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Training Manual on

Histopathological Techniques for Brackishwater Aquatic Animal Disease Diagnosis

18 - 23rd July, 2022

ICAR - Central Institute of Brackishwater Aquaculture
(ISO 9001:2015 certified)
Indian Council of Agricultural Research,
75, Santhome High Road, MRC Nagar, Chennai 600 028, Tamil Nadu

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Training Programme
on
Histopathological techniques for brackishwater aquatic animal disease diagnosis

Aquatic Animal Health and Environment Division

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Forward

During 90's *Penaeus monodon,* tiger shrimp, dominated Indian brackishwater aquaculture and now, this is being rapidly replaced by Pacific white shrimp *Litopenaeus vannamei*, following Indian Government's decision to import this species for production, consumption and export. Wide salinity tolerance, higher growth rate, and high suitability for higher density culture systems, are some of the major favourable factors that have made farmers comfortable with culturing *P. vannamei* which has now gained a popular ground in the industry. Intensification of aquaculture practices with the objective to achieve higher productions has contributed to emergence of disease problems. While vertical transmission of diseases to a large extent have been contained through use of imported SPF broodstocks and adoption of better management practices in the hatcheries, transmission of diseases through horizontal route remain a challenge to the sector. The havoc caused by shrimp viruses has raised numerous questions with regard to the management of animal health and pond management practices. Disease prevalence due to improper management of ponds and culture systems definitely provides food for thought as far as progress of aquaculture is concerned. Thus, better management practices have to be implemented without any compromise if sustainability is the goal. Needless to say, precautionary measures ring the bell and are hence, essential to meet the requirements for raising a disease-free crop for domestic and export markets. I congratulate the Aquatic Animal Health and Environment Division (AAHED) for conducting this training programme on "Histopathological techniques for brackishwater aquatic animal disease diagnosis" from the 18th to 23rd of July, 2022. This manual on histopathological techniques has been brought out to help the participants to know the histopathology and its application in various brackishwater aquatic animal disease diagnosis. I wish the participants all the best and hope that this timely training will shower immense benefits propelling the wheel of shrimp aquaculture towards a bright future. I also highly appreciate and acknowledge the efforts of scientists of AAHED in conducting this training programme.

(DIRECTOR)

Preface

Intensive culture practices followed in *P. vannamei* making it susceptible to several viral and bacterial diseases. Development of strategies for prevention and control of these diseases is vital for economic survival of the present shrimp industry and future finfish industry. Sensitive and cost effective diagnostic tools play a crucial role in early diagnosis of the diseases and to device suitable mitigation procedures. The training programme on histopathology is designed to make the participants aware of the problems related to health in the Indian culture operations. In addition to theoretical knowledge, participants will be provided with hands-on experience in basic methods from sample collection, processing to interpretation of the results. This manual contains the practical guide for the tests conducted during the training program. The manual has been prepared to help the disease diagnosis at the field level. AAHED is well equipped with advanced facilities and training personnel for conducting extensive research in disease diagnosis. I extend my heartfelt gratitude to the Director for his constant encouragement and support. I am grateful for all the inputs, suggestions and improvements contributed to the betterment of this manual by the scientists of CIBA and also for being a pillar of support in conducting this training programme.

M. Poornima

P. Ezhil Praveena

(Coordinators)

Overview of brackishwater aquaculture in India and importance of histopathology in disease diagnosis

