the pathogen load. Active ingredients and the mode of action of each chemicals should be known properly and accurate dosages should be used. Indiscriminate use of antimicrobials should be avoided to prevent stress on the animal and on the environment.

It is necessary to maintain good diversity of planktons and bacteria in the pond. This will avoid the multiplication of pathogens. Biodiversity can be

maintained through the use of good quality probiotics bacteria. Similarly, it is necessary to maintain proper plankton density in the pond and avoid bloom conditions.

4. People management

For all the people associated with either hatchery or farm, all the necessary biosecurity protocols should be put in place. However, this will be successful only when the people involved understand it clearly and practice it effectively. This involves management staff, workers and visitors those get a direct access to the aquaculture facility. Effective measures are required to prevent the entry of pathogens or spread through these people. Sensitive areas should be designated only for the authorised personnel through strict security arrangement.

Visitors from another farm are considered as serious risk factors and should be allowed after thorough sanitizing protocols.

Disinfectant foot baths, hand washing stations or spray bottles, net disinfection station, vehicle disinfection station and showers should be in proper places to avoid pathogen entry.

Sufficient and continuous awareness programmes should be arranged for all the employees to make them understand the basic principles and importance of biosecurity.

5. Aquaculture Quarantine Measures

Aquatic quarantine principle is applied to a cultured species coming to one area from the other area (within the country or another country). This is an important animal management and biosecurity measure. Through this procedure individual animals or population can be isolated and acclimatized to the new place for a specific period of time. During this period, these are observed for any abnormality or disease appearance. Even if these animals look healthy, these are tested for all possible known pathogens. If necessary, these are treated to make disease free. Once it is made sure that animals are free from pathogens, these are then either released to culture facility or live market. However, for exotic pathogens or presence of viral pathogens wherever treatment is not possible, the stock should be destroyed carefully making sure that the pathogen does not spread.

The quarantine facility should be well designed and located in an appropriate isolated place or ensure that the facility is physically separated from farms/hatcheries. The facility should have easy access for transportation of animals, get sufficient quality water and the discharge water should be handled properly. The facility should have well established

protocols and well trained staffs. This should have competent and readily available diagnostic support.

While animals are maintained in quarantine facility, these be sampled at the beginning, at the end and at point of disease appearance and all the necessary tests should be carried out. Usually random and non-lethal sampling is required to be done to determine the health status. In case of detection of any disease, the entire stock should be properly destroyed/disposed.

6. Conclusion

Aquaculture sustainability is the need of the time and effective measures should be put in place to achieve the same. Both biosecurity and quarantine measures are essential parts of a healthy aquaculture practice and avoid disease appearance. With the adoption of new culture practices involving high stoking density and with the import of several new species for aquaculture, it is expected to have more disease prevalence and culture loss in the future. Therefore, these two systems are very much important and should be well understood and established to avoid disease occurrence, mortality and thereby huge economic loss. This will also avoid unwanted situations due to introduction of exotic pathogens or appearance of emerging diseases. Therefore, both the practices should accurately and appropriately be followed.

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Application of Probiotics and Immunostimulants in Aquaculture and their Role in Aquaculture Disease Management

Prasanna Kumar Patil

ICAR-Central Institute of Brackishwater Aquaculture (CIBA), Chennai

E-mail of corresponding author: pk.patil@icar.gov.in

Aquaculture is an important economic activity of India providing livelihood, generating employment and earning export revenue to the country. Sector is facing economic loss due to deteriorating environmental conditions and stressful conditions leading to diseases and poor growth in intensive aquaculture operations. Prevention and control of disease is the key for successful farming operations. Probiotics protect against disease by activating both cellular and humoral immune defenses by increasing phagocytosis, respiratory burst and antibacterial activity in aquatic animals. Lactic acid bacteria are known to produce compounds such as bacteriocins that inhibit the growth of other microorganisms.

1. Probiotics

The probiotics for aquaculture are defined as 'live microbes with beneficial effect on fish/shrimp or improving the quality of pond environment'. Probiotics maintain the healthy balance between the helahty and pathogenic micboes in the culture environment. Probiotic play important role in aquaculture particularly in improving productivity, nutrient utilization, disease control, water quality and the impact of discharge on the sorrounding environment. Bacteria most commonly present in the probiotics for use in aquaculture include, lactobacillus, *Bacillus*, spp., photosynthetic bacteria and yeast. Probiotics are the best alternative to chemicals and antibiotics in aquaculture. Generally the probiotics are expected to perform in wide variety of culture environments, freshwater, brackishwater and marine in addition to varying pH, DO, turbidity etc.

The probiotic formulations used in aquaculture are classified as;

- Gut probiotics, applied through feed improve the health by improving digestion, immune response to diseases, resistance to stress and the common microbes are *Bacillus* spp.
- Water probiotics, broadcasted on the water surface improve the water quality through controlling Total Ammonia Nitrogen (TAN) and other

nitrogenous metabolic wastes. The common microbes are, *Nitrobacter* spp. *Nitrosomonas* spp.

- Soil probiotics, applied at the pond bottom to control toxic sulphur molecules. The commonly used microbes are Thiobacillus, spp.

2. Mechanisms of action of probiotics

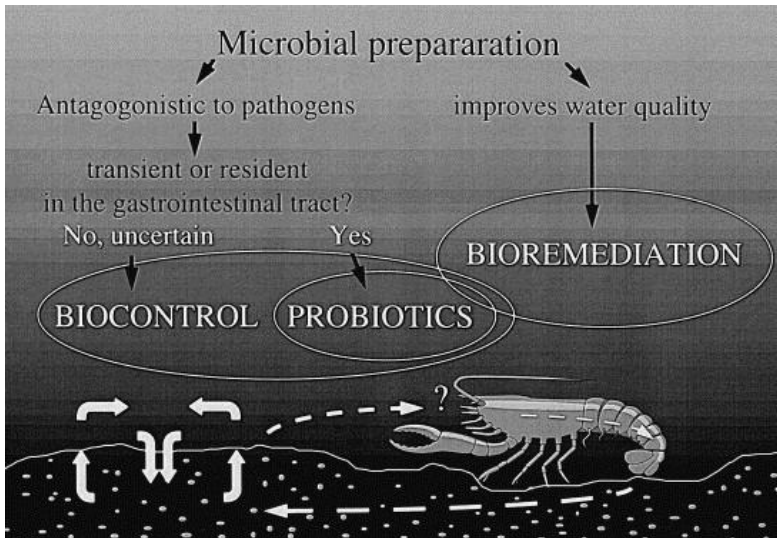
- production of compounds that are inhibitory to pathogens
- competing with pathogens for nutritional requirements and adhesion sites
- improving the digestibility through the production of enzymes and supplementation of micronutrients
- enhancing host immunity against both biotic and abiotic stresses
- improving the culture environment through bioremediation of metabolic toxins and interaction with phytoplankton
- clean up toxic metabolites through bioremediation
- degrade, transform or chelate various toxic chemicals

3. Is the intestinal environment of aquatic animals favourable to probiotics?

Since fish/shrimp larvae are released directly into the environment, microbes in the water director attack the young ones. Hence, the beneficial microbes are very much essential to protect the larvae at the early developmental stages. Since the fish drink water continuously intestinal microbes change very frequently depending upon the environment. So administration of probiotics at this stage will be helpful to improve the health of the larvae.

4. Application of probiotics to improve water quality

Mechanism of probiotics to controlling pathogenic bacteria is called biocontrol and the one improving the water quality is called bioremediation. Floating biofilters pre-inoculated with nitrifying bacteria decreased the amounts of ammonia and nitrite in the rearing water. *Bacillus* spp. formulations applied near pond aerators reduced chemical oxygen demand. Probiotics when applied to pond water reduce the levels of pathogenic *Vibrio* sp. Degradation of organic matter by *Bacillus* spp. improves water quality.



4.1. Antagonism to pathogens

Probiotic bacteria inhibit the pathogenic bacteria by production of antibiotics and inhibitory substances, like, organic acids, hydrogen peroxide and siderophores.

4.2. Intestinal colonization

Gut probiotics both bacteria and yeast, act by colonizing the host gut and also by transient presence when applied in high concentration.

4.3. Protection against pathogen challenge

Administration of probiotic bacteria confers protection against the pathogen challenge; improve survival in early life stages of fish/shrimp. This protection could be due to production of inhibitory substances that blocked bacterial growth.

4.4. Source of nutrients and enzymatic contribution to digestion

Probiotics benefit the host digestive process by supplying fatty acids and vitamins, extracellular enzymes, such as proteases, lipases, some growth factors.

5. Methods of probiotics application

- addition via live food
- bathing/immersion
- addition to culture water
- addition to formulated feed

6. Basic characteristics of a microbe in probiotic formulation

- Safe to the host and its environment
- Ability to administer through ingestion

- colonization and proliferation within the host

Generally the probiotic containing multistrains are much more effective than the single strain formulations. Probiotics are highly effective when applied in combination with prebiotics and immune stimulating agents. Enrichment of live feeds like, artemia, rotifier, copepods with probiotics as encapsulations is best practice in hatcheries. Probiotics increase the levels of lysozyme and respiratory burst, phagocytic and complement activity.

Application of probiotics in appropriate dose is very important as lower doses may be ineffective and high dose may lead to unnecessary expenditure without additional benefits. Application of probiotic formulations in several short spells is more effective than a single long duration. Prolonged application of probiotic formulations may lead to cost escalation without much benefit sometimes may also lead to immune suppression. Feeding probiotics in repeats of short-term-cycles gives higher benefits.

6.1. Enhance immune responses

Application of probiotics is known to increase leucocytes, lymphocytes, monocytes, erythrocytes, neutrophil adherence, migration of neutrophils and plasma bactericidal activity, complement activity, cytotoxicity, phagocytic and superoxide dismutase activities, total globulin, albumin, serum bacterial agglutination titres, serum peroxidase and blood respiratory burst activities, phagocytic activity, lysozymes, respiratory burst, antiprotease activity, peroxidase activity

Important criteria for selecting a probiotic product for application in aquaculture

- complete name of the microbes in the product
- strength of the product in terms of copy number (cfu/g)
- mode of application (through, feed, water or sand)
- combination of water and soil probiotic may be allowed but not the mixture with gut probiotics

6.2. Probiotics improve water quality

Probiotics have proven their effectiveness in improving water quality in different approaches. They enhanced decomposition of organic matter, reduced nitrogen and phosphorus concentrations, and controlled ammonia, nitrite, and hydrogen sulphide. Reduce organic matter accumulation, mitigated nitrogen and phosphate pollution in the sediments, reduced

metabolic wastes during transportation, reducing a number of pathogenic bacteria.

Probiotics improved digestibility: Administration of probiotics enhance the digestive enzymes like, alginate lyases, amylases and proteases carbohydrolases and lipases, generation of essential nutrients such as fatty acids, biotin and vitamin B12.

6.3. Specific probiotic species

There is no one good formulation for all purpose; farmers need to standardize the formulations based on the requirement mostly based on trial and error basis.

7. Limitation of probiotic effectiveness

Since microbes are very much sensitive to environmental parameters it is not necessary that products work effectively in different culture systems. Since the beneficial effect of the probiotic bacteria depends its interaction with host it is not necessary that probiotics work effectively in different cultured animals.

Since probiotics needs to be build up in the system for effective action regular application of the probiotic products ensures the beneficial effect. Since different bacterial compositions work in different site of actions like, gut, water and soil beneficial effect of the product can be achieved only when applied in appropriate route. Since there will be a completion between the opportunistic pathogen and the probiotic bacteria it is necessary that probiotic product needs to be applied in sufficient quantity.

8. Prebiotics

Prebiotics are the nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of probiotic bacteria in the gut and thus improves host health. Ex. Fructooligosaccharides, lactosucrose etc.

9. Immunostimulants

Enhancing the disease resistance power of the host is one of the crucial approaches for preventing diseases. Several substances of natural, chemical and microbial origins have been known to possess' immunestimulating potential. Though the administration of immune-stimulating agents for humans and animals' general health and well-being is a well-established practice, its importance has been recognized only recently in aquaculture. The development of immune stimulants for use in aquatic organisms was slow due to the lack of understanding of the basic immunology and the

efficient parameters to evaluate the immune response in crustaceans and molluscs. The information on the molecular immune system of aquatic invertebrates and tools for assessing the immune reactive molecules is emerging. This has made it possible to develop new molecules for effective stimulation of the immune system of aquatic organisms and the corresponding resistance to invading pathogens.

Similar to human and domestic animals, vaccination is the best possible disease protection method. However, the practical feasibility of this approach in aquaculture has led to the development of immune stimulating agents. Common immune stimulating agents include, polysaccharides, vitamins, different components of bacteria, biologically active materials, herbal and synthetic drugs.

9.1. Definition

Immunostimulants are naturally occurring compound that modulates the immune system by increasing the hosts' resistance against diseases'

Classification of immune stimulating agents based on

1. Their origin
2. Mode of action
3. Mode of administrated

9.2. Functions of immunostimulants

- activate the immune system
- enhance capacity of disease resistance

9.3. Components of cellular immunity in fish

Phagocytic cells, neutrophils, natural killer cells and lymphocytes

9.4. Components of humoral immunity in fish

Lysozyme, haemolysin, immunoglobulins and complement molecules, cytokines (interferon, interleukin 2, macrophage-activating factors)

9.5. Immune stimulation in crustaceans

Since the invertebrate immune system is less developed the immune stimulants act in crustaceans by increasing the phagocytosis of pathogens by

- activating phagocytic cells in the hemolymph
- increase the antibacterial and antiseptic properties of hemolymph,
- activate the prophenoloxidase system,

- mediate signal recognition and phagocytosis
- expression of immunostimulant genes.

9.6. Immune stimulants in fish

- expression of immunostimulant genes
- enhance the phagocytic capacity of neutrophils and lymphocytes
- stimulate the secretion of cytokines from lymphocytes
- coordinate cellular and humoral immunity
- evoke antibody and complement responses

9.7. Clinical observations of immune stimulating agents

- improved growth rates, survival rates and disease resistance
- immunostimulants are safer alternative to
- antibiotic to control bacterial infections
- chemicals for managing the environmental stress

9.8. The classification of immunostimulants

- Polysaccharides
- Nutritional supplements
- Oligosaccharides
- Herbs
- Antibacterial peptides
- Probiotics

9.9. Effect of polysaccharides on aquatic animals

Polysaccharides are from either plants, animals or microbial origin.

β-Glucans: activate phagocytic cells, improving phagocytosis and the ability of the cells to kill pathogenic organisms increase lysozyme and complement activity, phenoloxidase activity and respiratory burst activity, activation of the complement pathway.

9.10. Chitosan

Chitosan is a de-acetyl chitin, which is a type of alkaline polysaccharide found in the shells of aquatic animals such as shrimp, crab and shellfish. Chitosan is a natural polymer material that is edible, bio-compatible, bio-degradable, non-toxic and safe.

- promote the growth of aquatic organisms
- improve the immunity of aquatic animals
- inhibit the growth of aquatic pathogens
- purify the water used in aquaculture
- enhance the disease resistance of aquatic animals

9.11. Herbs

Indian and Chinese herbs have been used by people as traditional medicines and immune boosters for thousands of years. The non-specific immune properties of herbs include bacteriolytic activity and leucocyte function

Fish treated with herbs typically exhibit;

- enhanced phagocytosis
- increases in the respiratory burst and phagocytic activities
- increased survival relative to controls bacterial infection
- improve lipid utilization, lysozyme activity and stress recovery
- reduced total cholesterol levels
- appetite stimulants and to enhance the activities of digestive enzymes (protease, amylase and lipase

9.12. Vitamins

Vitamin C and Vit E are the most common immune stimulating agents for use in aquaculture. Vitamin C, also known as ascorbic acid, cannot be synthesized in aquatic animals and obtained from food only. Feeding of Vit C improves;

- immunity and resistance to disease
- increased lysozyme activity and increased the total number of white blood cells
- promotes the growth and early-stage development
- improved stress and disease resistance
- increases the non-specific immunity
- improves the quality of meat
- phagocytic activity
- antioxidant capacity
- enhance resistance to crowding stress

- higher percentage of spawning with a higher percentage of egg viability and hatching and a higher cumulative survival rate

9.13. Vitamin E

Vitamin E, also known as tocopherols is a biologically active phenolic compound

- enhance the generation of antibodies
- complement activity
- promote the proliferation and differentiation of lymphocytes
- cytokine production
- improve cytotoxicity and phagocytosis
- increased the total number of haemocytes
- improved physiological indexes: phagocytosis rate of blood cells, serum bacteriolytic activity, bactericidal activity and levels of the complement proteins C3 and C4.
- antioxidant capacities, resistance to crowding stress and growth
- protect tissues from lipid oxidation
- General improvement in growth and health

9.14. Carotenoids and astaxanthin

Carotenoids, the precursors of vitamin A. The natural carotenoids are abundantly available and cheaper to obtain, making them an attractive pigment source for feed supplementation. Carotenoids stimulate cell-mediated host defence and humoral immune mechanisms in fish, such as phagocytosis, non-specific cytotoxicity, serum lysozyme activity, serum complement activity that promotes larval growth and survival, and disease resistance.

9.15. Levamisole

Levamisole, generally used as an antihelminthic drug in humans and other animals, is a potential immunostimulant. Levamisole can enhance the cytotoxic activity of leucocytes, phagocytosis and macrophage-activating factors, increase in chemotactic ability, phagocytic activity and phagocyte chemiluminescence. macrophage activity.

10. Factors affecting the efficiency of immunostimulants

- Timing of immunostimulant administration-better to administer prior to expected stressful condition, like transportation, handling etc.

- Dosage of immunostimulant: need to standardize for species and type of immune stimulating agent, no 'fit-for-all' principle.

10.1. Mode of action

The oral administration method is non-stressful and allows for mass administration regardless of fish size.

- enhanced leucocyte function
- protection against infectious diseases
- bathing fish in an immunostimulant solution better used in larval stages
- activate phagocytosis and chemotaxis
- produce active oxygen in head kidney phagocytes
- enhanced protection against bacterial infection

10.2. Feed additives and immunological defences of fish

Functional feed additives act as immune stimulants by

- directly stimulating the innate immune system
- enhancing the growth of commensal microbiota

10.3. Medicinal plants as immune stimulants

The alternative herbal bio-medicinal products in the aqua cultural operations, which have the characteristics of growth-promoting ability and tonic to improve the immune system, act as appetite stimulators. They increase consumption, induce maturation and have antimicrobial capability and also anti-stress characteristics that will be of immense use in the culture of shrimps and other fin fishes without any environmental and hazardous problems. Several plants or their by-products contain phenolic, polyphenolic, alkaloid, quinone, terpenoid, lectine and polypeptide compounds, many of which are effective alternatives to antibiotics, chemicals, vaccines and other synthetic compounds) In addition, medicinal plants are rich in a wide variety of nutrients. They can be administered as a whole plant or parts (leaf, root or seed) or extract compounds, via water routine or feed additives, either singly or as a combination of extract compounds, or even as a mixture with prebiotics or other immunostimulants.

10.4. Organic acids

Organic acids are short-chain fatty acids, volatile fatty acids or weak carboxylic acids, such as formic, citric, benzoic and lactic acid generally used as food preservatives in livestock feeds due to their antimicrobial

properties. They are also known to enhance growth, nutrient utilization and disease resistance of aquatic animals.

In the intestinal tract of aquatic animals, organic acids inhibit the growth of Gram-negative bacteria. Organic acids and their salts can also contribute in nutritional ways; they are components in several metabolic pathways for energy generation, such as for ATP generation in the citric acid cycle or carboxylic acid cycle as dietary supplements to improve growth performance, feed utilization, nutrient digestibility, disease resistance as well as the alteration of the gut microbiota populations

10.5. Functional amino acids as immune stimulants

Amino acids like, arginine, glutamine, sulphur AA (methionine, cysteine and taurine), histidine and branched chain AA (leucine, isoleucine and valine) are known to increase disease resistance, immune response and reproduction in aquatic animals.

- Arginine effects on both the innate and adaptive immune through improved macrophage killing and phagocytosis abilities, increased the respiratory burst, increased nitric oxide production in head kidney leucocytes. Arginine also has anti-inflammatory activity
- Glutamate and glutamine modulate intestinal structure, protecting against oxidative damage and acting as energy substrate for the enterocytes. Act as immune stimulant through nitric oxide response of macrophages and act as energy substrate for leucocytes.
- Glutamine and arginine act synergistically to improve the immune response

10.6. Trace minerals as immune stimulants

General function of minerals includes constituents of the exoskeleton, balance of osmotic pressure, structural constituents of tissues and transmission of nerve impulse and muscle contractions. Minerals serve as essential components for enzymes, vitamins, hormones, pigments, and co-factor in metabolism, catalysts, and enzyme activators. Shrimp can absorb or excrete minerals directly from the aquatic environment via gill and body surfaces. So, the dietary requirement of minerals is largely dependent on the minerals concentration of the aquatic environment in which the shrimp is being cultured.

The micro-elements or trace minerals, such as chromium, cobalt, copper, iodine, iron, manganese, molybdenum, selenium and zinc, are required in small quantities and participate in a wide variety of biochemical processes. They are involved in cellular metabolism, formation of skeletal

structures, maintenance of colloidal systems, regulation of acid–base equilibrium, immunity enhancer, stress releaser, disease resistance and other physiological functions. They are important components of hormones and enzymes, and serve as cofactors and/or activators of a variety of enzymes.

Every trace mineral components are having their specific role in immunity of cultured animals, but the crucial trace metals that have been associated with an improvement in immunity or function that support immunity are Zn, Mn, Cu and Se. The immune system uses several methods to detoxify these foreign agents or antigens. The trace elements that have been combined with an improvement in immunity, or function that support immunity. The micro-elements have especially been strengthened by the importance of their roles in immune defence and antioxidative protection. Medicinal herbs administered in combination with micronutrients like selenium, boron or zinc provide greater benefits

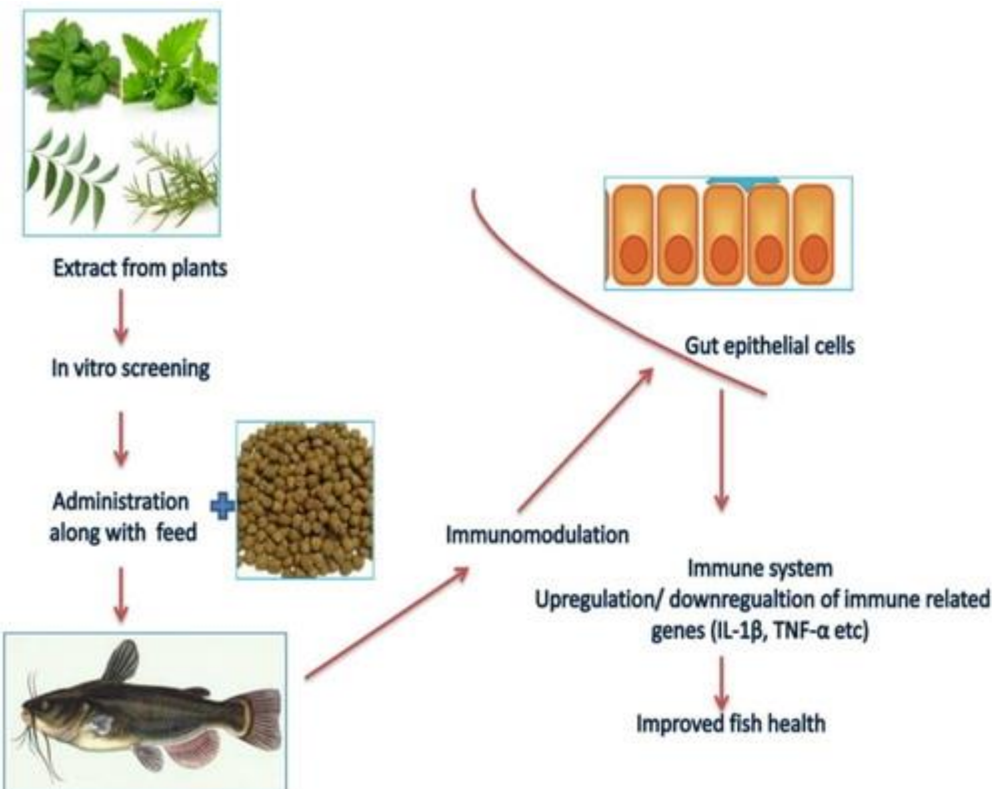


Figure 1. Overview of the possible outcome of action of herbal immunostimulants on fish immunity. Herbal extracts mixed with fish feed is administered into fish and its efficacy in modulating immune system is investigated. Gene expression studies reveal the genes that are more or less expressed.