Introduction

The world population is projected to reach 8.5 billion in 2030, and to increase further to 9.7 billion in 2050 and 11.2 billion by 2100. India is the home of 17.9% of the total world human population with an ever increasing trend and raising the demand for animal protein. The global animal protein supply is shared by egg (9.6%), aquatic meat (15.3%), milk (34%) and terrestrial meat (41.1%). The recommended dietary allowance (RDA) by Indian Council of Medical Research (ICMR) for Indians is 0.8 to 1 g protein kg^{-1} body weight (BW) day^{-1} for basic nutritional requirements. Hence, it needs to be harnessed with the advanced technical know-how to increase the supply of healthy protein and other nutrients from aquatic source. Fisheries and aquaculture plays a major role in employment generation, economic growth and food supply for million people around the world. Global growth in the supply of fish/shrimp for human consumption has outpaced population growth in the past five decades, increasing at an average annual rate of 3.2% during the period 1961–2013, double that of population growth. World per capita fish/shrimp consumption increased from an average of 9.9 kg in the 1960s to 19.7 kg in 2013, and reached a new record high of 20 kg in 2014 (FAO, 2016). This world aquaculture production is vulnerable to emerging disease outbreaks due to culture intensification, resulting in partial or total loss of production (Bondad-Reantaso et al., 2005). Ever increasing human population coupled with decreasing availability of space for land-based food production system has resulted shortage and inconsistent supply of quality food for large number of people across the globe. A realistic solution of the problem could be sustainable utilization of water resources which are lying unutilized or underutilized. Amongst a variety of food items present in the aquatic system, fishes are considered as the most important organisms suitable for human consumption. Aquaculture has developed rapidly over the last three decades and has become as an important economic activity worldwide. Fish and fishery products contribute around 15% of the animal protein supporting the nutritional security. The world fish production is in the order of 130 million metric tons, of which 50% is contributed by aquaculture. While capture fisheries is showing the signs of almost stagnation for more than a decade, aquaculture offers a vast scope of expansion. India is the second most populous country in the world and occupying only 2.4% of the world's landmass. In line with the global trend, greatest challenge the country faces is to ensure food security of the largely undernourished protein starved population in rural as well as urban areas, especially in the context of declining land resources available for agriculture and animal husbandry. Hence fisheries, mainly aquaculture sector would have to emerge as the rescuer to meet increased food demand. The aquaculture development was contributed by the top 10 species in 1990's and in recent years species diversification is increasing. India is a major maritime state and an important aquaculture country in the world. The sector has high potentials and development is imperative for rural development, domestic nutritional security, employment generation, gender mainstreaming and export earnings. It plays important role in the socio-economic development and human welfare in the country. It has a vast brackishwater area (1.2 million ha) available for aquaculture. The country also has an important role in global fisheries as the third largest producer of fish in the world and higher enhancement levels as compared to world fish production levels. The coastal aquaculture witnessed a phenomenal growth during 1980s and in the beginning of 1990s. But the growth is not progressed as visualized from the later part of 1990s due to socio economic, marketing and environmental issues coupled with the outbreak of uncontrollable diseases like white spot syndrome virus (WSSV), Monodon slow growth syndrome, loose shell syndrome, *Enterocytozoon hepatopenaei* (EHP), infectious myonecrosis virus (IMNV), etc. on shrimp industry. One of the reasons attributed for this is the unregulated development and unforeseen disease outbreaks.

With its limited resources of man and materials various central and state government agencies are making all out efforts to augment production by way of exploiting sustainably all potential sources of aquatic organisms in various kinds of water resources. Aquaculture offers vast scope for both horizontal and vertical expansion. As it plays vital role in socio-economic development in terms of income and employment, environment friendly aquaculture has been accepted as a tool for rural development. It also has huge potential for foreign exchange earnings. Initiatives have also been taken up to use the unutilized and underutilized resources in several regions of the country.

Brackishwater aquaculture its origin and development in India

Brackishwater farming in India is an age-old traditional system confined mainly to the '*bheries*' of West Bengal, *gheris*' in Odisha, *pokkali*' fields in Kerala, *kharlands*' in Karnataka and, *khazans*' in Goa coasts. These systems have been sustaining production of 500–750 kg/ha/year with shrimp contributing 20–25% with no additional input, except that of trapping the naturally bred juvenile fish and shrimp seed during tidal influx. In this traditional method, low lying areas near the banks of saline water rivers and creeks are encircled by peripheral dyke and tidal water is allowed to enter the impoundment along with natural seeds of various species of shrimp, crabs and fish. Water is retained with periodical exchanges during lunar cycles and the animals are allowed to grow. Commercial scale shrimp farming started after 1988 and the semi- intensive farming production reached 4–6 tons/ha. Area under shrimp farming increased substantially during 1990–1994 with remarkable growth rate till 1995 and the bust came in that year with the outbreak of viral disease called White spot syndrome virus. The fact that most of the farmers were new to commercial scale and high intensive shrimp farming, the general ignorance of good aquaculture practices and the lack of suitable extension services, led to a host of problems. Later with the advent of bio-secured closed culture technology using better management practices, semi- intensive and intensive shrimp farming again started to regain its lost glory. In India, till 2009 brackishwater aquaculture was mostly oriented to tiger shrimp, *Penaeus monodon* culture only. As tiger shrimp farming regressed by viral disease and profitability was decreasing due to abnormal hike in input cost and decreasing unit sale value, Indian farmers were looking an alternative. In 2009, the Coastal Aquaculture Authority of India (CAA) permitted the entrepreneurs to introduce a new species, *P. vannamei* (Pacific white leg shrimp) in India with prescribed guidelines. Since its introduction, vannamei farming showed rapid growth.

Various aquaculture farming systems

There are five different shrimpaquaculture practices mentioned in the literature, ranging from traditional to ultra-intensive techniques, but the most common techniques followed in India are traditional, extensive, semi-intensive, and intensive. Traditional culture practices completely dependent on the natural tidal entry for seed, food and water exchange. Furthermore, traditional systems are often characterized by polyculture with fish or by rotation with rice. In this method of aquaculture low lying areas near the banks of saline water rivers and creeksare encircled by peripheral dyke and tidalwater is allowed to enter in the impoundment along with natural seeds of various species of shrimp, crabs and fishes. Water is retained with periodical exchanges during lunar cycles and the animals are allowed to grow. After 3–4 months harvesting is done partially during lunar cycles. Producers rely on the tides to provide most of the food for the shrimp and as a means of water exchange. Feed for shrimp is naturally occurring, in some cases fertilizers or manure is added to promote algal growth. Low stocking densities result in modest yields. Land and labour are the principal inputs, which keeps operational cost at a minimum. The extensive farming is commonly known as improved traditional farming. This system involves construction of peripheral canals/ ponds of size ranging from 1–5 ha. Shrimp seed at the rate of 15000 – 20000/ha are stocked. Water management is done by tidal effect. The average yield is 1500 – 1700 kg/ha, including fin fishes. In most of the cases the stock is left at the mercy of nature and the predators. Supplementary feeding is not generally practiced as the entire production system is dependent on utilization of natural productivities. However some farmers use oil cakes and rice bran as supplementary feed. Semi-intensive cultivation involves stocking densities beyond those that the natural environment can sustain without additional inputs. Semi-intensive shrimp aquaculture relies on water pumps to exchange up to 25% of pond volume daily; however, mostly closed culture is practiced at present. The risk of crop failure increases with increasing farming intensity, which is mainly due to the impact on water quality exerted by the high stocking densities and supplementary feeding. All of the costs associated with semi- intensive production are much higher relative to those for extensive production, including a more complex system of ponds, installation of a pump system to regulate water exchange, skilled management, labour, purchased feed and seed stock, and increased energy usage for aeration and other purposes. The higher the culture intensity, the higher the capital required and risk involved. Thus, the increased capital inputs required for semi-intensive culture often preclude its adoption by small-scale producers. Tiger shrimp farming and low density pacific white leg shrimp farming in India falls under this category. Intensive grow-out systems utilize ample supplies of clean sea / estuarinewater, adequate infrastructure, and well- developed hatchery and feed industries. Intensive shrimp farming introduces small enclosures, high stocking densities, around-the-clock management, very high inputs of formulated feeds, and aeration. Aeration the addition of oxygen to the water permits much higher stocking and feeding levels. Yields range from 7 to 15 tons per hectare per year. The risk of disease can be serious in intensive culture, especially if water discharge from one pond or farm is taken into another to be reused. Most of the *P. vannamei* farming in India is conducted in this method using specific pathogen free (SPF) seeds under strict biosecurity protocol.