Common medicinal plants with immune stimulating activity

- Turmeric (*Curcuma longa*)
- Ginger (*Zingiber officinale*)
- Ashwagandha (*Withania somnifera*),
- Velvet bean (*Mucuna pruriens*)
- Aloe vera (*Aloe barbadensis*)
- Citrus, *Citrus limon* and *Citrus sinensis*,
- Mangrove plants are rich in polyphenols and terpenoids
- Guava (*Psidium guajava* L)
- African wormwood (*Artemisia afra)*
- Chamomile (*Matricaria chamomilla* L.),
- Pumpkin or calabaza (*Cucurbita mixta*) seeds
- Spanish dagger (*Yucca schidigera)*
- Oregano (*Origanum vulgare* L)
- Woodbetony (*Stachys lavandulifolia*)
- Peppermint (*Mentha piperita*)
- Spade flower (*Hybanthus enneaspermus*)
- Devil's horsewhip (*Achyranthes aspera*)
- Combination of plants

11. Conclusion and perspectives

The usage of antibiotics and chemotherapeutics in aquaculture and their subsequent undesirable consequences have made the researchers think about the safe alternative – probiotics and immune stimulants. Owing to the residual and side effects, antibiotics cannot be used in aquaculture industry, but probiotics and immunostimulants can be used to protect the crop against the abiotic and biotic stress conditions. Multiple bioactivities produced by probiotics and as components of immune stimulants help improving the immunity of aquatic animals. Use of these compounds play a key role in promoting economically viable and environmentally sustainable aquaculture.

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Role of Zero Water Exchange-based Technologies in Shrimp Culture with Special Reference to Prevention of Diseases

Sujeet Kumar

ICAR- Central Institute of Brackishwater Aquaculture, Chennai, India

E-mail of corresponding author: sujeetmicrobiol@gmail.com

1. Introduction

Shrimp aquaculture is an important commercial activity in Indian coastal ecosystem. Traditional aquaculture has evolved through centuries to create agricultural systems adapted to local environmental and cultural conditions. Under these scenarios, pond water is frequently exchanged to maintain the desirable water quality for shrimp growth. Nutrient laden effluent discharge causes environmental degradation and eutrophication of the nearby ecosystem. Growing attention of people, media and government over the environmental issues demand better culture practices which could have least negative impact over the environment. Further, over the last two decades, the global shrimp aquaculture is plagued with large number of diseases such as white spot disease (WSD), infectious myonecrosis (IMN), infectious hypodermal and haematopoietic necrosis (IHHNV), acute hepatopancreatic necrosis disease (AHPND), microsporidian infection by *Enterocytozoon hepatopenaei* (EHP) etc. Shrimp pathogens appear to be transmitted horizontally from effluent water. As no therapeutics or vaccines is available to control these diseases in shrimp culture, prevention becomes the ultimate remedies. Under these circumstances, recent development of zero water exchange, biofloc and periphyton based farming system will provide the necessary boost in disease control.

2. Zero water exchange

Global shrimp aquaculture is presently plagued with large number of diseases. In such scenario water exchange become a risky management practice. Central Institute of Brackishwater Aquaculture has developed a Biosecured Zero Water Exchange System Technology (BZEST). This system relies upon zero or minimal water exchange. The monsoon precipitations mostly take the care of evaporation loss. Zero or minimal water exchange coupled with stocking of disease free shrimp post-larvae and quality feeds help to realize the disease free shrimp culture.

Unlike the open system where water replacement is done as per the level of intensification, the zero water exchange shrimp farming system is a

closed system which provides a means to achieve higher degree of biosecurity. The biosecured system ensures the prevention of bacterial/viral contamination, which is the major bottleneck to have sustainable shrimp farming. Once the disinfected (with 60 ppm of chlorine) water is taken and cultured for optimum bloom, no further water is taken from the source and the evaporation loss etc. is compensated by treated water or the crop is so scheduled to take the advantage of rainwater for that purpose.

2.1. Role of zero water exchange in biosecurity

Biosecurity is the protection of living organisms by the Exclusion of Pathogens and Other Undesirables. The adoption of biosecurity protocols in shrimp aquaculture has resulted in the shrimp overall production increase. Success of establishment and implementation of biosecurity program in shrimp aquaculture system demands the pivotal role of cluster farming in which each individual farmer plays crucial role. Thus Zero water exchange system provides a means to achieve higher degree of biosecurity in shrimp culture system.

2.2. Methods adapted for disease control under zero water exchange culture system

Following culture procedure should be ensured for disease free rearing of shrimp;

- Preparation of pond: After harvesting pond bottom contains high load of organic matter, toxic compounds, bacteria, parasites, virus particles, EHP spores as well as many WSD virus carriers. Effective pond preparation include black soil removal by washing with high pressure water or scraping, drying for at least two weeks to kill all disease causing organism such as fungi, protozoa, bacteria and viruses by oxidation. Longer exposure to sunlight should be ensured in a pond which has recorded disease.
- Remove virus carriers such as wild shrimp, crabs, copepods and other crustaceans using nylon screen of 60-80 meshes/cm2. These meshes should be placed as three tier filtration at the inlet of the reservoir. Many animals like mudskippers, snakes, frogs could be kept out of farm by installing a fine net enclosure.
- Stocking of Specific Pathogen Free (SPF) post-larvae from well recognized and coastal aquaculture authority approved hatchery is the fundamental requirement to get disease free shrimp culture under zero water exchange. Insure that hatchery has supplied post-larvae to your farm after properly testing the major viral (WSSV, IHHNV, IMNV, TSV, YHV), bacterial (AHPND) and microsporidian (EHP) pathogens.

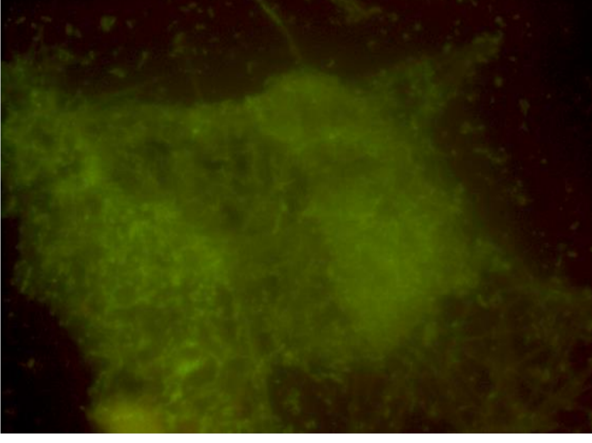
- Avoid very high density. Acclimatize the PL properly before release in pond at dawn or dusk.
- Avoid excess feeding and old feeds. Use check tray and monitor feeding as per the standing biomass and physiological condition of shrimp. Restricted feeding should be done during moulting. Never feed live crustaceans or trash fish and its frozen product. Use pellet feed with balanced nutrients.
- Oral probiotics and effective immunostimulants help to improve the host immunity by eliciting the non-specific immune response. This can be tried at regular interval.
- Avoid stressful conditions such as low water depth, overcrowding, poor water quality.
- Water quality monitoring and management with application of lime, gypsum.
- High temperature, no aeration, algal bloom or crash, and disturbing pH may increase the susceptibility of WSSV infection.
- Regular health monitoring and PCR testing of white spot virus, IMNV and EHP infection should be done.
- Virus can be inactivated by halogenous disinfectants including sodium hypochlorite or formalin - 0.25%, 0.5 ppm chlorine and 0.3 ppm Iodine.
- As EHP spores are typically resistant to a wide variety of environmental conditions, treat pond bottom with a very caustic material to bring the pH to 12. It has been recommended that earthen ponds be disinfected with very heavy use of calcium oxide, or quick lime, applied at a level of 6,000 kg/ha. Pond bottoms must be completely dry. Plough the quick lime into the dried sediments to a depth of 10 to 12 cm; then moisten the sediments to activate the lime. If the application is done properly, the pH of the soils will rise to 12 or more within days and then gradually return to normal as the quick lime becomes calcium carbonate. NACA also suggested >15 ppm $KMnO_4$ or >40 ppm chlorine to inactivate EHP spores in the soil.
- Treat pond effluents as per the norms set by aquaculture authority of India. Treatment of effluent is mandatory for bigger farms and collectively for smaller farms. This includes disinfection or biological filtration through cultivation of algae, sea weeds, clams, and filter feeders or omnivorous fishes to reduce the excess organic matter and pathogenic microorganisms.
- Every drop of intake water must be disinfected with 30 ppm calcium hypochlorite and left for 3-4 days. Minimize the water exchange.

3. Biofloc technology

Biofloc is a heterogeneous mixture of bacteria, algae, protozoa, zooplankton, food particles, organic polymer and dead cells. But generally, it is a bacterial dominated system, which often reaches in the order of 10^7 CFU/ml. This provides enough time for shrimp to engulf the floc particle.

3.1. Principle of Biofloc technology

Biofloc technology is based on the manipulation of carbon nitrogen ratio (C: N ratio) in aquaculture system. It has been suggested that C: N ratio of 10:1 is the best for biofloc production. The C: N ratio can be manipulated by application of various carbohydrate sources such as molasses, rice flour, tapioca powder etc. or by changing the protein level in the feed. As a thumb rule, for each 1 g of nitrogen, 20 g carbohydrate should be added. Otherwise, 30% protein level in the feed also works best for biofloc system. At high C: N ratio, heterotrophic bacteria immobilize the ammonium ions for production of biofloc. This helps to reduce toxic ammonia– N in aquatic system. At CIBA, our work suggests that biofloc improved the growth rate in juvenile and adult by 29.0 and 12.6%, respectively. Work conducted at Belize farm in Central America suggests that biofloc system in zero water exchange lead to 29% more nitrogen retention in *Penaeus vannamei*. Thus apart from faster growth rate, biofloc based system could help to reduce the feed quantity intake which will lead to reduced cost of production. Most of the farmers rely upon commercial probiotics application to improve the digestive performance of shrimp. The work conducted at CIBA suggests that biofloc improve the digestive enzymes and assimilative capacity of cultured shrimp. Similar results have come from the work conducted at many organizations worldwide. This shows the true potential of biofloc as probiotics to stimulate growth rate in cultured shrimp.



Biofloc under fluorescent microscopy

3.2. Contribution of biofloc in shrimp culture

3.2.1. Bioremediation of toxic ammonia: The biofloc system maintains adequate water quality especially toxic nitrogen metabolites. At higher C: N ratio, bacteria immobilize toxic ammonia into microbial protein within few hours as compared to slow conventional nitrification process which takes a month to get established. Thus, BFT is essentially a water quality management technique to minimize water exchanges through microbial degradation of nitrogenous toxic waste (ammonia and nitrite) either by conversion to less toxic nitrate or by being converted to microbial biomass.

3.2.2. Biocontrol agent: Numerous studies have reported that shrimps are healthiest and grow best in aquaculture systems that have high levels of algae, bacteria and other natural microbiota. Probiotics are viable microbial cells and have beneficial effect on health of shrimp by stimulation of immune system and microbial equilibrium in intestine, and by inhibition of pathogenic microbes. Microbes store poly- β-hydroxy butyrate (PHB) as a stored product of carbon and energy. Its synthesis is stimulated in the condition of limited nitrogen supply and with excess carbon supplementation. Condition available in biofloc system thus enhances its production. The PHB particles offer preventive and curative protection in *Artemia* nauplii against luminescent pathogenic *Vibrio campbelli*. This indicates that biofloc can serves as novel strategy for disease management on long term basis. Further, it has been reported that increase in C/N ratio from 5 to 20 reduced the Vibrio population. This suggests the possible role of biofloc system in the control of pathogenic microbes.

3.2.3. Healthy supplementary food: The protein content of bacteria is almost 60%. Therefore, its consumption becomes an alternate source of protein for aquatic animals like shrimp. The work conducted at CIBA revealed that biofloc is rich in several fatty acids such as palmitic acid (46.54%), cis-Vaccenic acid (15.37%), linoleic acid (10.67%) and oleic acid (9.19%).

3.2.4 Probiotics and immunostimulant: Biofloc is a microbial consortium, which has large number of bacteria, which could play a powerful role in digestive enzyme secretion and as immunostimulant. Our study indicated that biofloc system improves the load of *Bacillus* and *Lactobacillus* bacterium which is expected to play role in probiotics and immunostimulant effect. Our experimental trial on biofloc work carried at ICAR-CIBA and Kakdwip Research Centre of CIBA, West Bengal revealed that biofloc formation starts 24 hours after addition of carbon source when bacterial count reaches 10^6-10^7 cfu. Biofloc was mainly composed of bacterial aggregates, zooplankton

and phytoplankton. Our study indicated that biofloc alone or integration of substrate with biofloc system have profound effect on growth performance and immunity improvement in penaeid shrimps. The enhanced immunological parameters such as haemocyte counts, prophenoloxidase, super oxide dismutase, and catalase activity was observed in biofloc groups. Further, the challenge study carried out using pathogenic *V. harveyi* suggested that rice based biofloc system had 55.5% survival compared to only 11% in control. Similarly, Panigrahi et al. (2019) reported that biofloc treatment at 32 and 40% protein level significantly increased the transcription of antimicrobial peptides like crustin, MnSOD, hemocyanin, proPhenoloxidase (proPO), peroxinectin (PX) and serine protease (SP).

Several studies are also being carried out using probiotics such as *Bacillus, Lactobacillus* sp. in biofloc based system. The data from these studies reflects increase in growth potential and disease resistance abilities against pathogenic Vibrios. Recently, work conducted at ICAR- CIBA evaluated the effect of *Marinilactibacillus piezotolerans* and *Novosphingobium* sp. during the culture of Indian white shrimp, *Penaeus indicus*, under biofloc and clear water systems. They reported that bioaugmentation by probiotic bacteria significantly improved water quality, especially helpful in ammonia reduction. It also favorably changed the gut microbiota with reduced *Vibrio* population. There was an enhanced expression of digestive enzyme related genes such as trypsin, chymotrypsin, cathepsin L, cathepsin B and alpha amylase and immune genes such as peroxinectin and hemocyanin and antimicrobial peptide crustin.

3.3. Challenges in biofloc system

Though biofloc based system provide many opportunities over conventional zero water exchange system. But the practice is full of limitation and challenges. The biggest challenge a biofloc based system provide is excessive turbidity accompanied with high level of bacterial growth. This also leads to deposition of large amount of sludge at the bottom of the pond which needs regular removal. In *P. vannamei* ponds at Belize farm in Central America, sludge removal progressed from weekly interval in the mid phase of culture to everyday at the end phase of culture. This definitely limits its utility in semi-intensive farm, which most often exists in Indian condition. Another big challenge lies in maintaining the dissolved oxygen level. In routine, oxygenation in biofloc system should be done 24 hrs a day. The yard trial study conducted at CIBA indicates that oxygen drop is up to 1 ppm/hr in biofloc based system. This could be still higher in pond condition seeing the more depth. Thus more than one hr without oxygenation could be disastrous in biofloc system. Therefore, we suggest keeping generator back

up 24 hrs in ready condition. Further, regular checkup of biofloc volume should be done using Imhoff cone. Once floc volume crosses the critical level, addition of carbohydrate should be stopped and sludge removal should be initiated.

4. Periphyton based farming system

Periphyton refers to the entire complex of attached aquatic biota on submerged substrates. It comprises phytoplankton, zooplankton, benthic organisms and detritus. Natural biota associated with submerged substrates forms an excellent quality natural food for the cultured shrimps. Various kinds of substrates are used in periphyton based system. This includes bamboo, kanchi, jute stick, paddy straw, sugarcane bagasse, nylon, velon, etc.

4.1. Role of periphyton in shrimp health management

It has been reported that provision of substrate in the early growth stages of penaeid shrimp improves the survival even at high stocking density. The consumption of microbes and algal community present over submerged substrates enhances the growth of penaeid shrimp by providing quality natural food. The trials conducted at Kakdwip Research Centre of CIBA, Kakdwip yielded 17.9% gain in production and 22.3 % reduction in FCR compared to conventional culture practice.

Algal products and their cell wall components are widely used to elicit nonspecific defence mechanism in fishes and shrimp. Periphyton has been reported to enhance immune responses in shrimps like *P. vannamei*. Recently, work conducted at CIBA showed that periphyton powder as dietary supplement enhances immune response and disease resistance in *P. monodon*. Apart from improving productivity, periphyton based farming practice could also help in improving the shrimp immunity and health management.

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Pond Preparation and Management Practices with Special Reference to Prevention of Diseases in Brackishwater Aquaculture

Leesa Priyadarsani, Sanjoy Das, Prem Kumar

Kakdwip Research Centre of ICAR-CIBA, Kakdwip, West Bengal, India

E-mail of corresponding author: leesa.priya@gmail.com

In the last few decades, shrimp aquaculture has witnessed rapid growth. The expansion was driven by high profitability in shrimp farming that has attracted a wide range of investors starting from individual farmers to business houses. One among nine maritime states in India the state of West Bengal is endowed with vast and varied fisheries resources and considered as a sleeping giant. The brackish water potential resources are at the tune of 2.10 lakh ha which is distributed in three districts viz. Purba Medinipur, South and North 24 Pargana. With rapid expansion, it has been experienced that the outbreaks of various viral diseases in shrimp farming along with several environmental and social issues demand scientific management of shrimp farms. In this direction, Better Management Practices (BMP) is one method that can ensure efficiency and productivity by reducing the risk of shrimp health problems.

1. Pond preparation

The main objective of pond preparation is to provide the shrimp with a good rearing environment and optimum conditions for their growth and survival which will lead to successful shrimp farming. Improper pond preparation can affect the soil during the culture, which will release nutrients and toxic compounds and create stress for the shrimp and other environmental problems. Pond preparation is most important for disease control and should be a critical aspect of disease management strategy.

- Completely drain out the water from the pond. It helps in removing the disease-carrying fish and crustaceans from previous crops in the pond.
- Removal of bottom sludge - avoid seep back. The massive input from the previous crop has the potential to overload the organic load in sediments and cause deterioration of the pond bottom. Sludge releases toxic gases like ammonia and hydrogen sulfide in the pond leading to stress or death of shrimps. Pond sludge that accumulates on the pond bottom need to be removed before the next crop.

- Dry and plough the pond bottom - Ploughing of soil is to expose the black soil. Drying helps in oxidizing the organic matter thus reducing the risk from parasites and microbes from previous crop.
- Lime application - optimizes pH and alkalinity conditions of soil and water. The levels of lime application during pond preparation depends on the pH of the soil. Soil pH <5 - Quick lime; soil pH >5 – Limestone powder (LSP) / Agriculture lime (100kg/ha).

2. Water intake and preparation

- Use of water reservoirs.
- Water screening- Water screening is very important in keeping the disease carriers away. The pond should be filled with water using a double-layered mesh of 60 holes/sq inch at the water inlet point.
- Disinfection - Do not use pesticides to kill unwanted fish and disease carrying crabs in the pond. Use bleaching powder @ 60 ppm (600 kg/ha at 1.0 m water depth) to kill unwanted fish in the pond.
- Use inorganic fertilizers like dolomite @ 200 Kg/ha after 7 days of bleaching powder treatment. Add urea and single super phosphate @ 1:1 ratio i.e. 4Kg/ha each. This will help in improving the mineral content of the pond bottom in ponds with low soil fertility and also ponds that are in culture for more than a decade.
- Plankton bloom is essential in shrimp culture. It shades the pond bottom and prevents the growth of benthic algae, which provides a darker environment that the shrimp find less stressful.
- Make organic juice containing yeast, *Saccharomyces cerevisiae* (30kg paddy dust + 40kg molasses + 5kg yeast + 400L water/ ha). Ferment in air-tight incubation for 48hr. Spread the fermented mixture across the pond using, when the color of the water turns to green the pond is ready for stocking. Apply this mixture for 15 days intervals throughout the culture period to maintain plankton bloom. Manually remove if benthic or floating algae are present in the pond.
- Do not carry out netting through dragnet from the day of seed stocking until 45 DOC. After 45 DOC perform cast netting once a week.

3. Seed selection and stocking process

- Selection and stocking of good quality of shrimp seed (PL) in to the pond is necessary. Avoid wild seeds and uncertified hatcheries. Seed should be sourced from certified and reputed hatcheries. It should be specific pathogen free (SPF) stock.

- Quality tests should be conducted before packing
- Shrimp seed should pass a salinity stress tests and formalin test to access the seed quality. Seed should be packed at PL 10 – 12 at appropriate salinity.
- Transit time from hatchery to farm is around 12 -16 hrs
- Seed bags should be kept in pond for one hour to maintain the temperature.
- Before stocking the seed into the pond test the salinity of source water and pond in which seeds will be stocked. If salinity differs in source water, then slowly mix pond water into seed bag so that the salinity will be equal to pond water.
- After acclimatization, seed should be released in to the ponds with aeration.
- Stocking is generally carried out during cool hours of the day, i.e., after 8 AM or before 8 PM. Check the plankton bloom, if green water is visible then it is suitable for stocking. Avoid stocking if the pond has transparent water or dark green water.

4. Water quality management

- To reduce risk of disease through contamination, avoid water exchange from outside the farm. Water should be exchanged from reservoirs. Reservoir should be properly treated prior to use.
- Do not exchange water frequently. If necessary, water can be exchanged after 60 DOC but try to minimize water exchange as much as possible.
- Run the aerators for atleast two hours every day.
- Monitoring and management of salinity, pH, dissolved oxygen and microbial load should be done periodically.
- To control water pH within the optimum range of 7.5-8.0, apply lime to increase the pH. High pH results excess plankton bloom from over liming.
- If the pH is higher than 8.3, apply fermented juice to reduce the pH.
- Use agricultural lime after water exchange and after rain. Lime should be mixed with water and spread through out the pond. It acts as a buffering agent for water.
- The water depth at the shallowest part of the pond should be at least 80 cm.