Human resource development through brackishwater aquaculture

Employment opportunities in coastal areas have increased greatly with the development of shrimp farming. According to an estimate, the average labour requirement in shrimp farming has been estimated to be about 600 labour days/crop/ha. In contrast, labour days/crop/ha in paddy cultivation is 180, which is much lower than shrimp farming. Case studies carried out at a sea based farm in the Nellore District of Andhra Pradesh showed an increase of 2–15 percent employment and 6–22 percent income for farm labourer's following the establishment of shrimp farms. Hatcheries and feed mills in the brackishwater sector are also providing substantial employment opportunities. Jobs generated in the main and supporting sectors of the shrimpaquaculture sector was estimated to be over three lakhs in India.

Brackishwater aquaculture and research and development

Eight fisheries research institutes are there under ICAR, the nodal agency for aquaculture research in India, of which ICAR - CIBA, in Chennai is researches on brackishwateraquaculture and has regional centres located in West Bengal and Gujarat to undertake researchon problems of regional importance.The institutes transfer thedeveloped techniques and technology through research publications and on-farm demonstrations. To disseminate theemerging technologies, electronic media also play vitalrole.

Control of diseases through Good Aquaculture Practices (GAqPs)

During the last few years, shrimp production has experienced a global setback due to the outbreak of viral diseases. In India during 2018-19 loss due to EHP is ` 4183.08 crores ($ 567.62 M) and WSSV is ` 1756.37 crores ($ 238.33 M). Total annual employment loss due to diseases is 1.65 m man-days worth $ 7.07 m (`. 52.3 crores). The overall probability of infectious disease occurrence in the country is 49% leading to an annual loss of $ 1.02 b (`. 7543.67 crores). The viral diseases cannot be cured but could be controlled following GAqPs, which will avoid stress to the cultured shrimp. Lack of awareness, stocking PCR untested seeds, poor biosecurity measures, improper design and poor management of farms are the major predisposing factors for the outbreaks of any disease in the grow out system. Hence, adopting GAqPs will definitely pave way to the successful and sustainable aquaculture.

Importance of histopathology as diagnostics in aquaculture

Diagnostics play an important role in aquatic animal health management and disease control. Confirmatory diagnosis of a disease is often considered as complicated and costly which may be true in some newly emerging diseases. For instance, when Early Mortality Syndrome (EMS) outbreak in shrimp aquaculture was experienced in many Asian countries during 2012, it was great challenge to the global scientific community to identify the exact etiological agent. But not in all the cases with already standardized and validated diagnostics. Incorrect diagnosis can lead to ineffective or inappropriate control measures which may be even more costly. Disease diagnostics in food animals should be made available throughout the entire life cycle till they reach table for consumption. There are multifarious recent diagnostics available in aquaculture for disease diagnosis at different levels. Some diagnostics are used to screen healthy animals to ensure that they are free from any infection at asymptomatic levels with specific pathogens. This kind of screening is mostly done on aquatic animals which are transferred live or as products from one area or country to another. Such screening reduces the risk of carrying infectious agents including opportunistic pathogens which might proliferate during shipping, handling or change of environment (Bondad-Reantaso *et al.,* 2001). Further, it reduces the risk of resistant or tolerant animals transferring a significant pathogen to a susceptible population. Diagnostic tests may be applied to diagnose clinically diseased individuals, screening for specific disease surveillance, and as a confirmatory and calibration tests to validate the other diagnostics and procedures adopted. Valid laboratory results are essential for diagnosis, surveillance, and trade. In this context, histopathological investigation is inevitable gold standard diagnostic in making confirmatory diagnosis. The origin of histology as an academic discipline started during 19th century and paved a way for developments in histopathology. Histopathology is a branch of pathology ("histos - tissue", "pathos – suffering" and "logia – study of") refers to the microscopic examination of diseased tissues in order to study the manifestation of disease. It is an important tool in anatomical pathology to highlight the sequence of cellular changes during disease process. By examining stained sections, viruses, bacteria, fungi and parasites can be identified under microscope.

Way forward

Exports will remain the mainstay of the sector for years to come. Institutional agencies focused towards this end, must seek to examine the scope for diversification of markets, communicate to exporters and processors the niche markets that exist for exclusive markets for value added fish and shrimp. Domestic markets for fish and fish products not only provide an entirely new opportunity for growth but also can act as a buffer in case of gluts in the international markets. Mud crab farming in the brackishwater sector which was started recently to enhance the production of mud crabs as well as to uplift the socio-economic condition of coastal rural population. Efforts towards adoption of improved farming technologies like re-circulatory aquaculture system (RAS), improved polyculture, integrated multi trophic aquaculture (IMTA) may make brackishwater aquaculture more environmentally acceptable.

Conclusion

The importance of brackishwater aquaculture will become more apparent in the coming years with demanding pressure from an ever increasing world population. Brackishwater aquaculture has great potential for the production of food, alleviation of poverty and generation of wealth for people living in coastal areas, many of whom are under privileged. The assurance of mature and sound techniques in aquaculture is the most important channel towards a sustainable aquaculture industry. Therefore some strategies should be worked out. The first is to speed up the completion of unfinished current research on different aspects of aquafarming. The second is to formulate and implement systematic regulations and self-controls. Thirdly it is important to strengthen the diagnostic skills and exchange of technical know-how.

General sampling procedures for shrimp and fish disease diagnosis

Introduction

Aquatic animals are an integral part of our ecosystem. The intensification of aquaculture and global climate change in the last few decades has made Aquatic pathology, to gain prominence and recognition in the global arena over time. In spite of employing all the available modern technologies in disease diagnosis of domestic animal, laboratory animal and humans, in aquatic pathology, this branch still remains in its primitive stage because of the depth and breadth of knowledge of myriad diseases encountered by the aquatic species makes it lag behind our domestic animal disease diagnosis. But in the recent years the knowledge on the natural biology of aquatic species and the diseases is growing exponentially. Among all other diagnostic methods, histopathology plays very significant role in making confirmatory diagnosis i.e., presence of pathogen with sequential changes in the cells and tissues. Dead shrimp should never be collected to avoid the confusion in interpretation due to autolytic changes. Only live, moribund, or compromised shrimp should be selected for fixation and histological examination. The collected tissue should be processed following standard protocols (Bell & Lightner, 1986) and stained by Haematoxylin & Eosin and examined under microscope. Spore can be seen in the tubular epithelial cells as well as inside of the lumen of the tubules as ovoid bodies and wherever the epithelial cellular damage is more spores can be seen in clusters. Proper sampling is one of the pre- requisites for disease diagnosis. The aquatic health experts should take care at each and every steps of processing of samples for correct diagnosis of every disease. Every aspects of the sampling such as sample size, objective of the surveillance programme, choice of proper fixatives (as in case of histopathology), choice of proper media (as in case of microbiological analysis), proper incubation temperature should be taken into consideration for sampling procedure of both finfish and shrimp. Accurate disease diagnosis is an important aspect for health management. Since disease investigation facilities may not be available in many shrimp farming areas, it would be essential that the samples will have to be sent to diagnostic laboratories or the referral laboratories for diagnosis of the diseases. For investigation of disease in aquaculture, background information on the disease and appropriate samples are essential.

Dissection of shrimp for sampling

Depending upon the situation, lethal or non-lethal sampling can be done. For valuable animals or broodstocks or fully grown cultured shrimp, non-lethal sampling is preferred where only tip of the pleopod is cut and taken for testing. At the same time, if lethal sampling has to be done, the entire pleopod can be cut and taken as sample. As lethal sampling, many of the other organs can be taken as sample after dissecting out the animal. This depends on the type of pathogen to be analysed. For example: for WSSV and IHHNV, gill can be taken as a sample. If IMNV has to be detected, telson is preferred as a sample. For HPV, MBV, AHPND and EHP, hepatopancreas, stomach, midgut etc. are taken as samples. Lymphoid organ is a preferred sample for many of the shrimp viruses. Depending upon the requirement, samples can be collected either on alcohol, RNA*later* or Davidson's fixative. Proper sampling procedure is very much important for appropriate diagnosis of fish and shrimp diseases. Improper sampling may result in wrong disease diagnosis leading to incorrect interpretation. Sampling should be carried out in such a way as to provide the best possible likelihood that the sample will be representative for the population. Usually, the disease survey is done with the objective of exhibiting presence or absence of a certain factor (e.g. Disease) or to estimate the prevalence of any disease.