5. Pond bottom management

- Deteriorated condition of the pond bottom directly affects the shrimp. Removal of black and toxic bottom sediments is essential to maintain a healthy environment.
- Check weekly basis, especially at the feeding area for black soil, benthic algae and bad odour. Always take care not to accumulate leftover feed at the pond bottom because later on, it will deteriorate the soil.
- Do water exchange and reduce feeding in areas where the soil is black.

6. Feed management

- Feed management is one of the most important aspects of shrimp aquaculture as the feed accounts for 50 to 60% of the operating cost.
- Feed should be fresh and of good quality to maintain the health and growth of shrimp.
- Determine size and quantity of feed to be applied by following the feed chart. Check the feeding rate, reduce the feeding rate during periods of low DO, plankton crash, rainfall, moulting, extremes of temperature and during disease outbreaks.
- Excessive feeding is more dangerous than underfeeding. So, install feeding trays/ check trays to monitor the feeding. Based on the check tray reduce or increase the quantity of the feed.
- Do regular sampling of shrimps once a week after 45 DOC to determine growth rate and to calculate FCR. Feeding depends on the body weight of the shrimp.
- The method is being perfected based on experience and a standard chart is being prepared.
- Store feed in clean, cool and ventilated area, protected from sunlight.
- It is advisable to switch off the aerators just before feeding and up to 1 hr after feeding.
- First indicator of a serious disease problem: abrupt decline in feed consumption - Virulent infectious diseases and Low dissolved oxygen.

7. Routine Health monitoring

- Daily visual inspection of the animals- Shrimps should be sampled once a week by cast netting and should be checked for their general health conditions like external appearance.

- Note down the visual inspection of the animals like Gill fouling and discoloration, melanized cuticular lesions, abnormal coloration of appendages and chromatophores, soft shelled shrimp, opaque, whitish muscule, deformities and white spots.
- Take observation of pond conditions like shrimp swimming erratically at the surface, dead or moribund shrimp, birds circling overhead and phytoplankton crashes.
- Monitor water quality parameters once a week.
- Probiotics, immunostimulants, bioremediating agents can be working as prophylactic measures in grow out culture.
- Yeast based fermented organic juice can be applied to improve the overall pond microbial balance.
- Monitor for viral, bacterial, fungal and parasitic diseases.
- Diseases may be caused by a single species or a mixture of different pathogens
- Control workers' movement in and across the farm and minimize the number of workers in stocking, harvest, sampling etc.

8. Health and disease management practices during disease outbreak

When viral out-break is suspected need to quarantine the suspected pond. At the same time implement the following during disease outbreak:

- Stop peoples, motorcycles passing across the pond and stop sampling activities
- Check any abnormalities in water and soil condition
- To increase the carrying capacity of unaffected ponds increase number of aerators
- Emergency harvesting, if the mortality rate is increasing rapidly and shrimp are not feeding
- Apply bleaching powder in water to kill shrimps and run the paddle wheel aerators.
- Leave the pond with water for at least 7 days until the dead shrimps become red.
- Do not take paddle wheels out of the pond. Remove dead animals and bury them away from the ponds.
- Bleach pond water for 5 to 7 days before releasing into to the drainage. The pond water should be treated in an effluent treatment system.

- Neighbouring farmers should be kept well informed about shrimp disease problems, emergency harvesting and the time and date of water discharge.
- To avoid the cross contamination during periods of disease outbreak, surrounding farmers should try to avoid water exchange.
- Make sure all people and equipment involved in the quarantine process to follow the biosecurity protocol.
- Better management parctices includes reduced costs and improved Profits, reduced risk to small-scale farmers, increased co-operation and harmony among farmers, better organized farmer groups, reduced disease incidence.

9. Farm Record Maintenance:

- Record keeping is a key component to identify various risks and to rectify these problems at the earliest during the production cycle.
- Records are necessary for identifying water quality problems, monitoring day-to-day activities.
- Record keeping also helps the farmer to learn from past mistakes, thus reducing risk and minimizing costs of production in subsequent crops.
- Record keeping is for accruing the good and bad about the practices adopted.
- To know the net profit or loss.

10. Treatments and use of chemicals

- Antibiotics are very precious and primarily meant for treatment of infections in humans, so avoid using antibiotics
- Antibiotics resistance has become a common phenomenon by misuse of antibiotics.
- Later on, tissue residues may pose a serious challenge in treatment of human infections.
- Antibiotics residues hamper exports resulting in huge loss to farmers.
- Residue in farms affects biogeochemical cycles and productivity.
- Urgent regulation of antibiotic use in aquaculture is needed.

11. Harvesting

- Pre-harvest testing should be done for antibiotic residues.

- Harvesting is done only after the receipt of test report and the report is submitted to the buyer along with the shrimps harvested.
- Harvesting must be avoided during moulting period and agricultural lime can be applied 3-4 days before harvesting.
- Try to harvest in the early morning or evening. Harvesting should be done with a dragnet with minimum delay.
- Harvested shrimps are transferred to ice slurry for chill killing and thereafter shipped to the processing plant in insulated carriers packed in ice.
- The discharged water is routed to the ETS and discharged after ensuring the standards prescribed.

12. Conclusion

Impacts of BMP includes reduced costs and improved Profits, reduced risk to small-scale farmers, increased co-operation and harmony among farmers, better organized farmer groups, reduced disease incidence, reduced FCR and increased efficiency of resource use. Following strict management and biosecurity measures, higher survivability can be achieved. Biosecurity measures helps to prevent disease introduction and spread, protects your fish, your farm and your investment.

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Introduction to Crustacean Culture Activities Kakdwip Research Centre of ICAR-CIBA

Leesa Priyadarsani, Sanjoy Das, T.K. Ghoshal and Debasis De

Kakdwip Research Centre of ICAR-CIBA, Kakdwip, West Bengal, India

E-mail of corresponding author: leesa.priya@gmail.com

1. Introduction

Among all the brackishwater crustacean species, the major candidate species for culture in India includes tiger shrimp (*Penaeus monodon*), Indian white shrimp (*P. indicus*), **Pacific white shrimp** (*P. vannamei*), **kuruma shrimp** (*P. japonicas*), **red tail shrimp** (*P. penicillatus),* banana shrimp (*P. merguiensis*) and mud crab (*Scylla serrata* and *S. olivacea*). Shrimp Aquaculture has provided a tremendous opportunity for economic and social upliftment of rural communities in the coastal areas of our country. In India farming of *P. monodon* was predominant but due to disease, the production declined. Production was gradually compensated by *P. vannamei,* which was introduced in 2009 through SPF broodstock imported from other countries. Of the total shrimp produced in India, production of *P. vannamei* increased from 3,53,413 in 2014-15 to 8,15,745 MT in 2020-21, whereas black tiger shrimp (*P. monodon*) production reduced from 71,400 MT to 27,616 MT. Mud crab culture in India is limited due to the availability of crab seed for farming. ICAR- Central Institute of Brackishwater Aquaculture, Chennai has standardized the hatchery seed production cycle of *S. serrata* in late 1990s. A package of technology for mud crab culture, as well as production of seed in hatchery available for commercialization, encourages the scope for large scale crab farming in the country. *Scylla serrata* attains a maximum carapace size of 280 mm and 3.5 kg recording even 14–28 g/week weight gain. Scientific crab culture is in demand due to its high value in the live export market and minimum disease risk during the culture period.

2. Shrimp culture activities

For conducting any shrimp culture operation, it is important to follow strict biosecurity measures to prevent transmission of diseases in shrimp farm. The upper 25 to 75 cm layer of soil removed after complete draining and drying of pond. This top layer contains high organic content resulting from deposition of uneaten feed and faecal matter from the previous culture. High concentration of organic matter can lead to anaerobic sediment that can have adverse effect on shrimp growth and survival. Intake water filtered at the main sluice and at each pond feeder pipe with fine mesh screen filter

bag 60 µ mesh to prevent entry of vectors and pathogens that maybe present in the source water. The water disinfected with 60 ppm of active chlorine and left for at least a week. Inorganic fertilizers like dolomite @ 200 Kg/ha used after 7 days of bleaching powder treatment. Urea and single super phosphate @ 1:1 ratio i.e. 4Kg/ha each were added to the pond. Bird fencing and crab fencing were installed with separate implements for each of the ponds and taken care of biosecurity measures.

Regular monitoring of soil and water quality parameters like temperature, salinity, pH, dissolved oxygen, TAN, nitrate, nitrite, total suspended solids, etc. were done. *Penaeus vannamei* in particular is sensitive to oxygen stress and since a higher stocking density is maintained all along, aeration is very critical aspects for this species. Check tray were monitor at interval to avoid overfeeding or underfeeding of shrimp. Feeding is usually done @ 5% of total biomass at the beginning which is gradually reduced to 3.5% at the end of culture period. Shrimps were sampled once a week and checked for their general health condition and any other abnormalities like body colour, missing appendages, gill fouling, black gills and growth in terms of length and weight. All the detailed observations on pond preparation, seed and its stocking, feed management, water quality parameters, shrimp health and harvest were recorded.

2.1 Compensatory growth study of *Penaeus vannamei*

The use of a nursery system contributes to the rapid growth of cultured organisms. In the nursery phase in tanks, higher stocking densities are used so that shrimps will have decreased viable space. So, the growth will be stunted and when it will be stocked in ponds, it provides a higher growth rate and increased survival at the end of the growth period. The experiment was conducted in two phases to evaluate the compensatory growth pattern in *Penaeus vannamei.* During the first phase, *Penaeus vannamei* postlarvae were reared at densities of 5,000 shrimp/m^3 for 20 days in tanks. In the

second phase, the shrimp were then restocked for another 70 days and were maintained to confirm the presence or absence of compensatory growth. At DOC 90 including nursery rearing, shrimps were harvested. The survival rate was good. The weekly weight gain was more than normal shrimps. The shrimps are found to have a faster growth rate and the strategy of inclusion nursery rearing can be applied to the culture of *P. vannamei* to accomplish compensatory growth.

2.2. Experiment on *P. vannamei* with mustard oil cake and plankton^{plus}

Pond trial was conducted with feed incorporating three different level i.e., 0, 5 & 10 % of mustard cake in diet of *P.vannamei*. Plankton Plus was used at 30 ppm in each pond and shrimps were stocked @ 30 pcs/sq m. At the end of 120 days of culture, productivity was better and FCR was significantly ($P<0.05$) lower in all mustard cake supplemented groups. Phytoplankton and zooplankton populations in all the ponds were similar. It is concluded that mustard cake can be included at 10% level in diet for *P. vannamei* culture without affecting the production performance.

3. Crab culture activities

3.1. Crab fattening

Crab fattening is the technique where water crabs or newly molted are held for a period of few weeks until they are full of meat and ready to market. Generally floating cages or tanks used for fattening. Crabs are fed with bivalve meat / trash fish with a daily ration at about 5-10% of body weight. Carb fattening can be carried out at high densities provided with good quality of water, optimum feed management and health management.

3.2. Replacement of trash fish with formulated feed in crab culture

An experiment was conducted in mud crab to replace trash fish with formulated feed at KRC. The mudcrab were stocked into individual box during winter and fed with three feed combinations: daily feeding of trash fish (T1), feeding of trash fish and formulated feed in alternate day (T2) and

daily formulated feed (T3) for evaluation of growth performance. After 45 days, the highest average weight gain and average daily weight gain were achieved in T3 compared to T1 and T2. Survival during the 45 days culture was 100% in all treatments.

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Brackishwater Finfish Culture Technologies of Kakdwip Research Centre

Babita Mandal, Prem Kumar and *M.Kailasam

Kakdwip Research Centre of ICAR-CIBA, Kakdwip, West Bengal, India

** ICAR- Central Institute of Brackishwater Aquaculture, Chennai, India*

E-mail of corresponding author: sanjoy125@yahoo.co.in

1. Introduction

Farming in Indian brackishwaters has been a traditional practice for centuries. Brackish water of fisheries resources of India includes estuaries, coastline, backwater, mangroves etc. There are about 3.9 million ha of estuaries and 0.5 million ha coastal mangrove areas in the Country. However, potential brackish water area is estimated to 1.20 million hectare of which only 0.15 million hectares has been used for brackish water aquaculture. West Bengal has highest 405000 ha brackishwater area in the country of which only 14.5 % is used for faming activities. Brackish water aquaculture in the country has expanded steadily from a traditional activity scientific farming activity to reach the present commercial scale. Scientific brackishwater aquaculture started in India with tiger shrimp (*Penaeus monodon*) farming initiated during early 1990s. In addition, certain marine/ brackishwater fish such as, seabass, mullets, milkfish and pearlspot have shown a lot of promise. There is vast scope for sustainable development of brackishwater aquaculture in Sundarbans to meet the livelihood demand by utilizing the unused areas and adopting advanced farming practices. Hence, ICAR-CIBA has demonstrated various scientific farming approaches using different brackishwater candidate species for achieving sustainable development of this sector in the state.

2. Traditional grow out practices/Extensive farming

Traditional aquaculture/farming in bheries of west Bengal includes natural entry of fish/shrimp in the field and their growth which is mostly on natural flora and fauna and occasional feeding with formulated feed. In recent past, this practice has been improved with supplementary stocking, water quality management and feeding. This technology improved the production and with incorporation of more scientific methods and called as semi intensive farming. By adopting this method 3 – 4 tonnes/ha/crop can be achieved for seabass culture within 5 – 6 months. In addition to that farmers have achieved production of 2 tonnes/ha/crop of milkfish under monoculture systems.

Traditionally, Seabass culture in ponds has been carried out as an extensive type culture. Low-lying excavated ponds are stocked whenever the sea bass juveniles or available in the wild sea collection centres. Juveniles of sea bass are collected and introduced into the traditional ponds which will be already stocked with some species of fish shrimps and prawns. These ponds will have the water source from adjoining brackish water or freshwater canals, or from monsoon flood. The juvenile seabass introduced in the pond will pray upon the available fish or shrimp juveniles as much as available and grow. Since seabass by nature is a species with differential growth, on introduction into the pond at times of food scarcity, the larger may resort to feed upon the smaller ones reducing the number. Seabass are allowed to grow for 6 to 7 months of culture period till such time water level is available in these ponds and then harvested. At the time of harvesting there will be a large fish of 4-5 kg as well as very small fish. In this manner production up to 2 tonne/hectare/7 to 8 months has been obtained depending upon the number and size of the fish entered or introduced into the pond and the feed available in the pond. However, this practice is highly unorganised and without any guarantee on production or return.

3. Rice fish culture

Traditional systems are often characterised by poly culture of shrimp and fish or by rotation with rice. In this 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 into 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 utilising natural food. After 3-4 months, partial harvesting starts using tidal inflow during lunar cycles and continues up to 10 to 11 months. Productivity in this system ranged between 500 - 750 kg/ha/year of which about 30% is constituted by prawns/shrimps and 70% by other brackish water fishes.

4. Monoculture practices of different species

4.1. Asian seabass

Asian seabass farming is done in two phases nursery and grow out. Nursery pond size ranges from 500 - 2000 m^2 with water depth of 50 - 80 cm. A well prepared pond is important as predators and competitors can endanger the stocked fry. Fry ranging from 1 - 2.5 cm are stocked at the rate of 20 - 50 no/m^2. Fry are fed with chopped and grounded trash fish @ 100% of biomass initially and gradually reduced to 10% at the end in two equal males during morning and evening hours. The nursing period lasts for about

30 - 45 days until fingerling stage is reached. Hapa, is used for nursery rearing of seabass fry in pond, where 200 - 300 fry are stocked in series of hapas and fed with finely chopped trash fish in same way as in ponds. Fry in hapa rearing system can be fed with marine fish larval diet at 3 times a day. Fishes are graded in different size groups at 5 - 7 day intervals and restocked in thoroughly cleaned and sun-dried hapas. Better survival is achieved in hapa nursery rearing than in pond. For production of fingerlings, hapa is an ideal system for nursery rearing of Seabass. The grow out phase lasts for about 8 - 12 months. There are two culture systems employed in pond grow out of Seabass:

- The supplementary feeding system is followed in places with adequate supply of fresh trash fish at low-cost. Fingerlings are stocked at the rate 10,000 to 20,000/ha. Chopped trash fish is fed twice daily in the morning and evening @ 10% of biomass initially and gradually reduced to 5% at the end. A very recent development on improving the dietary intake of Seabass is the introduction of moist feed. A production of 2 - 3 tonnes/ha can be achieved in 8 - 12 months.
- Forage fish feeding system shows great promise. Abundant natural food is produced and selected Tilapia brood stocks are released in the pond at the rate of 5000 – 10000/ha. Sex ratio of male and female is 1:3. Tilapia is reared in pond for 1 – 2 months or until tilapia fry appear in sufficient number. Seabass juveniles (8-10 cm) from nursery are stocked at the rate of 10000 – 20000/ha. Fertilization is continued to maintain tilapia seed production. Seabass production of 2 – 3 tons/ha and tilapia production of 1 – 2 tons/ha is achieved in 8 – 12 months.

4.2. Grey mullet

Mullet (*Mugil cephalus*) culture is done in 2 phases: nursery rearing and growout culture. Fry are stocked in well – prepared earthen nurseries at high densities (upto 25/m^2), where they are fed on natural food in fertilized ponds. Periphyton based seed rearing is also a good option. Fry are kept in nursery ponds for 4 – 6 months until they are about 20 – 30 g in weight. Then fingerlings are stocked in monoculture/polyculture ponds. Prior to stocking ponds are sun dried and manured. Extruded feed is supplied to semi intensive ponds at 5 % of biomass initially and gradually reduced to 1 % at the end. The growing season is generally about 7 – 8 months. A production of 2.3 – 3.7 tons/ha can be achieved. In polyculture ponds they are stocked with Tilapia, Milkfish and pearlspot in brackishwater and with common carp and silver carp in freshwater. In this culture method 4.3 – 5.6 tons/ha/crop can be obtained.

Grey mullet can be farmed in monoculture ponds. The pond for monoculture is prepared first, following eradication of unwanted organisms and application of manures and fertilisers. Advanced fingerlings of >50 g size or stocked at 10,000 no/ha. Fish are fed with supplementary feed. In an 8 month culture, fish become 500 - 800 g with total production of 3 - 4 ton/ha.

4.3. Milkfish

Milkfish (*Chanos chanos*) is a highly potential candidate species for brackishwater aquaculture due to its fast growth, disease resistance, acceptance of formulated food. Recently, success in captive breeding has been achieved by ICAR-CIBA. Nursery rearing is carried out in small earthen ponds. Fry are stocked @ 10 – 25 m^2 and fed with 1:1 mixture of oil cake and rice bran as supplementary feeding at 20 % of biomass initially and gradually reduced to 6% at the end. Fishes attain 10 – 30 g in 2 – 3 months nursery rearing. Culture trails in KRC at stocking density of 1 no/m^2 and feeding with formulated pellet feed @ 2 – 6 % of fish biomass in non-aerated periphyton supported brackish water pond, and has achieved production of more than 4 tonnes/hectare in eight months. Fishes grew to 400 to 500 g during eight months period registering over 90% survival. This fish is a good candidate species for poly culture with shrimp as it has stronger mucosal immune properties compare to that of mullet and sea bass and may help in disease prevention.

4.4. Pearlspot

Pearl spot (*Etroplus suratensis*) is also known as “green chromide”, is cultured in the state of Kerala in traditional manner in the Pokkali fields. Farming of this species is practiced in West Bengal in a small scale. This fish is highly adapted to captive farming due to its ability to feed on variety of natural foods. Nursery rearing is done in small ponds and tanks system, ponds are sun-dried and limed before letting in water. Water is taken through fine mesh filters to avoid entry of predators. Organic fertiliser is applied at the rate 500 - 1000 kg/ha to boost up plankton bloom. After achievement of sufficient plankton bloom, fry of 1- 1.2 cm size are stocked @ 5 to 10 no/m^2 . Fishes are fed with mixture of rice bran and mustard oil cake (3:1) in fine powder form at 20% of biomass initially and gradually reduced to 5% at the end. After two months, fishes attain a length of 5 – 10 cm depending on availability of natural food in the nursery ponds. Most of them are harvested to be sold as ornamental fish. Grow-out monoculture is done in similar way with 2 – 5 no/m^2 and feeding is done at 5% of biomass initially and gradually reduced to 2% at the end for 6 - 8 months. Fishes attain an average body weight of about 100 g in this practice.

4.5. *Mystus gulio*

Mystus gulio is an euryhaline fish and suitable for culture in both fresh and brackish water environments. Wild M. gulio seeds are widely cultured in the paddy fields and brackish water areas of West Bengal and Odisha, and in sewage fed brackish water system of West Bengal. Moreover, this is a suitable species for poly culture with other fishes. Traditional cultural practises depend completely on the natural tidal entry of seed, food and water exchange. Furthermore, traditional systems are often characterised by poly culture with fish or by rotation with rice as practised in bheries of West Bengal and pokkalis of Kerala. In this culture system, low-lying areas near brackish water rivers and creeks and encircled by peripheral dyke and tidal water is allowed to enter 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 initiated partially during lunar cycles. Productivity in this system ranges between 500 to 750 kg/ha of which about 30% is constituted by prawns or shrimps and 70% by fishes including *M.gulio* as one of the species.

This species can be stocked for monoculture in ponds. In brackish water bhery it can grow up to 80 g, when stocked at 1 no/ m^2 in a year. However, high-density rearing with a commercial pellet feed at the rate of 4 to 6% of estimated biomass daily and densities of 8, 12 and 16 no/m^2 demonstrated highest productivity of 950 kg/ha at the highest density. CIBA has developed a monoculture technology of *M.gulio* in brackish water system. During seven months of farming period at the stocking density of 1 and 2 no/m^2, it attains an average marketable size of 58.3 g with production of 1000 to 1200 kg/ha.

M. gulio is a good species compatibility in poly culture with other freshwater and brackish water fishes and shrimp. Poly culture of *M.gulio* at a stocking density of 40 no./m^2 with *Oreochromis niloticus* 60 no/m^2 and *Rhinomugil corsula* 40 no/m^2 gave total production of 3867 kg/ha then monoculture total production of 1682 kg/ha in 120 days. Attend an average body weight of 50 g in a culture period of 4.5 months at the stocking density of 0.1 no.m^2 in poly farming with *Liza parsia* (0.1 no/m^2) and *Penaeus monodon* (15 – 17.5 no/m^2).

5. Cage culture

Cage culture system allows high stocking density and assured high survival rate. It is natural and eco-friendly and can be adopted to any scale. Feeding can be controlled and cages can be easily managed. Harvesting is not

expensive. Seabass is a good candidate species for cage culture. In the cages, seabass can be stocked @ 25 to 30 no/ m^3 initially when they are in the size of 10 to 15 g. As they grow, after 2 to 3 months culture, when they are around 100- 150 g, density has to be reduced to 10 to 12 no/ m^3 for space. Cage culture is normally done in two phases – till they attain 100 – 150 g size into 2-3 months and after words till the attain 600 to 800 g in 5 months. Fish in the cage can be fed with either extruded pellets or with low-cost fishes as per the availability and cost. Floating pellets have advantage of procurement, storage and feeding. Huge quantity of low-cost fishes are landed in the commercial landings in the coastal areas which fetch around Rs. 10-15/ kg only and can be used as feed for sea bass culture. Low-cost fish like tilapia available in freshwater and brackish water also serves as feed for sea bass in ponds and in many cage culture operations. The rate of feeding can be maintained around 20% initially and reduced to 10 and 5% gradually in the case of trash fish feeding and in the pellet feeding, the feeding rate can be around 5% initially and gradually reduced to 2 to 3% at later stage. In the feeding of low-cost fish, FCR is around 6 - 7. In the case of pellet feeding, FCR is claimed to be around 1 to 1.2. Production of 6 to 8 kg/m^3 is possible in the cages, under normal maintenance and production as high as 20 – 25 kg/m^3 is obtained in intensive cage management in the culture of Seabass.