Sampling methods for disease diagnosis of shrimp

The target organs in case of crustaceans depend upon the disease or pathogen to be tested, age and size of the animals and objectives of testing i.e. diagnosis of overt disease, detection of sub-clinical pathogens or carriers or sampling for targeted surveillance to show absence of specific diseases. For collection of shrimp samples, the proper record of sample is very much important and the sample should be properly labeled preferably with a sample code and record should be noted against the sample code. The record should include name and address of the farm, contact information, cultured species along with stocking density, seed source, water exchange information, days of culture (DOC), date and timeof commencement of disease symptom, details of samples (organs, collection media, etc.), water source, feed used, number of ponds, sampling pond number, water salinity, time of collection of samples and date of submission to the laboratories. When any specific disease is suspected based on symptoms and history, special consideration should be given targeting that particular disease.

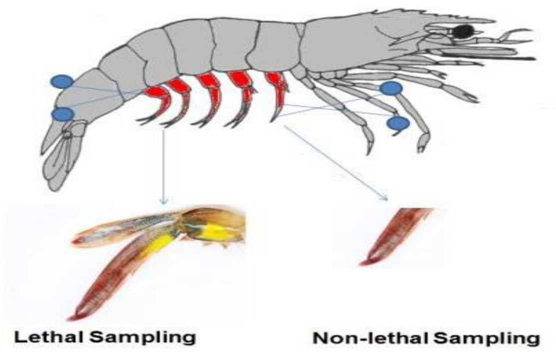


Fig. Depicting the areas where we can collect the samples for non - lethal method of testing

Sampling

The first step in disease diagnosis is sampling. The sample for histopathology should be collected in formalin based fixatives (either formalin 10% or Davidson's fixative) for specified time.

Sampling of shrimp

Sampling can be either lethal or non- lethal. Non-lethal sampling is usually carried out with broodstocks or cultured adult shrimp where a piece of pleopod is cut and used for PCR without sacrificing the animal. For lethal sampling, entire animal (in case of larvae) or any tissue material can be collected based on the type of viral pathogen needed for detection. Different parts those are usually collected for nucleic acid extraction include hemolymph, gill, muscle, pleopod, lymphoid organ, hepatopancreas, eye stalk and faecal matter. While moribund shrimp are usually preferred to detect the actual disease status, samples can also be collected from healthy shrimp to find out whether a particular virus is present or not. The collected samples may be transported in 95 % ethyl alcohol. If the samples are intended for detection of RNA viruses, then it can be preserved in any RNA preservation media (e.g. RNA later). For PCR analysis, the sample to be transported in frozen or chilled condition preferably in dry ice.

Name of the disease/Pathogen	DNA/RNA	Ideal tissue for sampling
WSSV	DNA	Larvae, Gill, Haemolymph, Gill, Lymphoid organ (LO), Pleopod, other ecto/mesodermal tissues
IHHNV	DNA	Larvae, Gill, Haemolymph, Gill, Lymphoid organ (LO), Pleopod, other ecto/mesodermal tissues
MBV	DNA	Larvae, hepatopancreas, gut, Faecal matter
HPV	DNA	Larvae, hepatopancreas, gut, Faecal matter
YHV/GAV	RNA	Larvae, Gill, LO, Pleopod
TSV	RNA	Larvae, Gill, LO, Pleopod
IMNV	RNA	Larvae, Telson, Pleopod Gill, Muscle, LO,