6. Polyculture

Modification of traditional brackishwater polyculture through selection of compatible species for sustainable better production and profitability were tested by KRC of ICAR-CIBA involving tiger shrimp (*Penaeus monodon*), Grey mullet (*Mugil cephalus*), Parsia (*Liza parsia*), and Milkfish (*Chanos chanos*) to assess the optimum species combination. Ponds are prepared following standard procedure and reared organisms were fed with commercially available sinking pellet feed (Crude Protein: 24%, Crude fat: 3%) @ 2-10% of fish biomass. Tiger shrimp, grey mullet, parsia and milkfish were stocked @ 2, 0.5, 0.5 and 0.25 no/m^2 , respectively. Best growth (44.17±1.53 g) of tiger shrimp was achieved in presence of all species with lowest survival (17.9±3.4%), and highest survival was observed in absence of milkfish (41.3±5.6%) with moderate growth (42.65±1.25g). Best growth of parsia was observed in absence of milkfish (54.16±6.34 g), while highest survival was found in absence of grey mullet (61.9±4.1%). Grey mullet grew best in absence of milkfish (487.17±20.42 g) and survived best in presence of all species (60.5±4.4%). Milkfish grew best in absence of grey mullet (382.91±18.39 g), and highest survival (71.1±6.8%) was found in presence of all species. Significantly ($p<0.05$) higher total harvested biomass (1931.64

kg/ha) was produced in presence of all species followed by treatments with absence of parsia (1794.11 kg/ha), absence of milkfish (1739.42 kg/ha), and absence of grey mullet (922.51 kg/ha). Highest total income (INR 520650) was achieved in species combination without milkfish followed by presence of all species (INR 410303), without parsia (INR 360362), and without grey mullet (INR 203099) with respective benefit-cost ratio of 2.798, 2.048, 1.824 and 1.242. Growth, production and economic parameters of the study indicated technical and economic viability of mullets-tiger shrimp polyculture. Mullets-milkfish-tiger shrimp polyculture was also viable but with lower profitability.

7. Organic Farming

Today, the demand in the importing countries for high quality and safe shrimps/fish/crab, raised in an ecofriendly manner by adopting good management practices, has become an essential pre-requisite for Indian seafood export. The brackish water area available in India for shrimp farming offers good potential for organic farming. This includes the vast traditional prawn filtration fields located in West Bengal and Kerala. The traditional type of prawn filtration system is highly environment- friendly as they use no chemicals, drugs or antibiotics. Organic aquaculture ensures that the farming activity is in harmony with the nature, raised with due care for the good health and welfare of the cultured organisms. Organic products have become very popular of late due to the rising awareness in health and food safety. There is a growing demand for organic products in the global market, especially in Europe, USA, China, etc. The purpose to shrimp certification is to enhance the market share for the shrimp produced by responsible methods, inputs and practices, that would meet the expectations of socially and environmentally aware consumers. Certification process has to be simplified and branding of shrimp produced through improved traditional methods as ‘organic’ may fetch better price. A trial towards organic farming of shrimp was carried out by ICAR-CIBA at KRC showed promising result with better profitability.

8. Periphyton based Farming

Artificial substrates for periphyton development have been widely used in fresh water aquaculture, particularly in carps, tilapia and giant fresh water prawn, to augment fish production. Similarly, promising results in terms of growth, survival and production were observed with periphyton in brackishwater penaeid shrimp, *P. monodon* and *P. vannamei*. Like biofloc, periphyton is also a heterogenous mixture of biota including bacteria, fungi, phytoplankton, zooplankton, benthic organisms, detritus, etc. But unlike biofloc-based system, here the mixture of biota is generally attached to any

submerged surface such as bamboo stick, plastic sheet, polyvinyl chloride (PVC) pipe, ceramic tile, fibrous scrubber, etc. Periphyton-based system also increases the aquaculture production and develops the resistance to different diseases by augmentation of immune response. Work carried out at Kakdwip Research Centre of ICAR-CIBA reported 17.9% gain in production and 22.3% reduction in FCR compared to conventional culture in case of *P. monodon*. The submerged substrates added into the aquatic system improve the water quality, and consumption of microbes and algal community present over submerged substrates enhances the growth of penaeid shrimp by providing natural food.

9. Multiple Stocking Multiple Harvesting model

For small farmers return from farming at regular intervals is most desirable to meet up their daily needs. Multiple stocking and multiple harvesting model are suitable for these farmers. Moreover, this method can augment productivity of small ponds by many folds. Milkfish farming model has been established with this model by KRC. Milkfish fingerlings (6 – 10 g) were stocked and reared in fertilized ponds (500 m^2) provided with formulated feed @ 3 – 5 % of body weight daily. After 100 days, harvesting was started when the fish attained at least 150 g and ponds were re stocked with same quantity of advanced fingerlings (25 to 50 g) at 15 day intervals keeping the total number of fish same to that of initial stocking. Higher production of 3.6 ton/ha has been achieved after 160 days of culture this model with higher density had a BCR of 1.66 suggesting its suitability over the lower density system with BCR of 1.50. Findings indicated that MSMH model with highest talking density of 15,000/ hectare can improve production and profitability in low input based milk fish farming. This model could be suitable for small and marginal farmers with several added advantages. The farmers do not require a large capital to meet up various recurring expenditure. After managing the ponds for a maximum 3 - 4 months, the farmers start earning, which is reinvested for purchase of various inputs required for further fish rearing. Therefore, a marginal farmers can also take up scientific milk fish farming with meagre resources by adopting this system and can meet their day-to-day needs from fish harvest at regular intervals. Moreover, netting at short intervals results in release of noxious gases and mixing of bottom nutrients with water, which enhances primary productivity of the pond.

10. Integrated Multi Trophic Aquaculture (IMTA)

IMTA is a farming practice which combines cultivation of fed aquaculture species (e.g. finfish or shrimp) with organic extractive aquaculture species (e.g. shellfish or herbivorous fish) and inorganic extractive aquaculture

species (e.g. seaweed/sea grass) in the appropriate proportions to create balanced systems for environmental stability, economic stability and social acceptability. The IMTA concept is very flexible and can be land based (pond/RAS) or open water systems (cage/pen), brackish water or marine system. IMTA is well recognised as a mitigation approach against the excess nutrients/organic matter generated by intensive aquaculture activities specially in brackish waters, since it incorporate species from different trophic positions or nutritional levels in the same systems. The preferred species for brackish water IMTA (BIMTA) are: fin fishes- Seabass, milk fish and mullets; shellfish-green mussel/oyster; seaweed-*Laminaria* sp., *Kappaphycus* sp. In a BIMTA model involving Mullets and tiger shrimp as fed species, and estuarine oyster and sea weed (Enteromorpha sp.), as extractive species was evaluated as a viable aquaculture option. Ponds are stocked with mullets and tiger shrimp at 10000 and 30000 no/ha respectively, *C. cuttackensis* at 1600 no/ha suspended with basket in water column and *Enteromorpha* sp at 200 kg biomass/ha. After 150 days of culture total production of 1707 kg/ha was obtained. Another model, involving Tiger shrimp @ 30000 /ha, mullets @ 12000 no/ha, water spinach 300 kg/ha and oyster was adopted. In this polyculture trial of 150 days, total production of 1510 kg/ha was obtained.

11. Way forward

The aquaculture development project should try to achieve the maximum possible yield, which is not currently possible with existing technology and infrastructure. Development of eco-friendly and cost-effective culture technologies of finfish targeting small-scale farmers is the need of the hour. Further steps to develop sustainable brackishwater aquaculture are expansion of brackishwater aquaculture in inland saline areas, bringing more areas under culture, species diversification. Adequate availability of quality fish seeds will also help in expansion of culture. Development of intensive farming technologies like re-circulatory aquaculture system (RAS), improved polyculture, biofloc based farming, IMTA, organic farming, periphyton based culture will help to develop sustainable and eco-friendly brackishwater aquaculture.

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Introduction to Hilsa Research Activities of ICAR-Central Institute of Brackishwater Aquaculture

Debasis De

Kakdwip Research Centre of ICAR-CIBA, Kakdwip, West Bengal, India

E-mail of corresponding author: dedebasis47@gmail.com

1. Introduction

The Indian shad (*Tenualosa ilisha*) is popularly known as Hilsa. Hilsa is one of the most important commercial fishes of the Indo-Pacific region. This fish is nostalgia and an important part of rituals in Bengali culture, thus make it a delicacy on plates. The Government of West Bengal, the state where the maximum catch and sale of Hilsa takes place in India, reported that catch has declined by a staggering 67% from almost 80,000 tonnes in 2001 to 26000 tonnes in 2017. Hydrological alteration in the form of barrage and dam across the east and West Coast River blocked the migratory route of hilsa to its breeding ground resulting into decline of hilsa fisheries due to recruitment failure and intensive exploitation of adult as well as juveniles of the species. Mean size at capture was 356 mm in 1960 to 263 mm in 2016-17. Therefore, to conserve and propagate this species, research activities have been taken up by six ICAR Fisheries Research Institutes since 2012. ICAR-CIBA has been a party in this collaborative research project. The chapter attempts to highlight the research progress made on feed development, breeding and culture of hilsa in brackishwater pond ecosystem.

2. Body composition analysis of hilsa from different sources

Nutritional composition of hilsa, *Tenualosa ilisha* of different size groups were analyzed in an effort to develop artificial feed for hilsa . Proximate composition revealed significant ($P<0.01$) reduction in crude protein and carbohydrate content and increase ($P<0.01$) in total lipid as the fish grew. Arginine, methionine and glycine contents were significantly ($P<0.05$) higher in fish below 5 g whereas fish of higher size groups (> 800 g) had higher ($P<0.01$) leucine and isoleucine contents. Saturated fatty acids and mono-unsaturated fatty acids were lower ($P<0.05$) in fish below 5 g size as compared to bigger ones, whereas docosahexaenoic acid was higher ($P<0.01$) in fish below 5 g size.

Total carbohydrate was significantly ($P<0.01$) higher in smaller size groups as compared to larger size groups. Nitrogen free extract (carbohydrate) content (%) was maximum in <5g size groups and decreased

as the weight of fish increased. Among the minerals, calcium, phosphorus, magnesium, zinc, manganese, chromium and iron content were significantly (P<0.01) higher in smaller size groups compared to other groups. Calcium and phosphorous contents (%) varied between 0.37 to 0.99 and 0.32 to 0.65, respectively. In the present study, crude protein, carbohydrate and fatty acids mainly DHA content in fish muscle of small size groups of hilsa indicated higher nutrient requirement due to higher metabolic activity of small fish. When dietary energy exceeds the energy requirement as normally happens in adult fishes when metabolic activity comes down, energy is stored as lipid in body as was observed in large size groups of fish, where lipid content increased.

3. Preferred feed for Hilsa in different stages of growth

Index of preponderance (%) and frequency of occurrence(%) of gut contents indicated that diatoms were the major items in the gut of fishes below 5 g size and *Coscinodiscus* sp. was the most prevalent diatom . In 5-100g size copepod, diatom and filamentous algae were the major food item and *Coscinodiscus* sp. *Biddulphia* sp. and *Nitzschia* sp. were dominant among diatom. In 101-200 g group, *Coscinodiscus sp.* and *Biddulphia sp.* followed by *Pleurosigma* sp., *Diatoma* sp. and *Nitzschia* sp., were dominant among the diatoms, and among filamentous algae *Ulothrix* sp. was dominant. In 201-400 g group *Coscinodiscus* sp. followed by *Nitzschia* sp., *Asterionella* sp., *Pleurosigma* sp., *Diploneis robustus, Biddulphia* sp., *Rizosolenia setigera* were dominant among diatom and among filamentous algae *Spyrogyra* sp. followed by *Ulothrix* sp. were dominant. In 401-600 g group diatom and copepod were the major food. *Coscinodiscus sp.* followed by *Diatoma* sp., *Nitzschia* sp., *Pleurosigma* sp. and *Asterionella* sp. were dominant among diatom, *Ulothrix* sp. was dominant among filamentous algae and *Coccolithophore sp.* was dominant among unicellular algae. In 601-800 g group also diatom and copepod were major food. Among diatom *Coscinodiscus sp.*, *Nitzschia sp.*, *Pleurosigma normanii, Rhizosolenia crassipina, Thalassiothrix sp.* were predominant items. In above 800 g size group, diatom was major food item and *Coscinodiscus* sp., *Diatoma* sp. *Nitzschia* sp., were dominant among diatom. The study indicated that hilsa (*Tenualosa ilisha*) prefer diatom and copepod in all stages of growth. Form gut content analysis it was found that that in their early stages they mostly prefer copepod and other zooplankton and shift their preference towards diatom and filamentous algae when they grew beyond 50 g size. Indoor experiment on preferred live food for hilsa indicated that hilsa fry preferred mixed zoo plankton consisting of copepod, mysids and rotifer and gained maximum weight in tank.

4. Nutritional requirement of Hilsa

Fish need energy and essential nutrients for maintenance, movement, normal metabolic functions and growth. Fish can obtain their energy and nutrients from natural food in ponds, from feed supplied by the farmer or from a combination of both sources. The feed requirements of fish vary in quantity and quality according to their feeding habits and digestive anatomy as well as their size and reproductive state. Feed requirements are also affected by environmental variations such as temperature and the amount and type of natural food available. The major components of feeds are water, protein, lipid, carbohydrate, minerals and vitamins. Among these, protein, lipid and carbohydrate are called as macronutrients and minerals and vitamins are called micronutrients.

Protein is composed of amino acids. There are 10 different amino acids that cannot be synthesized in fish at rates sufficient for maximum growth and development and have to be supplied in the diet. These are the "essential amino acids". There are many others that can be synthesized from the essential amino acids by fish. The exact requirements for essential amino acids vary between species and life stages.

Lipids are composed of fatty acids and some of these are essential for some species of fish. Lipids are important in the diet as a source of energy, essential fatty acids, sterol, phospholipids, and carriers of fat soluble vitamins. Fish oil and soya oil are generally used as lipid source during feed formulation.

Carbohydrates include fibre, starches and sugars and while not usually considered essential, they can be an effective source of energy and improve food conversion efficiency when included at moderate amounts. Carbohydrates are usually the cheapest sources of energy although different species of fish differ in their ability to use carbohydrates. Carbohydrates can also help to bind a diet together.

Table 1. Nutrient requirements of Hilsa

Nutrient	***Tenualosa ilisha***
Energy (Kcal/kg)	4000-4500
Protein %	35-40%
Lipid %	12%

Minerals are important for normal skeletal development of fish but some also have a vital role in the functioning of enzymes and other metabolic functions. The ash content of an ingredient is the total amount of minerals (or inorganic matter) present within a food.

Vitamins are complex organic compounds required in small amounts for normal growth, reproduction, health and general metabolism. Diets lacking adequate levels of vitamins and minerals can result in growth and development disorders and death in severe cases of deficiency. Many vitamins and especially Vitamin C (ascorbic acid) are easily damaged by heat, light and humidity and this reduces their usefulness to fish.

Hilsa eat primarily to satisfy energy requirements. If there is too much energy compared with protein, animals will stop eating before they consume enough protein for maximum growth. Too much energy from dietary fat or carbohydrate can also lead to high body fat, low dress out yield and poor shelf life in market size animals. If there is too little energy compared with protein, part of the dietary protein will be used for energy. It is therefore important to determine the optimum ratio of energy to protein for hilsa. This ratio can also be affected by the size of the animal. Generally the ratio of energy to protein increases as the animal gets bigger.

Nutrient requirement study of Hilsa fry using RAS

For nutrient requirement study, formulated floating feed with different levels of protein and fat were prepared by locally available ingredients by twin screw extruder. And it was found that 35 % protein and 12 % lipid was optimum for growth of hilsa fry. After optimizing protein and lipid requirement, optimal level of methionine and lysine required in diets of hilsa fry were also determined.

5. Feed Formulation

After optimizing the protein, lipid and amino acid requirement, feed (floating and slow sinking) for hilsa were formulated using locally available ingredients. The main objective or aim of feed formulation is development of a nutritionally balanced mixture of feed stuffs which will be eaten in adequate amounts to provide optimum growth of the cultured hilsa at an acceptable cost by utilizing knowledge of nutrient requirements, locally

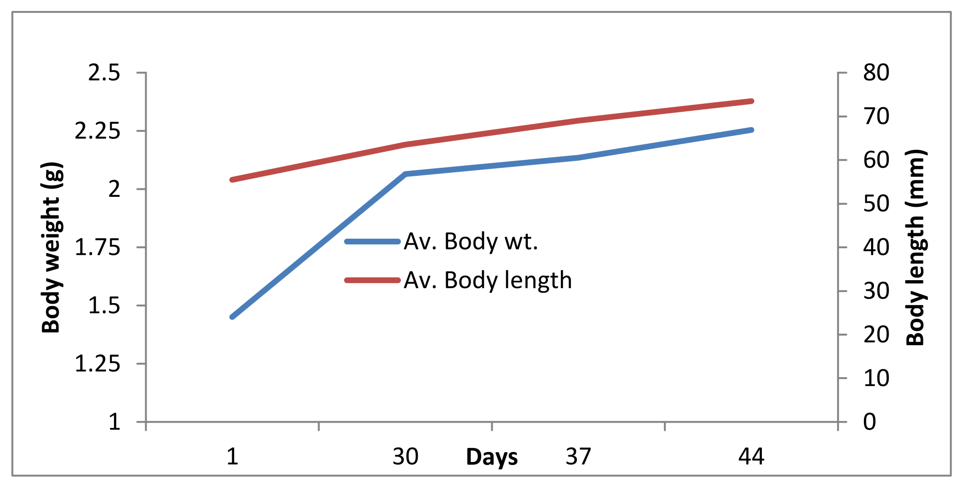
available feed ingredients and digestive capacity of the fish. Before proceeding with formulating a feed, the ingredients are to be selected from available sources. No single ingredient can be expected to provide all the nutrient requirement. Each ingredient in the diet should be included for a specific reason i.e., either to supply a specific nutrient or physical property to the diet. Seasonal availability of ingredients, proximate composition, digestibility and nutrient availability should be taken into account during selection of ingredients. Constraints of using some feed ingredients having antinutritional factors must also be kept in mind. Additional cost for ingredient handling, processing prior to mixing or pelletizing and transportation cost is also to be considered.

Formulation of a feed by the nutritionist is only the beginning of a process that ends when the feed is finally consumed. Feed formulation is essentially a recipe making process keeping in mind the nutritional requirement of particular species, palatability and growth promoting ability of that feed. These objectives can be achieved by judicious selection of feed ingredients, mixing them in proper proportion and presenting them in a most acceptable form.

6. Acceptance of Formulated feed

6.1. Acclimatization and weaning

Hilsa fry were acclimatized with live feed in fibre reinforced plastic (FRP) tank for 1 month. Fishes were weaned with dried copepod powder replacing live feed for one week. Dried copepod powder was gradually replaced by formulated micro particulate feed by one week. After 45 days fishes were completely weaned and there was 55.86% increment in growth during weaning.



Growth curve of hilsa during weaning

6.2. Larval feed for Hilsa

Larval feed are micro-particulate feeds of different particle sizes (300 to 800 micron) which were prepared to contain 40% crude protein and 12% lipid, using premium ingredients enriched with EPA and DHA. The larval feeds were tested in institute's own research facility. The results revealed that the feed have better attractability and palatability for hilsa larvae of different stages with respective particle sizes.

Micrparticulate particulate larval feed

6.3. Grow-out feed for Hilsa- Hilsa^{Plus}

CIBA has developed the cost effective balanced feed **Hilsa*^{Plus}*** using indigenous ingredients. After optimizing the protein, lipid and amino acid (lysine and methionine) requirement of hilsa fry, formulated feed (floating and slow sinking) with 35 % protein and 12 % lipid for hilsa were prepared using locally available ingredients. The feed is being used for culture of hilsa in brackishwater as well as fresh water ponds.

This feed has been tested in brackishwater and freshwater ponds and was found to have good attractability, palatability. Using this growout feed Hilsa seeds (52.97±5.50 mm/1.37±0.18 g) stocked @ 8800 nos./ha in brackish water ponds grew to 360g/ 330 mm in 21 months with 30% overall survival.

6.4. Broodstock feed for Hilsa -Hilsa Brood^{plus}

Institute has developed a unique formulation for Hilsa Broodstock using novel and speciality ingredients and the feed has been found to be effective for getting reproductive maturity of hilsa in captivity. Proteins, energy, lipids and fatty acids, minerals and vitamins have been incorporated according to the specific need of the maturing hilsa. The formulation ensures complete utilisation of all nutrients by the fish for maintaining all round health and rapid gonadal growth. High quality raw ingredients such as high quality fish meal, fish oils, phospholipids, combination of high quality vegetable proteins

were incorporated to provide balanced amino acids, Fatty acids (EPA, DHA), minerals and vitamins. Using this feed males reached milting stage in 1.5 years and females developed mature oocytes (Stage V+) in 2 years (October-November and January- February) in brackish water ponds, males started milting within the same period. However, the oocyte stages did not reach beyond this stage, which needs further trials for achieving. Mobilization pattern of different fatty acids from muscle to gonad and retention of specific fatty acid in gonad (viz., C14:0, C18:0, C20:5, C22:6 and C20:4) needs to be taken in to consideration while formulating the functional feed for broodstock.

Maturation of female and male hilsa in brackishwater pond

6.5. Feeding behavior of hilsa

Feeding behaviour of pond reared hilsa was studied by observing the fullness of gut at 4 hrs interval and through gut content analysis. Fishes were collected from pond at 7.00 AM, 11.00 AM, 3.00 PM & 7.00 PM and fullness of gut was examined. It was found that at 7.00 AM gut was empty, at 11.00 AM 50 % of gut was filled, at 3.00 PM 75 % of gut was filled and at 7.00 PM 100 % of gut volume was filled with planktons available in the ponds. Result indicated Hilsa fingerlings feed during day time and they are nonselective feeder.

7. Breeding attempt

Hilsa could be successfully bred by dry stripping of brood fish (male 225-280 g / 262.8 265 mm and female 450-660.5g/ 376.5-380 mm) collected from the Hooghly River using gill net at Godakhali, West Bengal. The sex ratio of female and male was 1:3. Clean and aerated bore well water was sprinkled over the mixture and mixed gently. Several washing were carried out to remove extra milt and ovarian fluid. After 1 hour of post stripping, the eggs were transported to KRC of ICAR-CIBA, Kakdwip with oxygen packing and incubated at 23±1.0°C in natural photoperiod. Embryonic developmental stages were observed at different hours. Fertilization rate

varied from 62.67 to 86.33% and hatching rate was around 57%. The embryonic and larval development was also investigated.

8. Larval Development

Digestive enzymes in the larvae were estimated at different days after hatching, and it was found that no digestive enzyme activity was detected before the 4th day after hatching. On 5th day post-hatching, amylase, cellulase, acidic protease, alkaline protease, and lipase activities were detected. Breeding trial with different sources of freshwater (river water, potable drinking water and freshwater from bore well) suggested that the hardness of the water used for fertilization and incubation of eggs should be similar to source water where from brooders were collected.

9. Culture of Hilsa in brackishwater pond

Culture in brackishwater was reported for the first time by Kakdwip Research Centre of ICAR-CIBA in 2020, where the wild-collected seeds were acclimatized and stocked in brackishwater pond and culture was done using formulated feed (crude protein 35%, ether extract 12%). At the end of 6 months of grow-out culture, the survival rate was around 40% in grow-out pond. After 6 months of grow-out culture, sub-adults (66.76 g, 184.75 mm) were collected through drag nets and restocked in broodstock ponds (1000 m^2) at 3500 nos./ha for broodstock development. The survival rate was 20% in captive broodstock pond.

10. Further Readings

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Feed Management in Brackishwater Aquaculture with Special Reference to Aquatic Animal Health

T.K. Ghoshal and Debasis De

Kakdwip Research Centre of CIBA, Kakdwip, South 24 Parganas 743347

E-mail of the corresponding author: ghoshalciba@gmail.com

1. Introduction:

Aquaculture has now been recognized as an important area for fisheries development to meet the food needs of the people and also to create employment opportunities. In recent years there has been a great emphasis on enhancement of aquaculture yield through approaches such as the use of genetically improved fish varieties and also complementing the natural food with supplementary diets, which meet all the nutritional requirements of the cultured species of fish and shellfishes. Of late the use of formulated feeds have become unavoidable especially under high stocking densities as observed in practices of intensive and semi-intensive rearing of fish and shrimp. Hence feed is a major expenditure for fish and shrimp farmers. Good feed management can reduce the overall culture cost, improve fish farm environment and ensure healthy growth of fish stock. Fish feed management includes choosing the right feed, using a correct feeding method, calculating the feeding cost and ensuring the cost effectiveness of fish farm. Poor feed management not only reduces profit to farmers but also leads to serious environmental pollution. Fish faecal waste and uneaten food can cause water quality deterioration and the buildup of anoxic sediments leading to environmental pollution. Provision of well balanced feed is important as it not only help to attain genetic potential for growth but also to hasten recovery from diseases and aid in overcoming environmental stress. Thus proper feed management helps in reducing the cost of culture operations since in farming operations cost of feed accounts to about 55-60% of the total operating costs.

2. Feed management in fish culture systems

Feed management is the judicious use of feed so that feed utilization is optimum and wastage is minimum, hence negligible impact on environment, achieving best feed conversion ratio, maximum growth and production of fish. A very good quality feed can also give poor result with improper feed management, whereas, a moderate feed can produce very good results with proper feed management.

The foremost critical factor is selection of appropriate feeds and planning of optimal feeding regimens. Suitable feed should fulfill the nutritional requirements of 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 that the diet remains intact in water before ingestion and nutrients are prevented from dissolving. The general categories of feed 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 percent. Since problems are associated with the distribution, handling, utilization, storage and quality of wet feeds and moist feeds, more and more dry feeds are manufactured either by steam pelleting or by extrusion pelleting. Advances in fish feeds and nutritional studies mean that many commercial feeds satisfying a wide range of options are now available.

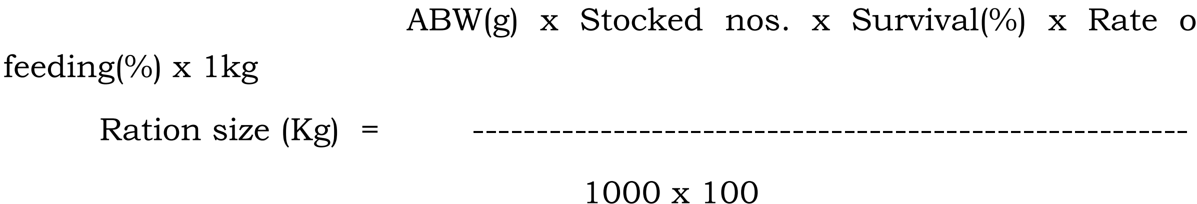
Following points should be strictly followed while feeding the fish for maintaining good pond hygiene and to reduce wastage of feed and to avoid accumulation in pond bottom.

1. Pond biomass should be assessed regularly and ration should be offered as per biomass of the pond.
2. Time and method of feeding should be proper.

2.1. Ration size

The allotted quantity of feed for a period of 24 hours of a candidate species is the ration size for that fish. The optimum quantity of feed determination for aquaculture species is a difficult task. The size of daily food ration, the frequency and timing of meals are the key factors influencing the growth and feed conversion. Hence, the optimal feeding regimens must be determined as per the feeding behaviour, appetite and functioning of the digestive systems and the various specific chemical substances, which act as feeding stimulants for fishes. Fish lose weight when their food intake falls below that required for maintenance. When ration size increases, the growth rate increases. Generally the method of calculating the daily ration is based on the body weight of fish. The quantity of ration varies from 100% of body

weight for larvae and fry and gradually reduced to 50 %, 20%, 10%, 5% and 2-3% as the fish grow marketable size. Ration size may also differ because of physiological stress created due to fluctuation of water quality parameters, particularly temperature. Water temperature higher or lower than the optimal range may affect the feed intake and hence ration size needs to be adjusted to minimize the feed wastage. Generally the method of calculating the daily ration is based on the body weight of fish.



$$\text{Ration size (Kg)} = \frac{\text{ABW(g)} \times \text{Stocked nos.} \times \text{Survival(\%)} \times \text{Rate of feeding(\%)} \times \text{1kg}}{1000 \times 100}$$

Ration size is also estimated by various methods using the feeding charts, feed equations, growth prediction and check try etc. Feeding rates for seabass of 1-2 g is 20 %, 2 -20g is 15-7 %; 21-55 g is 6-4 %, 56-180 g is 4-2 % and when body weight is more than 180 g, feeding rate is 2 % of biomass (De et al. 2012). For assessing the average body weight, sampling needs to be done on regular basis i.e., weekly or fortnightly to recalculate the amount of feed to be given. Use of sensor based demand feeder may help for precise estimation of ration size which may be helpful in large scale farming. Besides the food ration size, the optimal food particle size also affects the growth and feed conversion efficiency. Large fish can ingest small particles, but it requires more energy to capture the required equivalent weight or smaller food particles. This results in measurable reduction in food conversion efficiency (Goddard, 1996). Attention should also be given to the influences of feed shapes, colors and textures of pellets on ingestion rates.

2.2. Feeding methods

Production of high quality fish at least-cost depends on an effective feeding method. Various techniques exist, from hand feeding to mechanized feeding. They depend on diverse range of factors such as labour costs, scale of farming, species under farming, the type of holding system and hatchery or grow out systems. Often farmers use a combination of feeding methods such as hand feeding to mechanized feeding. Feed bag suspended at different places in ponds is most common method of feeding to the fish. For improving feed utilization efficiency floating feed can be given in net enclosure and sinking pellet may be given in feed tray. Finfish are easily conditioned to feeding and react to the first appearance of food, making

them suitable for broadcast feeding (De Silva & Anderson, 1995). Sometimes feed are offered in the dough form kept on a platform just below the water surface. For Asian seabass, primarily fry should be weaned with formulated feed in hapa, cement cistern or fibre tank so that feeding can be monitored easily. They should be fed with little patience as it takes longer time to feed. When the fishes are habituated with pellet feed they should be transferred to culture pond. In grow out pond weaned seabass are offered feed by broadcasting the feed in 4-6 places. Feed should be broadcasted as long as fishes stop taking the feed. In mechanical feeding system, demand feeder is used in which fish approaches to the feeder for its feed requirements when they feel hungry. It was observed that fish quickly learn how to obtain feed. The growth of fish is good with best FCR and minimum wastage of feed in self-demand feeding system. A reliable and least- cost feeding system should ensure the effective distribution and spread of adequate feeds in aquaculture ponds.

2.3. Schedule and frequency of feeding

The total feed required in a day should not be fed at a time. Scheduling and frequency of feeding greatly help in successful feed management. Determination of feeding frequency is a researchable area in aqua nutrition and it differs from species to species and culture system. Species which require more time for evacuation of stomach require less number of feeding compared to species in which passage rate of feed in gastrointestinal tract is faster. Generally young fish are fed more often and the frequency of feeding decreases as the fish grow. As a thumb rule fish should be fed at 1 % of body at each meal. For example if the ration size is 5 % of biomass, fish should be fed five times each at 1 % of biomass. Most of the brackishwater fishes are fed 3-4 times a day. Higher feeding frequency reduces starvation and stunting and results in uniform growth of fish. Time schedule for feeding the fish may be fixed in such a way that larger ration may be given when the fish is expected to be most hungry. If night feeding is limited the morning feeding should have larger ration. There should be a minimum of three time schedules of feeding in a day - morning, noon and evening. Species which are having nocturnal feeding habit should get comparatively larger portion of the ration in the evening/night. Frequent feeding of small portion of ration help in better utilization of the feed and thereby lead to efficient FCR. There must also be a mechanism in each case to monitor the feed consumption and offering of next dose of feed should be regulated on basis of consumption from the previous feed offered.

2.4. Feeding management in shrimp culture system

As feed alone costs 50-55% of total culture expenditure, strict supervision on feeding is required. Shrimp find their feed mainly by chemosensory mechanism rather than vision. The chemoreceptors are concentrated on the anterior appendages, antennae and antennules. Once the smell is detected, the shrimps move towards the feed and quickly grasp it with their chelate pereiopods. As the shrimps are sluggish feeders, it is practically difficult to feed them up to their satiation. Hence, shrimps are fed following prescribed schedule based on culture system. Due to benthic feeding behavior, sinking pellet feed should be offered to shrimp. 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 pond bottom.

- Proper feeding guidelines should be followed to fix ration size for shrimp culture pond
- High quality feed should be used
- Daily ration should be offered in 4/5 meals
- Feed intake should be checked through check trays (6 nos/ha)
- Feed should be reduced up to 50% during moulting period
- Feeding should be avoided during heavy rain
- Feed of proper pellet size should be offered

Appetite of shrimp 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 pollutes the water. As shrimps are the nocturnal feeder, larger doses may be offered in the evening and during night. Unlike other shrimps, white leg shrimp (*Penaeus vannamei*) are more active during day time and hence major share of the ration is offered during day time. Generally during new moon and full moon moulting of shrimp takes place and they become physiologically less active and reduce the feed intake. Quantity of feed offered should be reduced at the extent of 30-50 % during that period. Regular observations and experience helps in mastering the management of feeding in a culture farm.

2.5. Ration size, feeding frequency and time of feeding

Generally, the method of calculating the daily ration is based on the body weight of shrimp (Table 2 & 4). Blind feeding is generally practiced during first fifty days of culture (Table 1 & 2). Daily ration is divided and offered 2 to 5 times a day (Table 3 & 5) depending on stages of culture. 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 should be 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 vary as per body weight of shrimp (Table 6). Feed should be broadcasted evenly in a periphery of about 2 meters from dyke in all sides of the pond.

Table 1. Ration size for first fifty days of tiger shrimp farming

Age (Days)	Feed increment /day (g)	No. of meals /day	Feed (Kg) /day / lakh PL_{20}
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. Ration size after 50 days of culture in tiger shrimp based on check tray performance

Days of culture	Expected ABW (g)	% of biomass as feed	Feed % in check tray	No. of meals / day
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
132-138	33-36	2.0-1.8	3.7	5

Table 3. Feeding schedule for tiger shrimp

Feed type	Shrimp weight (g)	Time of feeding				
		6.00 AM	11.00 AM	6.00 PM	10.00 PM	2.00AM
Starter	Up to 4.0	30 %	-	35%	35 %	-
Grower	4 – 25	25 %	15 %	30 %	30 %	-
Finisher	> 25	25 %	15 %	20 %	25%	15%

Table 4. Ration size for *Penaeus vannamei*

Age in days	Feed increment/ day	Feed(kg)/day/lakh PL_{15}
1	-	2.0
2-10	400	2.4-5.6
11-20	500	6.1-10.6
21-30	600	11.2-16.6
31-50	700	17.3-30.6
After 50 days (based on body wt. g)	% of biomass	Feed in check tray (g/kg/tray)
5-10	5.5-4.5	2.4-2.8
10-15	4.5-4.0	2.8-3.0
15-20	4.0-3.5	3.0-3.3
20-25	3.0-2.5	3.3-3.6
25-30	2.5-2.0	3.6-4.1

Table 5. Feeding schedule in *Penaeus vannamei* farming

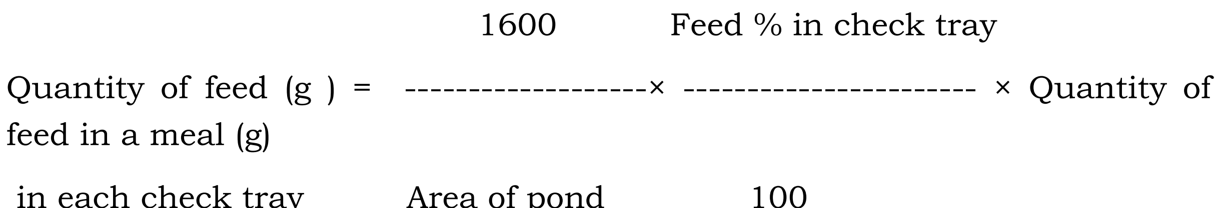
	Percentage of daily ration in meals				
	6 AM	9 AM	12 PM	3PM	6 PM
1st month	40	-	-	60	-
2nd month	40	-	-	30	30
3rd month	20	20	-	30	30
4th and 5th months	15	15	10	25	35

Table 6. Recommended pellet size for tiger/white leg shrimp

Feed type	Size of shrimp (g)	Pellet size
Starter	0-4.0	0.5-1.0 mm crumble
Grower	4.0-25.0	2 - 2.3 mm x 4 - 5 mm
Finisher	>25	2-2.5 mm x 6 – 8 mm

2.6. Check tray monitoring

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



$$\text{Quantity of feed (g) in each check tray} = \frac{1600}{\text{Area of pond}} \times \frac{\text{Feed \% in check tray}}{100} \times \text{Quantity of feed in a meal (g)}$$

The check trays should be observed after 2 hr 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, shortage of dissolved oxygen and stressed condition due to heavy rain, high temperature, unfavourable pond bottom and water quality. Feed adjustment for shrimp should be done by check tray observation (Table 7).

Table 7. Feed adjustment for shrimp by check tray observation

Average amount of unconsumed feed remaining in trays (%)	Feed adjustment in subsequent meal
0 (zero)	5% increment
<5	No change
5-10	5% reduction
10-25	10% reduction

If tray monitoring is done properly and check tray feed is consumed within 2 hours, survival % can be accurately estimated by the following formula

$$\text{Survival \%} = \frac{\text{Total quantity of feed consumed per day (g)}}{\text{Stocked shrimp} \times \text{ABW (g)} \times \text{\% ABW feed}} \times 100$$

For example in a culture pond of 1500 sq m having total stocked shrimp of 45000 nos consuming 18.225 kg per day and body weight of shrimp is 10 g, assuming feed requirement of 4.5 % ABW, survival % would be

$$\text{Survival \%} = \frac{18225}{45000 \times 10 \times 0.045} \times 100 = 90\ \%$$

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, tiger shrimp can attain average weight of 30-35 g with survival up to 70-80 % in culture duration of 120 days, whereas exotic white leg shrimp could achieve 20-25 g with a survival of 80-90% in 100 days culture period. Progressive farmers may form co-operative society and

can have small scale feed mill to prepare shrimp feed using locally available feed ingredients for tiger shrimp/ vannamei shrimp/Indian white shrimp culture and may get a good economic return.

2.7. Handling and storage of feeds

Prepared feed for fish and shrimp are perishable products. They are also more or less fragile, depending on the type of feed. Feed processors attempt to formulate and manufacture aquaculture feeds to extend their shelf life and improve durability. For reasons of cost and convenience, dry diets are presently the most widely used feeds in aquaculture. These include extruded feeds, hard pellets, crumbles, and flakes. The general rule for preservation of these feeds is to store them in a dry, well-ventilated area that affords some protection from rapid changes in temperature. Optimizing handling and storage procedures on farms is an essential component of good management practice. High quality feed can readily spoil and denature if stored under inadequate conditions or for too long a period. Incorrectly stored feeds 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 behaviour, poor feeding response and growth. Hence different feed types such as wet feeds, moist feeds and dry feeds must be handled and stored under appropriate conditions. A practical knowledge of the most important factors that contribute to feed degradation and a little attention to maintaining proper storage conditions can significantly minimize loss of vitamin potency, mold growth, fat rancidity and infestation by insects and rodents. different feed types such as wet feeds, moist feeds and dry feeds must be handled and stored under appropriate conditions. Wet feed having moisture content of more than 70% should ideally be stored at temperature of -30 ^{o} C or lower. Common freezer temperature of -20 ^{o} C are inadequate for long term storage. Dry feed should be stored under cool, dry condition, ideally at temperature below 20 ^{o} C and at relative humidity of below 75 %. A normal storage time of 1-2 months is recommended for dry feeds stored in tropical condition (Table 8).

Table 8. Maximum permissible storage time for different feed stuff

Feed stuff	Storage time
Ground ingredients	1-2 months
Whole grain and oil cakes	3-4 months
Compounded dry feed	1-2 months
Vitamin mix. (kept cool)	6 months

Source: New (1987)

Improper storage may lead to oxidation of fatty acids in feed resulting in rancidity. Poly unsaturated fatty acids and pure lipid are very much prone to oxidation. Rancid fat results in less palatability and may produce toxic peroxide compound that inhibits growth of fish/shrimp.Chemicals produced in the deteriorated feed may reduce availability of amino acids and vitamins, vitamin C being most susceptible. One thing to remember is that storage never enhances feed quality, but proper storage reduces the rate of deterioration of feed.

2.8. Water quality:

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 survival can be ensured. When the water quality parameters fall below optimal levels, feeding and growth will be impaired and the species under culture will be stressed due to 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. Judicious feed management is an important factor in achieving good feed efficiency and reducing wastage. Selecting feeds, which are freshly prepared, quality assured and proven with best potential FCR, could reduce waste production. Poor quality and water unstable feeds, which have lost their nutritional potency and are poorly accepted by the fish, should be rejected. Appropriate particle size of the feed should be designed for a particular stage. The ration size and feeding schedules should be regulated with reference to feeding guides, response of fish and environmental conditions.

3. Conclusion

Judicious feed management is the most important factor in achieving good feed efficiency, reducing feed wastage and thereby reducing stress and disease incidence of cultured fish/shrimp. Freshly prepared good quality feed proven with best potential FCR, could reduce feed waste. Feed with poor water stability, which have lost their nutritional potency and are poorly accepted by the fish or shrimp should be rejected. The ration size and feeding schedules should be regulated with reference to feeding guides, response of fish and environmental conditions.

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Simplified Seed Production Technology of Brackishwater Catfish, *Mystus gulio*

Prem Kumar, Babita M, Leesa P, Sanjoy Das and T.K Ghoshal
Kakdwip Research Centre of ICAR-CIBA, Kakdwip, West Bengal, India
E-mail of the corresponding author: prem.cife@gmail.com

1. Introduction

Brackishwater catfish locally known as "nuna tengra", (*Mystus gulio*) is an important farmed fish of the Sundarban delta. *M. gulio* can be farmed in wide range of salinities ranging from fresh water to brackishwater. This fish easily accept floating and sinking feed. This is an important candidate species for aquaculture diversification because of its hardy nature, delicious taste, excellent nutritional value and high market demand. To meet the seed demand, Kakdwip Research Centre of ICAR-Central Institute of Brackishwater Aquaculture has developed a simplified, improved farmers friendly seed production technology of *M. gulio*, which comprising of captive breeding, larval rearing and nursery.

2. Broodstock development

In captivity fish attain sexual maturity at the age of 6-8 months. However, the small size brooder (50-70 g) and will be low fecund (5000-8000). It is also advisable to collect wild adult (>50 g) during month of February to March that is before the onset of peak spawning season (May-August) and reared in small earthen pond (1000 m^2) at the density of $2/m^2$. During broodstock development fish should be fed with high protein pellet diet (30 % protein, 8% lipid) and/or liver @3% of biomass.

3. Induced breeding

Breeding season (May-August)

Selection of matured male and female fish in a sex ratio of 2:1

Selection of matured male and female fish in a sex ratio of 2:1

Female with swollen belly (>900 μm oocyte diameter), reddish open vent and round bulge anal opening. Male having elongated papillae with pink or red tip are selected.

Prophylactic treatment with 50 ppm formalin for 2 min

Breeding tank: Circular or oval-500-1000 L, provision of water flow (8-10 L/h) and shower.

Egg collector: Made up of bunch nylon fibers with sinker tied at one end

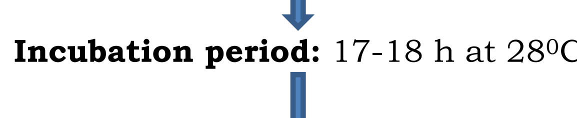
Hormone dose: commercial hormone@ 30-50 μl/female and half the dose to male

Hormone Injection: Intramuscular (I/M) during afternoon (3:00 PM)

Latency period: 8-12 h at 28^0C

Fertilized eggs are sticky and transparent

Incubation: Size- 250-500 L with provision of water flow. Sticky eggs along with egg collectors are transferred to incubation tanks.



Incubation period: 17-18 h at 28^0C

Removal of egg collector after 6-12 h of hatching

Newly hatched larvae are shifted to larval rearing tanks after 12-15 h post hatching

4. Larval rearing

Due to bottom dwelling behavior of larvae, larvae are shifted to larval rearing tanks after 12-15 h post hatching, which reduces handling stress. After two days post-hatching (dph), larvae were fed with freshly hatched *Artemia spp.* nauplii measuring 150- 175 µm in width and 500-580 µm in total length. Optimum larval stocking density is 25 nos/ L. Larvae are fed with *Artemia* nauplii at the density of 3000 nos/ L for four times in a day for initial 7 days. *Artemia* nauplii and crumbled feed from 8 dph and exclusively with crumbled feed from 15 dph. In 30-35 dph, fry attain 48-50 mm size and cost of production for one fry varied from 30-40 Paise only.

5. Further Readings:

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PRACTICAL

Collection, Preservation and Processing of Fish and Shellfish Samples for Bacteriological Examination

Leesa Priyadarsani, Sanjoy Das, *M. Poornima and *K.P. Jithendran

Kakdwip Research Centre of ICAR-CIBA, Kakdwip, West Bengal, India

** ICAR- Central Institute of Brackishwater Aquaculture, Chennai, India*

E-mail of corresponding author: leesa.priya@gmail.com

1. Introduction

Bacterial diseases represent one of the major obstacles to sustainable aquaculture. Routine screening for bacterial pathogens at various life stages of fish and shellfish is important to minimize their introduction into production systems before they can cause serious diseases and spread to other farms. During disease outbreaks or mortality, samples from moribund animals should be taken rapidly as part of the disease diagnostic investigation. Bacteriology is the culture and identification of bacteria growing under specific conditions. The diagnosis of bacterial fish diseases has progressed from traditional culture-dependent methods involving the recovery of pathogens on agar-containing media and identification by examination of phenotypic traits. Newer approaches centre on culture-independent approaches.

2. Materials

Materials required for fish and shrimp sampling are vials for collection of samples, sampling sheets, marker pen, dissection set, gloves, cotton, tissue paper, alcohol, alcoholic Bouin's fixative, Davidson's fixative, camera for documentation and notebook for details of the sampling.

3. Fish samples for bacteriological examination

3.1. Collection and preservation of fish samples

Collect a minimum of 60 diseased fish samples for microbial analysis at the site as per Office International des Epizooties (OIE) guidelines. Do thorough examination and record behavioural abnormalities, gross and clinical signs on the sampling sheet for the sampled fish. Note down the clinical signs like sluggish behaviour, erratic movement, loss of scales from some areas of the skin, excessive mucus all over the body surfaces, petechial haemorrhages, fringed gill, gill rot with discolouration, necrotic lesions extending all over the body, exophthalmia, discoloured and liquefied internal organs, reddening of body, ulceration, focal gill necrosis, fin rot and tail rot.

Collect the fish with typical disease symptoms for bacteriology and histopathology as per standard protocol. Dissect the fish and infected organs like kidney, liver and muscle and collect aseptically for bacteriology. Fix the organs in alcoholic Bouin's fixative for histopathology. Also, transfer the tissues from affected external parts and kidney of morbid fish into the sterile transport medium (soft agar in tubes) and bring them to the laboratory in an insulated container for further analysis. Transport the live diseased fish samples aseptically to the laboratory in sterile jars containing water for microbiological analysis. Keep the boxes in in an insulated container to maintain temperature.

3.2. Media for bacteriological analysis

The most common bacteriological media include Mueller Hinton Agar (MHA) or tryptone soya agar (TSA) or Nutrient agar (NA), tryptone soya broth (TSB), thiosulphate citrate bile salt sucrose agar (TCBS agar) for brackishwater microbes. Adjust the pH of the media to the required level using 1 N sodium hydroxide solution or 0.1 N hydrochloric acid solutions before sterilization. Prepare normal saline solution (1% (w/v) sodium chloride (NaCl) solution) as a diluent (saline) for the enumeration of bacteria and suspending the bacterial cells.

3.3. Bacteriological analysis of fish

Disinfect the fish body surface with 70% alcohol, then dissect under aseptic condition. Take inocula aseptically from the lesions or affected external parts such as gills and body surface, and from the kidney of morbid fish. Label plates with date, fish number, and sample site. Streak onto TSA, MHA and TCBS media then incubate at 30±2^{0}C for 24-48 h (Austin and Austin, 2012).

Directly inoculate samples of fish obtained from skin, muscle, intestine and liver in nutrient broth and incubated at 37^{0}C for 6 hours. Inoculate loopful from each broth culture onto MHA or NA, TCBS. Incubate the plates at 37°C for 24-48 hours. Suspected colonies onto the surface of these media to be identified by studying characters of the colonies as well as Gram's stain morphologically.

3.4. Isolation and identification of bacteria

Randomly pick up one single distinct representative colony, based on dominance and distinct colony morphology, from TSA, MHA and TCBS agar plates. Purify the isolates by repeated streaking on TSA to obtain pure culture for further identification. Maintain the pure culture on TSA slants. To identify the bacterial isolates up to the genus level a series of biochemical

reactions as described by Collins *et al.* (2004) and Austin and Austin (2012) is to be performed. Taxonomic keys to be followed for respective targeted bacteria as per Bergey's Manual.

4. Shrimp samples for bacteriological examination

4.1. Collection and preservation of shrimp samples

Note down gross clinical signs like behavioural abnormalities, change in appearance, colour, lesions, etc. In dead shrimp autolysis takes place immediately, so most appropriate sample is the moribund shrimp. Collect ten samples of each diseased as well as normal shrimp aseptically early in the morning and transported immediately to the laboratory using sterile polyethylene bags with oxygen. Pack these bags in an insulated container to maintain the temperature. Place a couple of ice pack or gel pack while sending samples long distance. Water sample (100 ml) for bacteriological analysis should be collected in sterile container and transported in ice. Label properly with complete history of disease, place and all the details related to it. Take proper photographs for documentation.

4.2. Media for bacteriological analysis

The most common bacteriological media include Mueller Hinton Agar (MHA) or tryptone soya agar (TSA) or Nutrient agar (NA), tryptone soya broth (TSB), thiosulphate citrate bile salt sucrose agar (TCBS agar) for brackishwater microbes. Adjust the pH of the media to the required level using 1 N sodium hydroxide solution or 0.1 N hydrochloric acid solutions before sterilization. Prepare normal saline solution (1% (w/v) sodium chloride (NaCl) solution) as a diluent (saline) for the enumeration of bacteria and suspending the bacterial cells.

4.3. Bacteriological analysis of shrimp

Collect ten samples of each category aseptically early in the morning and transport immediately to the laboratory using sterile polyethylene bags with ice. Divide the shrimp samples into three parts - head, body and tail. Then homogenize 10 g of all parts of the samples through blending with 90 ml peptone water individually in a sterile automatic blender. Incubate the broth at 37°C for 24 hours. Then do serial dilution up to 10^{-6} (Cappuccino and Sherman, 1996).

4.4. Isolation and identification of bacteria

To enumerate total viable bacteria (TVB), spread aliquot 0.1 ml of the sample onto NA and TCBS. After spreading, incubate the plates at 37^{0}C for 24 hours. Randomly pick up one single distinct representative colony, based on dominance and distinct colony morphology, from TSA, MHA and TCBS

agar plates. Purify the isolates by repeated streaking on TSA to obtain pure culture for further identification. Perform Gram's staining for morphological identification. Identify the pure isolates using different biochemical tests like Kligler's Iron Agar (KIA) test, Indole production test, Methyl Red (MR) test, Voges-Proskauer (VP) test, Citrate utilization test, Motility Indole Urea (MIU) test, Carbohydrate fermentation test and salt tolerance test.

5. Further readings

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Collection, Preservation and Despatch of Finfish and Shrimp Samples for Histopathological Analysis

Sanjoy Das, Leesa Priyadarsani, Prem Kumar and *P. Ezhil Praveena

Kakdwip Research Centre of ICAR-CIBA, Kakdwip, West Bengal, India

** ICAR- Central Institute of Brackishwater Aquaculture, Chennai, India*

E-mail of corresponding author: sanjoy125@yahoo.co.in

1. Introduction

Proper sampling is one important crucial step for disease diagnosis. Histopathological analysis of various organs of aquatic animals is important for diagnosis of various diseases in them. For efficient histopathological analysis, proper sampling is very much important. Further, improper sampling may result in wrong and incorrect diagnosis leading to ambiguous interpretation. In such cases adaptation of corrective measure will be difficult.

2. Sampling methods for disease diagnosis

Sampling should be done in such a way that it should represent the population under study. The simple random sampling/probability sampling, is one of the most common sampling techniques employed by all. When this is not possible, the sampling should provide the best practical generating optimal inferences about disease patterns in the target population. For correct and efficient sampling, the suitable target organ(s) should be collected based on the disease suspected, age and size of the animals and objectives of testing i.e. for diagnosis of *Enterocytozoon hepatopenaei* (EHP), hepatopancreas and gut are considered as the most suitable organs. The samples should be labelled properly and proper record of the sample should be kept. The sample should be recorded preferably with a 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 time of 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.

3. Sampling media for histopathological analysis

3.1. Shrimp

Moribund diseased shrimp is the ideal sample for histopathological analysis of shrimp. The shrimp should show the visible symptoms of the disease

suspected and is about to die. The most suitable media for preservation of shrimp samples for histopathological analysis is Davidson's fixative. As compared to finfish tissue, the shrimp tissue is more delicate and it liquefies very rapidly. Among the shrimp tissue, the hepatopancreas is the most delicate tissue and it needs to be preserved by injecting the Davidson's fixative directly into the hepatopancreas during live condition. After injection into the hepatopancreas, the remaining portion of the fixative should be injected at different parts of body of the shrimp. The shrimp should be injected with Davidson's fixative (Fig. 1) at the rate of 10 % of the body weight. If the weight of the shrimp is 30 g, then 3 ml of Davidson's fixative should be injected.

Fig.1 Fixing of Penaeus monodon shrimp for histopathological analysis

Following injection of the fixative, the cuticle of the shrimp should be silted along the midline to ensure easy penetration of fixative. The preserved sample should ultimately be stored under 10 volumes of Davidson's fixative. For example, if the size of shrimp is 30 g, then the sample should be stored in 300 ml of Davidson's fixative. Now, the sample in this condition is ready to be transported to laboratory with proper labelling.

3.2. Finfish

Like shrimp, for finfish also the live or moribund fishes are suitable for histopathological analysis. In some cases, for better penetration and fixation, the euthanasia of fish should be done just before fixation. For preservation of finfish for histopathological examination, 10 % neutral buffered formalin (NBF) is the preservative of choice. However, Davidson's fixative or Bouin's fixative may also be used, especially when rapid penetration is required. Both of them are acidic fixatives with slight decalcifying action. Bouin's fixative is suitable for fixing very small fish and also for preservation of skin of large fish. Before fixation, the scales may be removed for easy penetration and fixation. The total requirement of the fixative is approximately equal to 10 times of volume of the tissue. Use of less amount of fixative may cause autolysis. For fixation of gill infested with

parasite, the Davidson's fixative is preferred. If finfish sample is fixed with Davidson's fixative, then it should be replaced with 70 % alcohol after 48-72 hours. Long storage of finfish tissues with Davidson's fixative may result in excessive hardening of tissue. If the finfish sample s very small (Length < 5 cm), the same can be fixed as a whole. But fish larger than 5 cm should not be fixed as whole until internal organs are well-exposed. In case of large fish, the organ of choice may be preserved separately in the suitable fixative (Fig.2). The selection of the organ depends upon visible symptoms (if any), disease suspected and the site of suspected lesion.

Fig.2. Dissection of fish and fixation of organs for histopathological analysis

The preferred sample/organ of some important diseases of finfish and shrimp have been enlisted in Table 1.

4. Sampling for diagnosis of specific suspected disease

The appropriate sample to be collected for diagnosis of specific disease has been summarized in Table 1.

Table 1: Samples to be collected for different diseases

Disease	**Causative agent**	**Preferred sample**
	Shrimp diseases	
Vibriosis in shrimp	Different species of *Vibrio*	1. Haemolymph aspirated directly from heart on TCBS or ZMA media for isolation and identification of *Vibrio* colony 2. Gut and hepatopancreas for isolation of bacteria 3. For histopathology: Shrimp fixed in Davidson's fixative
Acute hepatopancreatic necrosis syndrome	Specific strain of *Vibrio parahaemolyticus*	For histopathology: Shrimp fixed in Davidson's fixative.

White spot disease	White spot syndrome virus (*Whispovirus*)	1. Gills and cuticular epithelium 2. Shrimp fixed in Davidson's fixative
Infectious hypodermal and haematopoietic necrosis (IHHN) disease	Viral infection caused by *Brevidensovirus* (Parvoviridae family)	For histopathology: Ectodermal and mesodermal tissues including gill, cuticular epithelium, connective tissue, haematopoietic tissue, lymphoid organs, antennal gland, etc.
Black gill disease	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.
Loose shell syndrome	Unknown etiology	Hepatopancreas, lymphoid organs and muscle.
Hepatopancreatic parvovirus (HPV) infection	*Brevidensovirus* (Parvoviridae family)	Hepatopancreas of the affected shrimp
Yellow Head Disease	Yellow head virus (*Okavirus* under Roniviridae family)	Cephalothorax tissue of moribund shrimp, tissues of ectodermal and mesodermal origin for finding of cytoplasmic inclusion bodies.
Taura syndrome	Taura syndrome virus (*Aparavirus* under family Dicistroviridae)	Cuticular epithelium, appendages, gill, hindgut and subcuticular connective tissue
	Finfish diseases	
Epizootic Ulcerative syndrome	*Aphanomyces invadans* (An oomycetous fungus)	Muscle tissue at the edge of the ulcer
Iridovirus infection	Viral infection (*Lymphocystivirus* and *Ranavirus*)	Liver and spleen
Vibriosis in brackishwater finfish (e.g. Asian seabass)	Different species of *Vibrio* (e.g. *Vibrio anguillarum*)	Kidney, Liver and spleen
Viral Nervous Necrosis (VNN) or Viral encephalopathy and retinopathy (VER)	Betanodavirus	Brain, spinal cord and retina for finding of vacuolation and intra-nuclear inclusion bodies

Aeromonas infection	Different species of *Aeromonas* (*A. hydrophila*, *A. caviae* and *A. punctata*)	Spleen and other affected areas including tail, fin, etc. for bacterial isolation
Septicaemia in pearl spot	*Klebsiella pneumoniae*, *Proteus vulgaris* and *Pseudomonas aeruginosa.*	Gill tissue, spleen and lymphoid organs for isolation of bacteria
Tilapia Lake virus	Tilapia lake virus	Liver and Brain

5. Composition of Fixatives:

Davidson's fixative:

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

10 % Neutral buffered formalin

40 % Formaldehyde	: 100 ml
Distilled water	: 900 ml
NaH_2PO_4	: 4 g
Na_2HPO_4	: 6 g

Bouin's fixative

Picric acid (Saturated aquatic solution)	: 75 ml
Formalin (40 % w/v)	: 25 ml
Glacial acetic acid	: 5 ml

6. Further readings

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Collection, Preservation and Processing of Fish and Shellfish Samples for Molecular Detection

Vidya Rajendran
ICAR- Central Institute of Brackishwater Aquaculture, Chennai, India
E-mail of corresponding author: v.rajendran@icar.gov.in

1. Introduction

The rampant disease often affects the culture irrespective of period, stage, or species. Fish and shellfishes can be infected with small prokaryotic viruses and bacteria to eukaryotic ciliates to metazoan parasites. Identification of infectious agents is based on scientific principles and methods. Accurate diagnosis always depends on the freshness of the sample, collection, and preservations. Preservation of samples varies with the type of pathogens involved. Infectious agents are detected at the molecular level using DNA or RNA extracted from the preserved samples. Viruses, bacteria, and parasites can be detected through polymerase chain reactions using specific primers designed based on the genetic material of infectious agents. The selection of genetic material for extraction depends on the nature of the infectious agent. DNA is usually used to detect viruses, bacteria, and other eukaryotic agents, including parasites. RNA is specifically used for RNA viruses. There are many viruses and bacteria affecting fish and shellfishes; Noda virus and Tilapia lake virus (Tilv) are RNA viruses affecting brackishwater and marine fishes, Infectious myonecrosis virus and Yellowhead virus are RNA viruses that affect shrimps. Grouper iridovirus is a DNA virus affecting brackishwater fishes, white spot syndrome virus (WSSV), infectious hematopoetic hepatopancreatic necrosis virus (IHHNV) are the commonly found DNA viruses in the shrimps. *Vibrio parahemolyticus* with pir gene is a bacteria responsible for Acute Hepatopancratic Necrosis Disease (Early mortality syndrome) in shrimps, and Enterocytozoon hepatopenaei, the causative agent of hepatopancreatic microsporidiosis are also detected at the DNA level.

2. Methodology

2.1. Sampling

Sampling must ensure an accurate representation of the health status of the population or individual. Sampling can be either lethal or nonlethal. Nonlethal sampling is usually carried out with broodstocks, where the tip of the pleopod or fecal threads of shrimps are used for polymerase chain reaction based molecular detections without sacrificing the animal. For

lethal sampling, entire larvae or any tissue material such as hemolymph, gill, muscle, pleopod, lymphoid organ, hepatopancreas and eyestalk collected based on the type and tissue specificity of the viral pathogen to be detected. Egg and larvae (~ 150 numbers of egg or larvae- nauplii to mysis) or ~10 PL depending on size/age) can be taken as a whole and pooled to make a representative sample. In case of late PL, it is preferred to cut the head and take abdominal portion to avoid PCR inhibitors. The diseased or moribund shrimps of three to six numbers and an equal number of normal shrimps should be collected and packed separately for diagnosis.

The selection of a particular tissue type is mandatory in the accurate diagnosis of viral infection because shrimp viral pathogen infects particular cells and tissues of a host, which support the growth of a particular virus. Some viruses have a broad tissue tropism and can infect many types of cells and tissues. Other viruses may infect primarily a single tissue. For example, the White spot syndrome virus (WSSV) infects ectodermal and mesodermal origin tissues such as the epidermis, gills, pleopod, and hemolymph. However, monodon baculovirus(MBV) infects only endodermal origin tissue hepatopancreas. The selected tissue of the organism should be relatively free of compounds potentially damaging to the nucleic acid or interfering with PCR. For example, Eyeballs are known to contain PCR inhibitors.

2.2. Preservation of tissue

Fresh material from live animals consistently provides the highest yield and quality of nucleic acid for amplification. The live or moribund animals can be frozen in dry ice and rapidly placed in the cold and away from light. The tissues should be packed in plastic cryotubes or Ziploc bags, excluding as much air as possible to avoid cross-contamination. The tissue samples can be stored and transported in 95–100% ethanol at ambient temperature. The larger size or exoskeleton of the animal does not allow the penetration of ethanol of the tissue and causes degradation of the tissues. These samples should be injected with ethanol dissected into smaller pieces to allow the ethanol to diffuse directly into the internal tissues. There should be about ten volumes of ethanol to one sample volume for the proper preservation of the sample. Ethanol should be replaced after the initial fixation and periodically at a regular interval. Long-term storage conditions should minimize variation in temperature. The animal tissues will remain indefinitely stable for extraction of nucleic acids at -70 to -80 °C. This will allow the archiving of samples for reanalysis. There are also several commercial preservatives available specifically to preserve nucleic acid in the tissue.

2.3. Collection and preservation of samples

2.3.1. Materials required

95% ethyl alcohol for DNA virus, RNA Later for RNA virus, dry ice, scissors, forceps, gloves, Aluminium foil, Anesthetizer, freshly dead or moribund fish and shrimps, Postlarvae

2.3.2. Technical procedure

Collect the moribund fish or shrimps from the affected pond or farm. Euthanize the fish using suitable methods such as an anesthetizer (>250 mg/L buffered MS-222) or sacrifice the fish or shrimps on ice under sterile conditions. Using sterile scissors and forceps, cut the opercula of fish and dissect out the gills and fix in 95 % ethyl alcohol for detecting DNA viruses. Cut open the cranium and remove the brain and fix in commercial transport medium (RNALater) or store at -80 °C for RNA extraction to detect Noda virus infection in fishes. Whole post larvae and fish larvae (N=10 to 20) can be directly fixed in fixatives or frozen at -20 °C in the tightly capped container and must be submitted to the disease diagnostic lab at the earliest. These fixed samples may be kept in refrigerator (4 °C) till the day of transportation to avoid evaporation of fixatives. Frozen shrimp packed in zip-lock bags should be shipped immediately on dry ice or ordinary crushed ice flakes with the case history of disease incidence.

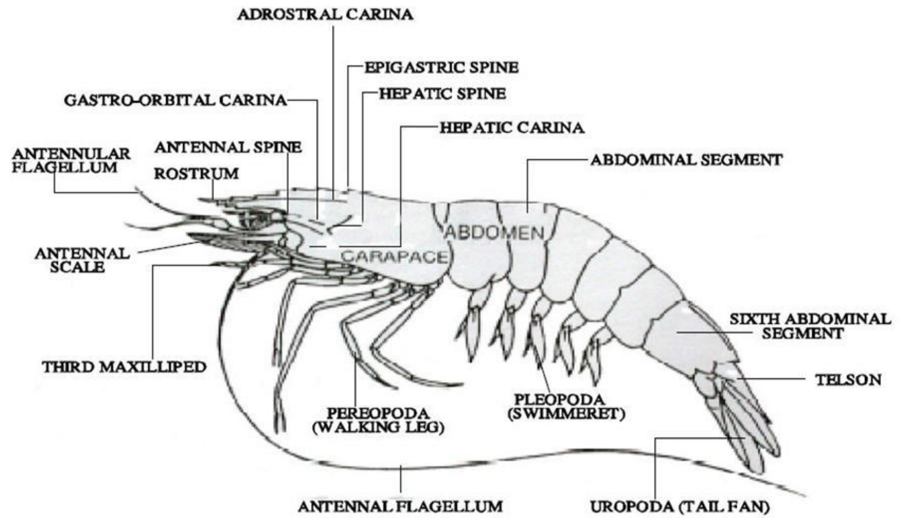
Population size	Prevalence (%)						
	0.5	1.0	2.0	3.0	4.0	5.0	10.0
50	46	46	46	37	37	29	20
100	93	93	76	61	50	43	23
250	192	156	110	75	62	49	25
500	314	223	127	88	67	54	26
1000	448	256	136	92	69	55	27
2500	512	279	142	95	71	56	27
5000	562	288	145	96	71	57	27
10000	579	292	146	96	72	57	27
100000	594	296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

The number of samples collected at different prevalence rates and stocking densities from a stocked pond or farm

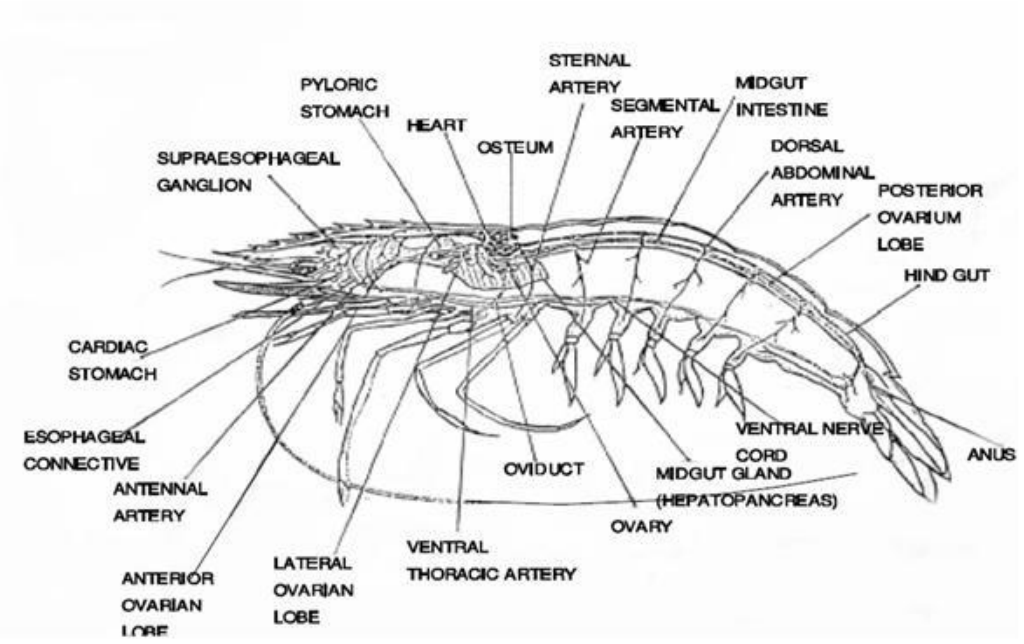
Target organs of DNA, RNA viruses, bacteria and Parasites infecting shrimp

DNA Viruses	Abbreviation	Target organs	Genome
White spot syndrome virus	WSSV	larvae, Pleopod, gill, hypodermis , hemocytes	dsDNA
Monodon baculovirus	MBV	larvae, hepatopancreas	dsDNA
Baculoviral midgut gland necrosis virus	BMNV	larvae, hepatopancreas	dsDNA
Baculovirus penaei	BP	larvae, hepatopancreas, anterior midgut	dsDNA
Spawner isolated mortality virus	SMV	larvae, hepatopancreas,midgut	ssDNA
Hepatopancreatic parvovirus	HPV	larvae, hepatopancreas	ssDNA
Infectious hypodermal haeamtopoietic necrosis virus	IHHNV	larvae,Pleopod, gill, hypodermis, haematopoietic tissues, lymphoid organ	ssDNA
RNA Viruses	**Abbreviation**	**Target organs**	**Genome**
Yellow head virus	YHV	larvae, gill, gut, gonads, pleopod,hemocytes, lympoid organ	(+)ssRNA
Taura syndrome virus	TSV	larvae, gill, gut, striated muscle, pleopod, hypodermis, lympoid organ	(+)ssRNA
Infectious myonecrosis virus	IMNV	larvae, Skletal muscles, lympoid organ, hemocytes	(+)ssRNA
Macrobrachium rosenbergii nodavirus	MrNV	larvae, gill,pleopod, muscle, ovary	(+)ssRNA
Mouriliyan virus	MoV	larvae, gill, lympoid organ, cuticular epithelium	(-) ssRNA
Gill associated virus	GAV	larvae, gill, gut, gonads, pleopod,hemocytes, lympoid organ	(+)ssRNA
Lymphoid organ vacuolization virus	LOVV	larvae, lympoid organ,	(+)ssRNA
Laem singh virus	LSNV	larvae, gills, lympoid organ, nervous tissues	dsRNA
PARASITE	**Abbreviation**	**Target organs**	**Genome**
Enterocytozoon hepatopenaei	EHP	larvae, Hepatopancreas	ds DNA
Bacteria	**Abbreviation**	**Target organs**	**Genome**
Necrotizing hepatopancreatitis	NHP	larvae, Hepatopancreas	ds DNA
Acute hepatopancreatic necrosis disease	AHPND	larvae, Hepatopancreas, bacterial culture from gut tissue of live shrimp	ds DNA

List of RNA and DNA viruses and their target organ in shrimps



Morphology of shrimps



Anatomy of shrimps

Lethal Sampling

Non-lethal Sampling

MG HP ST

Gills

Telson

Anterior artery

Lymphoid organ

Heart

Hematopoietic tissues

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

Organs of adult shrimps for disease investigation

Egg or larvae ~ 150 nos

Dissected fish displaying internal organs

2.4. Processing of samples

Samples received in good condition will be processed for molecular detection of infectious agents. The processing of samples varies with the nature of the virus. DNA viruses were detected by extracting the DNA from samples based on the target organ of infection. As DNA viruses are more stable than RNA viruses, preserving them in 95% alcohol or in ice is easy, whereas samples from the RNA virus should always be preserved in RNA later solution or must be kept at -80 °C. The processing of virus samples should be accurate enough to reduce the cross-contamination as the processing contamination will produce false-positive reactions in polymerase chain reactions.

2.4.1. Instruments required

Refrigerated centrifuge, Deep freezer, Ice Flaker, Shaking water bath, Spectrophotometer, Vortex mixer, Autoclaved Milli Q water, Adjustable micropipettes, Micro-centrifuge tubes, Homogenizer, Disposable gloves.

Sample material: Specific tissue from shrimp, fish, or bacterial colonies for DNA isolation.

Method I: DNA extraction using the lysis buffer-phenol-chloroform method

2.4.2. Materials required

10mM Tris HCl pH 8.0

25mM EDTA

0.5% SDS

100mM NaCl

Makeup to 100ml with dd water.

Add 0.5 µl proteinase K (20mg/ml) to the lysis buffer just before use to make the working concentration of proteinase K @10 µg/ml.

2.4.3. Technical procedure

- Take 20 to 50 mg tissue (PL, pleopod, gill, or 50 ul hemolymph samples) in a 1.5ml microcentrifuge tube. The frozen tissue should be thawed directly in lysis buffer, and ethanol preserved tissue should be blotted dry with fresh tissue and add tissue to a 1.5 ml microcentrifuge tube containing 150 µl lysis buffer and homogenate gently and entirely with the disposable pestle and then add 350 µl lysis buffer.
- Incubate the tissue homogenate at 95°C for 10 min with intermittent mixing. Allow the homogenate to cool down to room temperature.
- Centrifuge at 12000g for 10 minutes at 4° C and transfer 200 µl of the transparent upper solution to a fresh 1.5ml tube with 400 µl ethanol.
- Centrifuge again at 12000g for 5 minutes at 4° C and carefully discard the supernatant and air dry the pellet.
- Dissolve the pellet with sterile Milli-Q water or TE buffer.

2.5. Method II: DNA extraction by CTAB method

2.5.1. Reagents

- Extraction (CTAB) Buffer: 4 M Sodium chloride, 100 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0),-2% E-Mercaptoethanol,2% CTAB
- Isopropanol
- Saturated phenol pH 8.0 4.
- Chloroform : isoamylalcohol (24:1) mixture
- Tris:EDTA (10mM:1mM) pH 8.0
- RNase A (10mg / ml): Dissolve RNase A in 10mM Tris-Cl, pH 7.5, 15 mM NaCl. Heat at 100°C for 15 min. Cool to room temperature and store as aliquots at -20° C.
- 70% ethanol

2.5.2. Procedure

- Take 25 to 100mg tissue (PL, pleopod, gill, or 50 ul hemolymph sample) in a 1.5ml microcentrifuge tube. The frozen tissue should be thawed directly in CTAB buffer, and ethanol preserved tissue should be blotted dry with fresh tissue and add tissue to a 1.5 ml microcentrifuge tube containing 150 µl CTAB buffer and homogenate gently and entirely with the disposable pestle and then add 800 µl buffer.

- Incubate the tissue homogenate at 65°C for about one hour with intermittent mixing. Allow the homogenate to cool down to room temperature.
- Vortex briefly the homogenate and then add 0.7 ml of chloroform, vortex for another 20 seconds, and then centrifuge at 12000 g for 5 minutes.
- Transfer 700 µl upper aqueous phase to a new 1.5 microcentrifuge tube.
- Add 630 µl (0/9 volume) isopropanol and mix gently and allow the DNA to precipitate for 30 min by keeping it in a – 20° C deep freezer.
- Centrifuge at 12000g for 5 minutes, then wash the pellet with 600 ul of 70% ethanol.
- Centrifuge again at 12000g for 5 minutes at 4° C and carefully discard the supernatant and air dry the pellet.
- Re-suspend in TE buffer.
- Add RNAse A and incubate at 37° C for one hour.
- Add an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), mix appropriately for at least 5 min, and centrifuge at 12000 g for 10 minutes. Extract twice with chloroform: isoamyl alcohol.
- Precipitate the DNA by adding 1/10 volume of 3M sodium acetate and 2 volume of ice-cold ethanol. Mix gently and spool out the DNA or precipitated by centrifugation at 12,000 g for 10 minutes.
- Remove extra salts by washing the pellet with 70% ethanol. Air-dry the pellet and resuspend the pellet with TE buffer and store at – 20° C.

2.6. Isolation of RNA using TRIZOL reagent:

2.6.1. 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.

2.6.2. Technical procedure

- **Tissue Homogenization**: Homogenize the tissue samples with 1 ml of TRIZOL reagent per 50 to 100 mg of tissue using a disposable sterile Teflon homogenizer. The sample volume should not exceed 10% of the volume of TRIZOL Reagent used for the homogenization. Incubate the homogenized sample for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes and centrifuge to remove cell debris. Transfer the supernatant to a new tube.
- **Phase separation**: Add 0.2 ml of chloroform per 1 ml of TRIZOL Reagent. Cap sample tubes securely. Vortex samples vigorously for 15 seconds and incubates at room temperature for 2 to 3 minutes. Centrifuge the samples at no more than 12,000 x g for 15 minutes at 2 to 4°C. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. Transfer the upper aqueous phase carefully without disturbing the interphase into a fresh tube. Measure the volume of the aqueous phase (The volume of the aqueous phase is about 60% of the volume of TRIZOL Reagent used for homogenization).
- **RNA precipitation**: Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization. Incubate samples at 15 to 30°Cfor 10 minutes and centrifuge at not more than 12,000 x g for 10 minutes at 2 to 4°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube. G: RNA WASH: Discard the supernatant completely. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization. Mix the samples by vortexing and centrifuge at no more than 7,500 x g for 5 minutes at 2 to 8 oC. Repeat the above washing procedure once. Remove all leftover ethanol.
- **Redissolving RNA**: Air-dry or vacuum dry RNA pellet for 5-10 minutes. Do not dry the RNA pellet by centrifuge under vacuum. It is important not to let the RNA pellet dry completely as this will significantly decrease its solubility. Partially dissolved RNA samples have an A260/A280 ratio < 1.6. Dissolve RNA in DEPCtreated water bypassing solution a few times through a pipette tip.

2.7. Fractionation of nucleic acid by agarose gel electrophoresis

2.7.1. Agarose Electrophoresis

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

2.7.1.1. Agarose gel preparation

- To prepare a gel, required quantities of agarose are weighed and added into the wide mouth glass conical flask with 1x TAE buffer and melt the mixture in the microwave oven until it becomes clear without any gel particle.
- Cool down the clear molten agarose gel at room temperature and add 1 µl ethidium bromide (10mg/ml) and slowly pour the gel into the gel mold without air bubble. The volume of the gel varies from the size of the gel mold. The height of agarose gel only has to go above the bottom of the gel comb for about 0.3~0.5 cm, and thickness is suggested to be no less than 0.8 cm.
- When agarose gel is completely solidified. Carefully remove blockers at both sides of the gel mold and place them in the gel tank containing 1x TAE buffer. After few minutes, the comb will loosen up in the gel and can be carefully removed without damaging the wells. This agarose gel is ready for electrophoresis.

2.7.1.2. Electrophoresis

- Add 1X TAE buffer over the gel box until the buffer level submerges the gel.
- Load 5 µl each of the "PCR product-loading dye mixture" into each well. The mixture will sink to the bottom of the wells because its density is higher than the buffer. This step should be carefully handled in order to avoid cross-contamination between the adjacent wells.

- DNA marker is loaded at the extreme end of the gel. The DNA molecular weight marker is served as a reference to predict the size of the PCR product.
- After loading all the samples, the gel is electrophoresed at a constant voltage between 70V~100V.
- The loading dye Bromphenol Blue gives deep blue color; Xylene Cyanol gives light blue color. When the dark blue dye approaches 1/2 to 2/3 of the gel, stop the electrophoresis. Then, remove the gel from the gel mold to observe under UV light.

2.7.1.3. Staining and visualization

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

3. Further Readings

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Detection of Shrimp Pathogen by PCR and Real time PCR

T.Sathish kumar, M.Poornima, Subhendu Kumar Otta and J. Joseph Sahaya Rajan

* *ICAR- Central Institute of Brackishwater Aquaculture, Chennai, India*

E-mail of corresponding author: sathishkumar.t@icar.gov.in

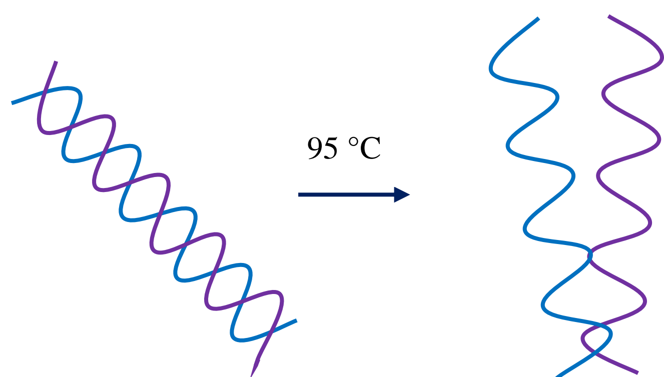
1. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is most commonly used in the detection of viral, bacterial, fungal and parasitic infection in shrimp. It was discovered in 1985 by Kerry Mullis, and become both essential and routine tool in most biological laboratories. In PCR, undetectable quantity of DNA from the sample can be amplified to produce detectable quantities of the target DNA. The PCR involves DNA polymerase mediated enzymatic amplification of DNA complementary to the offered template strand using specific oligonucleotide primers designed against target DNA sequence, and d**eoxynucleotide triphosphates** in a buffer system. The resultant PCR product may then be compared to a known standard using gel electrophoresis.

1.1. Thermal cycling reactions

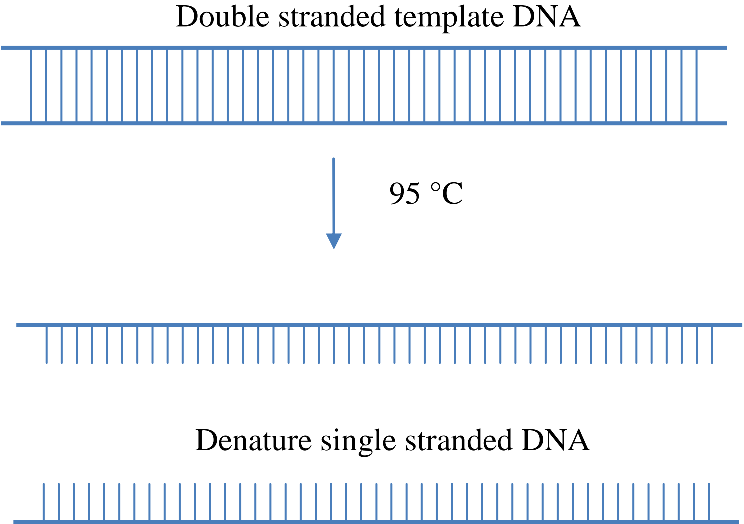
1.1.1. Initial denaturation

The reaction mixture is incubated at 95 °C for 2–5 min. This is to ensure that all complex, double-stranded DNA (dsDNA) molecules are separated into single strands for amplification. The duration of this step depends on enzyme characteristics and template complexity.



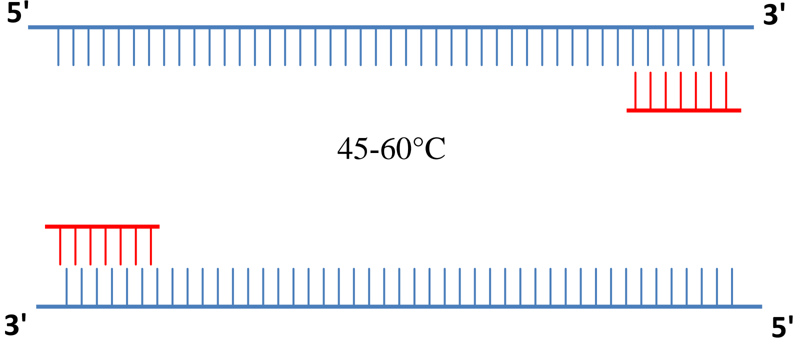
1.1.2. Denaturation

This step involves incubation of reaction mixture at 94°C for 15-30 seconds. During this step, the double stranded DNA is denatured in to single strands due to breakage of weak hydrogen bonds.



1.1.3. Annealing

The reaction temperature is rapidly lowered to approximately 5°C below the melting temperature (T_m) of the primers and often 45-60°C for 20-40 seconds. This promotes the binding of primers to their complementary sequence in the template DNA.

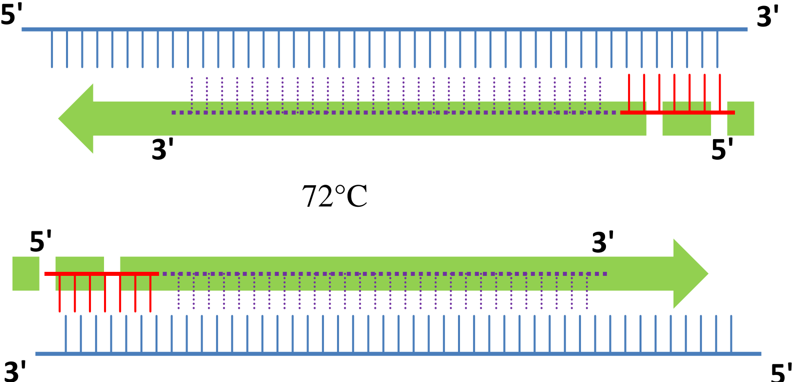


1.1.4. Elongation

The temperature is increased to 72 °C, which is optimum for DNA polymerase activity. In this step, the polymerase enzyme sequentially adds bases to the 3′ each primer, extending the DNA sequence in the 5′ to 3′ direction at the rate of 1,000 base pair per minute. 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',

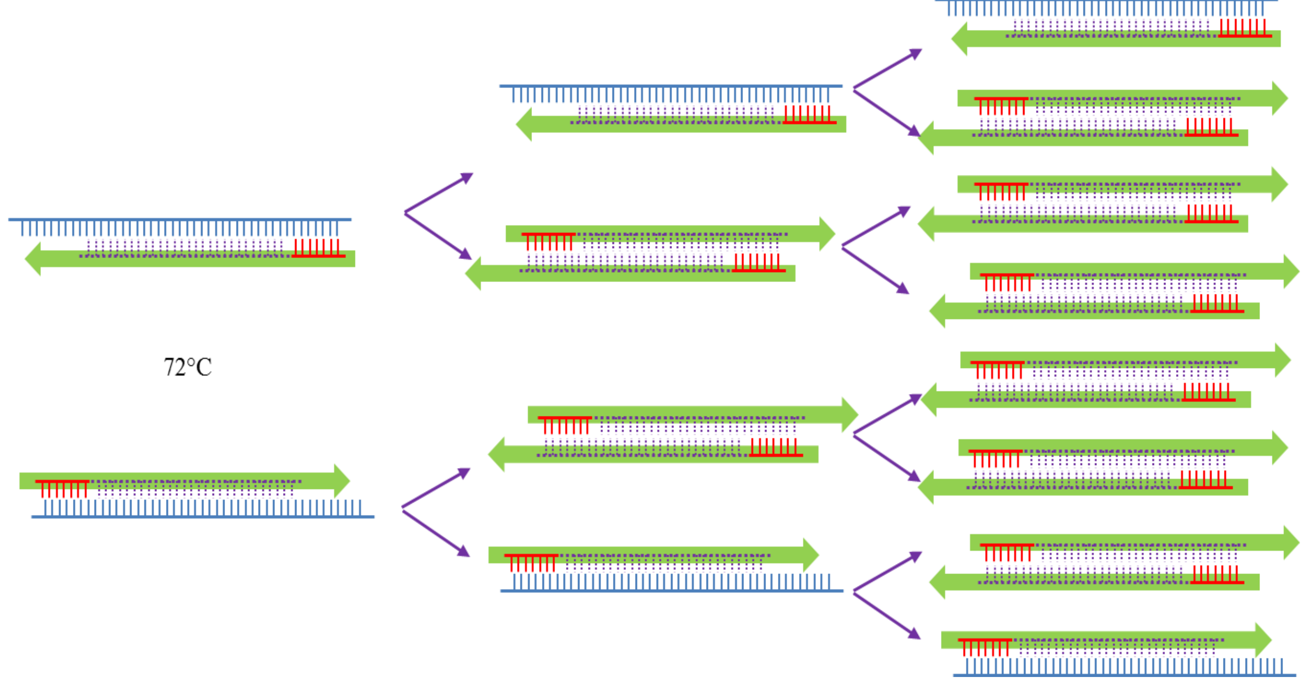
reading the template from 3' to 5'. Because both strands are copied during PCR, there is an exponential increase of the number of copies of the gene. But, polymerization is not strictly doubling the DNA at each cycle in the early phase. The number of PCR cycles depends on the amount of template DNA in the reaction mix and on the expected yield of the PCR product. Usually 25–35 cycles are sufficient.

Steps 1–3 are performed in a cyclic manner, resulting in exponential amplification of the target region.



1.1.5. Final elongation

This single step is optional, but is performed at a temperature of 72°C for 5–15 minutes after the last PCR cycle to ensure the elongation of remaining single-stranded DNA.



1.2. PCR Protocol

1.2.1. Sample collection

Samples such as fresh or ethanol-preserved samples of shrimp tissue or post larvae (PL) may be used. In certain cases (ie) for detecting EHP, fresh fecal sample may also be used as non-lethal sample for analysis.

1.2.2. DNA Extraction

- Take 20-30 mg of sample in 150 µl lysis buffer (50 mMTris pH 8.0- 606 mg, 1mM EDTA- 37.2 mg, 500 mMNaCl- 2.922 gm, 1% SDS – 1 gm make up to 100 ml with Proteinase K @ 10 µg/ml) and homogenize the sample with the micropestle.
- Add 350 µl of lysis buffer to the homogenate and mix well by vortexing.
- Incubate the sample at 95°C in dry bath for 1 min.
- Cool the lysate to room temperature and centrifuge the sample at 12,000 rpm for 10 min.
- Collect 200 µl of supernatant into fresh tube with a micropipette and 500 µl of 70% ethanol and mix by invert mixing.
- Centrifuge the tube at 12,000 rpm for 10 min and decant the supernatant without disturbing the DNA pellet.
- Invert the tube on a clean dry tissue wipe and air dry the pellet.
- Reconstitute the DNA pellet in 200 µl of water or TE buffer (1 ml 1M Tris (pH 8.3) and 200 µl 0.5 M EDTA (pH 8.0) and adjust the volume to 100 ml of de-ionized water).
- Use 1 µl of this sample as DNA template for the PCR.
- Quantification of DNA
- (Purity 1.8-2.0 (260/280), concentration may be adjusted to 50 ng/µl)
- The extracted DNA can be kept for long period at 20°C until further use

1.2.3. Setting up the PCR Reaction

Choose the appropriate substrate DNA and PCR primer sequences as mentioned below:

Reagents	**Vol (µl) per reaction**	**Vol (µl) (X) no. of reactions**
Water	9.5	
2x Master Mix (contains Buffer with $MgCl_2$, dNTPs, Taq DNA polymerase)	12.5	
Forward primer (10 µM)	1	
Reverse primer (10 µM)	1	
DNA template	1	
* Original Rxn. Vol. 25 µl	25 µl	

The reaction mixture can be kept as specified reaction conditions (Initial denaturation, Denaturation, annealing, Extension) in PCR machine/Thermocylcer.

Note:

- Take care not to cross-contaminate the reagents, especially the templates and primers
- Pipetting order in general; we may add water first and the enzyme last.
- For a large number of reactions, it is good practice to first set-up a master mix of the common reagents and then aliquot them, rather than to pipette the reagents separately for each individual tube.

1.2.4. Observations and Documentation

- After completion of the PCR, 1.6% agarose gel 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).
- Ethidium bromide is added to the molten agarose (0.5 µg/ml final concentration)
- Then hot agarose gel cooled to 45-50°C and then poured into the casting tray.
- Once the gels are solidified, it is submerged in the tank with the same buffer.
- A total volume of 5-10 µl amplified product is directly loaded in sample wells and 4 µl of the 100 bp DNA ladder loaded to the marker well in 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 and visualise under UV transillumінator.

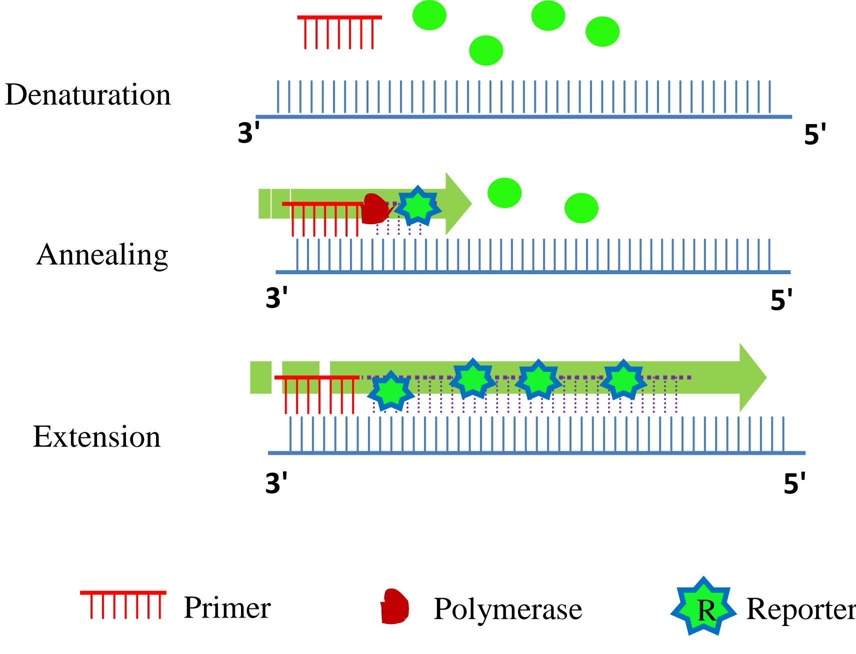
2. Real-time PCR

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. A real-time PCR or quantitative PCR monitors the amplification of a targeted DNA molecule during the PCR in real time. It can be used to quantify the target sequences in the sample. 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.

2.1. SYBR Green I Assay

2.1.1. Principle

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 the double stranded DNA.



SYBR Green I Assay

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 or Ct values at different DNA concentrations, quantitation of target DNA in any sample can be made.

2.1.2. Protocol

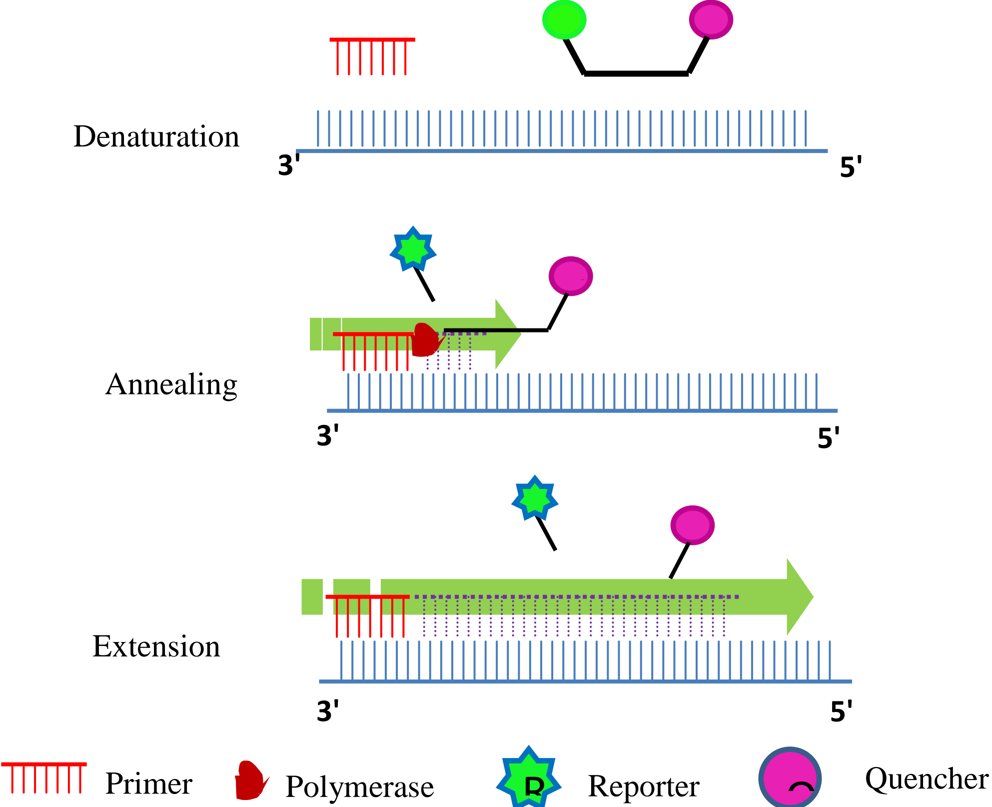
- Real-time PCR amplifications can be carried out in a total volume of 25µl containing 12.5 µl of MaximaTM SYBR Green qPCR Master Mix (Fermentas, India), 0.5µl of (18 pm) each primer (forward and reverse), and 1 µl (equivalent to 50 ng) of DNA.
- Amplification can be carried out in an specified conditions in an Real time PCR system
- Melting curve analysis of amplification products can be performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected.
- The target amplicon cloned in plasmid was diluted from 10 to 10^6 used as a standard in the real time PCR
- For each template, three well replicates should be used.
- Ct values determined can be used to confirm the quantitative estimate of the copy number of pathogen

2.2. TaqMan assay

2.2.1. Principle

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 amplification 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.



TaqMan assay

2.2.2. Protocol

- Real-time PCR can be performed in 25 µL PCR mixture
- Reaction mixture includes 2x master mix containing a high-performance Taq antibody, hot start real-time PCR enzymes and a buffer with 0.25 µM of forward primer, 0.25 µM of reverse primer and 0.125 µM TaqMan probe and 50 ng DNA as template.
- Amplification can be carried out in specified conditions in an Real time PCR system
- The target amplicon cloned in plasmid was diluted from 10 to 10^6 and used as a standard in the real time PCR.
- The threshold was automatically detected and the resulting in a fractional cycle number (Ct value) assigned to each individual sample.
- Regression of the log of viral copy number and the Ct value was used as a standard curve for determining viral load.

Table 1. Primer sequence for OIE listed aquatic pathogens of shrimp

Sl. No.	Pathogen	Primer Sequence 5' to 3'	Amplicon size (bp)	
			First step	Nested
1	WSSV	F: ATCATGGCTGCTTCACAGAC R: GGCTGGAGAGGACAAGACAT	982	570
		Fn: TCTTCATCAGATGCTACTGC Rn: TAACGCTATCCAGTATCACG		
2	IHHNV	F:TCCAACACTTAGTCAAAACCAA R: TGTCTGCTACGATGATTATCCA	309	-
3	MBV	F:CGATTCCATATCGGCCGAATA R:TTGGCATGCACTCCCTGAGAT	533	361
		Fn:TCCAATCGCGTCTGCGATACT Rn:CGCTAATGGGGCACAAGTCTC		
4	HPV	F:GCATTACAAGAGCCAAGCAG R:ACACTCAGCCTCTACCTTGT	441	-
5	IMNV	Fn:CGACGCTGCTAACCATACAA Rn:ACTCGGCTGTTCGATCAAGT	328	139
		Fn:GGCACATGCTCAGAGACA Rn:AGCGCTGAGTCCAGTCTTG		
6	YHV	F:CCGCTAATTTCAAAAACTACG R:AAGGTGTTATGTCGAGGAAGT	135	-
7	TSV	F:AAGTAGACAGCCGCGCTT R:TCAATGAGAGCTTGGTCC	231	-
8	NHPB	F:CGTTGGAGGTTCGTCCTTCAGT R:GCCATGAGGACCTGACATCATC	379	-
9	AHPND	F: ATGAGTAACAATATAAAACATGAAAC R: ACGATTTCGACGTTCCCCAA	1269	230
		Fn: TTGAGAATACGGGACGTGGG Rn: GTTAGTCATGTGAGCACCTTC		
10	EHP	F:CAGCAGGCGCGAAAATTGTCCA R:AAGAGATATTGTATTGCGCTTGCTG	779	176
		Fn:CAACGCGGGAAAACTTACCA Rn:ACCTGTTATTGCCTTCTCCCTCC		

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

Table 2: Trouble shootings in PCR

Observation	**Possible Cause**	**Possible solution**
No PCR product	Insufficient number of PCR cycles	Replace the PCR vials and run an extra 5 cycles
	DNA-template degenerated	Check DNA quality by electrophoresis

	Thermocycler programme is not correct	Check temperatures and cycle time
	Inhibitors present, which slow down the PCR	Reduce the volume of sample in the reaction mix; carry out another ethanol precipitation with the samples
	Missing reaction component	Check the reaction components and carry out a new PCR
	Suboptimal reaction conditions	Optimize Mg2+ concentration, annealing temperature and elongation time. Always vortex PCR buffer. Primers should be present in equal concentrations
	PCR vials not autoclaved	Autoclaving PCR vials prevents contamination, while inhibitors can not interfere with the reaction
	Nucleotides degenerated	Store nucleotides in frozen batches, thaw quickly and keep on ice. Prevent frequent freeze/thaw cycles
	Error in gel analysis.	Check that the gel was loaded correctly and stained properly
Amplification with low yield.	Enzyme activation not long enough (or too long) or the temperature not high enough	Check manufacturer's recommendations
	Annealing temperature too high or too low.	Optimise the annealing temperature using a gradient if possible.
	Annealing or extension time too short.	Increase the hold times. For long products (>2kb) use incremented hold times on the extension step.
	Not enough cycles	Increase the number of cycles
	Not enough	Increase the amount of

	template.	template
No template control (NTC) shows amplification.	Amplicon or template contamination of one of the reagents.	Repeat the assay with fresh reagents. Separate PCR set up from analysis. Use filter tips
Non-specific products: primer dimers	Short non-specific products amplified in preference to the target.	Reduce primer concentration; reduce $MgCl_2$ concentration; use a hot start enzyme; use a touchdown PCR protocol; re-design primers
Non-specific products: smeared bands on gel	Degraded template and/or reactions conditions too permissive.	Check template integrity. Increase stringency of reaction – use a touchdown PCR method
	Long products not amplified completely.	Increase extension time – use an incremented hold time on the step. Ensure the mix contains enough reagents such as dNTPs.
	Primers not specific	Check primer specificity
Non-specific products : Multiple bands	Premature replication	Use a hot start polymerase, such as OneTaq Hot Start DNA 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
	Incorrect Mg++ concentration	Adjust Mg++ in 0.2–1 mM increments
	Contamination with exogenous DNA	Use positive displacement pipettes or non-aerosol tips Set-up dedicated work area and pipette 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
	Primer concentration high	Check primer concentration and decrease concentration, if necessary

3. References

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Assessment of Soil and Water Quality Parameters of Shrimp Culture Ponds

Leesa Priyadarsani, Sanjoy Das, Prem Kumar, Babita Mandal

Kakdwip Research Centre of ICAR-CIBA, Kakdwip, West Bengal, India

E-mail of corresponding author: leesa.priya@gmail.com

Shrimp farming is one of the profitable business, at the same time, this industry is facing several problems like poor water quality and feeding management. Among these, water quality management is a crucial part of shrimp farming. Hence, water quality parameters such as pH, dissolved oxygen, ammonia, nitrite, hardness and alkalinity should be maintained at optimum level throughout the culture period for successful shrimp farming.

1. Water quality parameters

1.1. Temperature

The surface water temperature can be measured by using a mercury centigrade thermometer (0 to 50^{0}C) with 0.10^{0}C graduation at the station itself. Water temperature greatly affects the condition of shrimp, especially their appetite. The higher the water temperature will be higher the metabolic processes in the body of the shrimp. Conversely, if the water temperature is very low metabolic processes in the body of the shrimp are inhibited so the shrimp do not want to eat. The optimal temperature for the growth of shrimp is 28-30 °C.

1.2. pH

The pH of water samples can be measured by a digital pH meter following the electrometric method or by a pH paper. The optimal pH value for shrimp culture is 7.5 to 8.5. Lower pH can be treated with lime application whereas higher pH can be treated by the application of alum or gypsum (if high alkalinity and low calcium hardness).

1.3. Dissolved Oxygen (DO)

Dissolved oxygen of water samples will be estimated following the azide modification method (APHA, 1998). Collect water sample in a 300 ml sampling bottle avoiding any air bubbles, then add 1 ml of Winkler's A (Manganous sulphate solution) followed by 1 ml of Winkler's B (Pottasium iodinate). When precipitate has settled sufficiently to a level clear supernate above the manganese hydroxide floc, add 1 ml of concentrated H_2SO_4 till the colour turned to golden. Then take 200 ml of sample in a conical flask and add a few drops of the starch indicator to it. Then titrate the sample against

N/40 Na2S2O3 (Sodium thiosulphate) till the sample became colourless. Then record the volume of the titrant and the result is expressed as mg/l.

Calculation of DO as follows

For titration of 200 ml sample, 1 ml 0.025 Na2S2O3= 1 mg DO/l.

If the DO level is too low, the growth of the shrimp will be slow. A minimum level of dissolved oxygen of 4 to 5 ppm is recommended.

1.4. Total Alkalinity

Total Alkalinity calculation will be done through the titration method (APHA, 1998). Take 50 ml of water sample in a conical flask. Then add 2-3 drops of phenolphthalein indicator to it. If pink colour developed, titrate the sample against 0.02 N H2SO4 till the colour disappears. Note down the burette reading down and to that sample, add 2 drops of methyl orange indicator and the solution will turn into orange colour. Continue the titration with 0.02 N H_2SO_4 until the orange colour turns pink. If the pink colour did not appear with the phenolphthalein indicator, titrate the sample against 0.02N H_2SO_4 after adding methyl orange as an indicator. Note down the total burette reading and the result expressed as mg/l.

Calculation of Alkalinity as follows,

Phenolphthalein alkalinity = A × 1000 × t/ C

Where, A= ml of H_2SO_4 used

C= ml of sample

t= titer of stander acid, mg $CaCO_3$/ ml

Total alkalinity = D × 1000 × t/ C

Where, D= ml of total H_2SO_4 used

C= ml of sample

t= titer of stander acid, mg $CaCO_3$/ ml

1.5. Total Hardness

The total hardness of water was estimated through the complex metric method (APHA, 1998). Take 50 ml of water sample in a conical flask. Add 2 ml of ammonium buffer solution and a few drops of Erichrome Black-T indicator to the sample which turn it to a wine red colour. Then titrate the sample against the 0.01N EDTA (Ethylene diamine tetraacetic acid) until the initial wine red colour of the sample changed to ink blue as the endpoint. Record the volume of the titrant consumed and the result is expressed as mg/l.

Calculation of Hardness as follows,

Total Hardness = A × B × 1000/ C

Where, A= ml titration for sample

B= mg $CaCO_3$ equivalent to 1 ml EDTA titrant

1.6. Phosphate-Phosphorous (PO4-3)

Estimation of phosphate-phosphorous will be done spectrophotometrically following the stannous chloride method (APHA, 1998). Take 10 ml of filtered water sample in a 25 ml test tube and add 2 drops of phenolphthalein indicator. If the solution turned pink, add strong acid solution (H_2SO_4 + HNO_3) dropwise until the pink colour disappears. Then add 0.4 ml of ammonium molybdate [(NH_4) MO_7O_{27}] reagent and 0.05 ml of stannous chloride ($SnCl_2$) indicator subsequently and mix thoroughly till blue colour develops. Take the absorbance of the solution after 10 minutes and before 12 minutes of addition of stannous chloride through spectrophotometer at 690 nm wavelength. The Phosphate-phosphorous concentration of the water sample was computed from the standard curve and the result is expressed as mg/l.

1.7. Ammonium-nitrogen (NH4-N)

Ammonium-nitrogen of water sample will be analyzed by phenate method (APHA, 1998). Take 25 ml of sample in 50 ml conical flask. Then add 1 ml phenol solution followed by 1 ml sodium nitroprusside solution and 2.5 ml oxidizing solution and mix thoroughly. Cover the sample with plastic wrap or paraffin wrapper film and keep in a dark room at 22 to 27^{0}C temperature for at least 1 hour for colour development. The colour remains stable for 24 hours. Simultaneously prepare a blank and standards. Measure the absorbance of the samples with a spectrophotometer at 640 nm wavelength and compare with the standard curve. The concentration is expressed as mg/l.

1.8. Nitrite-Nitrogen (NO_2-N)

To an aliquot of sample solution containing 0.50-20 μg of nitrite (0.02-0.80 ppm when diluted to 25 ml) in a 25 ml calibrated flask, add 1 ml of 0.1% p-aminophenylmercapto-aceticacid solution and 2 ml of the 1% N-(1 naphthyl) ethylenediamine dihydrochloride solution. Set aside for a minimum of 15 min for full colour development and dilute to the mark with distilled water, a bluish violet colour develops and remains stable for 48h. Measure the absorbance at 565 nm in 1 cm cuvettes against a reagent blank prepared in the same manner but containing deionized water Prepare a calibration graph for nitrite in this manner. The concentration is expressed as mg/l.

1.9. Nitrate-Nitrogen (NO3-N)

Nitrate concentration is estimated by the EPA approved Brucine Method (Bain, 2009). Take 5 ml of sample in a 50 ml conical flask and dilute with deionized water to make the total volume 10 ml then add 10 ml sulfuric acid solution and mix thoroughly. Cooldown the conical flask in flowing water and then heat at 100^0C in a water bath for 25 minutes. After that, cool down the sample with flowing water and dilute up to 25 ml with deionized water. Simultaneously prepare a blank and standards. Measure the absorbance of all samples with the spectrophotometer at 410 nm wavelength and compare with a standard curve. The concentration is expressed as mg/l.

2. Soil quality parameters

The soil samples were collected from the four corners and the centre of the pond. All these sediment samples were mixed to form the composite sample of the respective ponds and packed in zip covers. Similarly sediment samples were collected from 10 ponds and brought to the laboratory. The samples were dried in the shade and ground to powder. The powdered soil sample is sieved through a 2mm sieve and finally through 80µ mesh sieve and packed in cloth bags for subsequent analysis

2.1. Soil Organic Carbon (Walkley and Black, 1934)

Weigh 1 g of 0.2 mm soil sample into 500 mL dry (corning/borosil) conical flask. Add 10 mL of 1*N* $K_2Cr_2O_7$ and 20 mL of concentrated H_2SO_4.For blank, pour 10 mL of 1 *N* $K_2Cr_2O_7$ in dry empty 500 mL conical flask and add 20 mL concentrated H_2SO_4 to it slowly. Leave the blank and sample solutions undisturbed for 30 minutes in dark. Add slowly 200 mL of distilled water and 10 mL of orthophosphoric acid. Add 1 mL of diphenylamine indicator. Take 0.5*N* ferrous ammonium sulphate solution in 50 mL burette. Titrate the contents until the colour changes from bluish to greenish. If the titre value is less than 6, repeat the procedure taking 0.5 g soil sample.

$$\text{Organic carbon (\%) in soil} = \frac{10 \times (B - S) \times 0.003 \times 100}{B \times Wt._{soil}}$$

Where B and S stand for the titre values (mL) of blank and sample, respectively.

The quantity of soil organic matter (SOM) is estimated through the determination of soil organic carbon which assumes that 58% of the SOM was formed by carbon. So SOM content in soil is calculated as

SOM (%) = 1.724 x Organic carbon (%) in soil

1.724 is called as Van Bemmelen factor

2.2. Total Nitrogen Content of Soil

Weight 5 g of processed soil sample (1 g of muck or peat or 20 g of sandy soil) a 500 mL Kjeldahl flask. Add 30 mL of conc. H_2SO_4 and 2 g of salicylic acid. Shake the content of the flask until thoroughly mixed, and allow to stand for, at least, 30 minutes with frequent shaking. Add 5 g of sodium thiosulphate and 1 g of the digestion accelerator, and digest the content on a digestion block by setting the temperature at about 370-410 °C till light green colour solution is obtained. The reagents, viz., H_2SO_4, $CuSO_4$, and K_2SO_4, sometimes, contain impurities. So run a blank with the same quantities of reagents, and subtract the blank value from the value of the soil digest. Cool the content, and dilute to about 100 mL with distilled water. Add a few glass beads to prevent bumping. Fit the distillation tube to the nitrogen distillation unit. Pipette out 20-25 mL 4% boric acid containing mixed indicator or 0.1N H_2SO_4in a 250 mL conical flask and place it in distillation unit in such a way that the outlet of the condenser is dipped inside the acid. Add about 50-60 mL of 40% NaOH solution which can be done by setting the distillation unit manually. Adjust the distillation time for 5 minutes. Run the instrument and allow the NH_3 liberated to be absorbed, in the boric acid or standard H_2SO_4. The NH_3 absorbed in boric acid can be determined by titrating with standard 0.1 N H_2SO_4 solution till green colour change to pink or by back-titrating the excess of the 0.1 H_2SO_4 acid with 0.1 N NaOH solution, till the pink colour change to yellow.

Observations

Weight of the soil or plant sample = W gm

Normality of H_2SO_4 used = N/10

Volume of acid taken = V mL

Normality of NaOH used = N/ 10

Volume of NaOH used = V_1 mL

Calculations

Milliequivalents (Meq.) of H_2SO_4 taken = 0.1 X V

Meq. of NaOH used in the titration = 0.1 X V_1

Meq. of H_2SO_4 used in sample titration = (0.1 X V) - (0.1 X V_1)= 0.1 (V- V_1) = S

Meq. of H_2SO_4 used in blank titration = B

Thus, corrected meq. of H_2SO_4 used in absorption = (S-B)

Now, 1 mL of 0.1 N H_2SO_4 (= 0.1 meq. H_2SO_4) = 0.0014 g of N

(Since, 1 meq. of H_2SO_4 = 14 mg of N)

So, (S-B) meq. of 0.1 N H_2SO_4 = (S-B) X 0.0014 g of N

Thus, the W gm sample contains = (S-B) X 0.0014 g of N

Hence, the 100 gm sample contains = (S-B) X 0.0014 X (100/ W)

Therefore, total % N in soil or plant = (S-B) X 0.0014 X (100/ W)

2.3. Soil pH

Place soil sample about three fourth full in sample jar and add distilled water to cover soil. Cap the jar and shake the soil vigorously a few times. Let mixture stand 10 minutes to dissolve the salts in the soil. Calibrate the pH tester with a pH 7 and a pH 10 buffer solution. Remove the cap and place the pH tester into the wet soil slurry. Measure pH and record the measurement. pH directly affects the availability of essential nutrients.

3. Further readings

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ICAR - CENTRAL INSTITUTE OF BRACKISHWATER AQUACULTURE

KAKDWIP RESEARCH CENTRE

Kakdwip, South 24 Parganas, West Bengal - 743347

Phone : +91 3210 255076, Fax : +91 3210 257030

E- mail : krckakdwip@gail.com / krckakdwip@yahoo.co.in

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