For histopathological analysis

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

Composition of the fixatives

1. Davidson's fixative 1000ml

   37% formaldehyde (Formalin): 220ml

   95% ethanol: 330 ml

   Distilled water (Tap water if distilledwater not available): 335 ml

   Glacial acetic acid: 115 ml

2. Neutral Buffered formalin 1000ml 37% formaldehyde (Formalin): 100ml

   Distilled water (Tap water if distilledwater not available): 900 ml

   Sodium phosphate monobasicmonohydrate: 4.0 g

   Sodium phosphate dibasicanhydrous: 6.5g

3. 95% Ethyl alcohol 1000ml Absolute alcohol: 950 ml Distilled water: 50 ml

The shrimp should be injected with Davidson's fixative at the rate of 10% of the bodyweight. If the weight of the shrimp is 20 g, then 2 ml of Davidson's fixative is required. At first, the fixative should be directly injected into hepatopancreas to ensure rapid fixation. Then the remaining portion of the fixative should be injected at different parts of body of the shrimp. Then the cuticle of the shrimp should be silted along the midline to ensure penetration of fixative. Then the sample should be placed in a container with 10 volumes of fixative. If the size of shrimp is 20 g, then requirement of fixative will be 200 ml. Now, the sample in this condition is ready to be transported to laboratory. When diagnosis has to be done for specific disease, the target sample will vary depending upon the disease suspected.

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

Live shrimp: Aquatic animals in live / moribund state will be best samples for diagnosis. Sending correct number of samples is important. If samples are collected from a pond, it should represent the entire pond and therefore samples should be collected from four corners and from middle by cast net. The number of samples to be collected from a pond or hatchery tank depends on the stock. Affected and control animals should be packed separately in poly packs with oxygen, placed in a Styrofoam box and additionally some ice/gel pack should be placed to control temperature. All the samples should be labelled properly and then sent by courier. Such live moribund samples are ideal for collection of haemolymph samples and microbiological investigation.

Fixed samples: Samples for molecular biology and histopathological analysis can be fixed and sent for laboratory for analysis. Affected moribund samples and apparently healthy looking samples can be fixed by a variety of fixatives (Davidson's fixative is highly desired for shrimp samples) for histopathology. For molecular diagnostics, dissected tissues should be fixed in 95% ethyl alcohol for detection of DNA viruses and in RNA later for detection of RNA viruses. Shrimp larval samples can be fixed in these fixatives whereas bigger animals have to be fixed in a proper manner. For histopathology and PCR analysis, individual organs can be dissected out and fixed in the fixative. Samples should be packed separately in tightly capped containers and have proper labelling. Leakage of fixative solution (which generally contain some alcohol or acid), may affect the labelling of the container when marked by a marker pen. Therefore, pencil marking is preferred. All the containers should be sealed, put in a box and sent to laboratory in shortest possible time. Fixed samples are not suitable for bacteriological or other microbiological analysis. Some of the essential background information is required to be recorded provided to the diagnostic laboratory. History of the events prior to disease outbreak and information on the clinical signs (appearance, colour change etc.), behaviours (feeding pattern, swimming pattern, active / lethargy etc) will help arriving at diagnosis.

General Precautions:

1. Accurate fixation protocol shouldbe followed.
2. Samples should be tightly packed and sealed properly.

   Accurate and detailed labelling of the samples is very important. Label the samples with pencil. Containers marked with permanent markers will erase due to the fixatives.

3. Avoid using glass containers as these may break during transport.
4. The sample should be accompanied with the proforma as required by the diagnostic laboratory.

Proforma for sample collection:

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

Sampling for finfish

Although sampling procedures for tissue fixation should vary depending on the animals and situations, an example is described here for fin fish. In general, the skin and gills should be sampled first, before being dried or damaged by handling. Then, the visceral organs are sampled. Subsequently, the kidney and heart are sampled. Lastly, the brain is sampled, since the brain is covered with a cranium and it often takes time to sample the organ. Agitate the fixative briefly, at each time you put a piece of tissue into the fixative. The overall sampling should be carried out as quickly as possible, since postmortem autolysis proceeds even while you are dissecting the fish. Wet several overlapping soft paper towels, and wring them so that the water doesn't drop. Do not wring them dry. Anesthetize a single specimen from the group of fish you are going to sample. Place the fish on the wet paper towels and draw blood from caudal artery/vein as much as possible. Alternatively, you can bleed the animal by severing the caudal peduncle or ventral aorta. Otherwise tissue sampling would be hampered by blood when you open the abdominal cavity. Furthermore, blood often interferes in fixation process or in observations of tissue sections. It is also difficult to identify blood congestion, if the blood has not been removed. In addition, blood causes the deposition of formalin pigment, if you use formalin as part of the fixative without neutralization. You can make blood smear preparations here, if necessary. Remove a few gill arches and put them in the fixative. If there is a macroscopic lesion on the skin, the portion should be sampled together with the underlying muscle and fixed Open the visceral cavity as widely as possible to sample visceral organs. For a small fish, the cluster of whole visceral organs should be removed by severing the digestive tract at the oesophagus and anus. Do not separate organs from each other. Hold the organ cluster with forceps on wet paper towels and cut it by moving the blade of a scalpel between the tips of the forceps. Move the blade rather horizontally. Do not compress tissues with the blade. Cut out a 5mm-thick tissue block by cutting the visceral organs transversely. Be sure that the tissues you need are included in the block. Cut out multiple tissue blocks if necessary. In this way, the fixative enters the lumen of the digestive tract quickly. This is important since the post-mortem autolysis proceeds very rapidly in the epithelium of the digestive tract where active digestive enzymes are often present. If the fish is large, a piece of tissue is sampled from each organ. For such a case, a piece of intestine should be less than 1 cm for fixation. Do not remove attached tissues such as the mesentery. Lesions often develop on the mesentery or in the intraperitoneal fat. Also, the pancreas is scattered in the mesentery. If the fish has many pyloric caeca, a bunch of them should be cut out into 5 mm-thick block by cutting perpendicularly to the caeca without separating each one of them, so that you can observe the mesentery, fat, and pancreatic tissues among the caeca. If there are any lesions, such as hemorrhage, in the lateral muscle, a piece of muscle should be sampled including the lesion. The muscle is usually cut out as a 5mm-thick section perpendicular to the muscle fibres. If the fish is small, the lateral muscle should be sampled with skin. To cut out the muscle, only scalpels or razor blades should be used. Avoid using scissors, which may destroy substantial part of the dissected musculature.

Fix the body kidney. If the fish is large, a piece of the tissue can be cut out from the organ. If the fish is small, the whole body kidney should be fixed along with some muscle and vertebrae surrounding the kidney. Further trimming can be done after fixation. Remove the heart (atrium, ventricle, and bulbus arteriosus) and put it in the fixative. If possible, the heart should be cut into two pieces along the midsagittal plane so that the lumen of the ventricle and atrium comes in direct contact with the fixative. Severe the head from the body at the posterior end of the operculum. Remove the lower jaw. Remove tissues surrounding the cranium as much as possible. Carefully make a small opening in the cranium and expose part of the brain, so that the fixative enters the brain case through the opening. Now, the brain can be fixed together with the cranium. If the fish is smaller, the whole severed head can be directly placed into the fixative solution, although it is still a good idea to remove the lower jaw from the head since the oral cavity may contain air. If the fish is large, a meat cleaver or a thick-bladed carver for dressing fish is useful. To dissect out the whole brain, remove the muscle and connective tissue attached to the cranium using crown scissors or kitchen scissors as possible as you can. Then, remove the upper and side part of the cranium to expose the brain as much as possible. Hold the posterior end of the medulla oblongata or the anterior end of the spinal cord with a pair of tweezers and lift it gently as you sever brain nerves from posterior to anterior. Remove the brain completely by cutting the optic nerve and olfactory nerve (or olfactory tract). The pituitary is usually left on the base of the cranium. If needed, the brain may be cut into smaller parts before fixation.

Picture showing the postmortem in a flat fish

Hematology of shrimp

Haemolymph collection

Shrimp is handled following the ethical principles and restrained properly between fingers. Haemolymph (0.2 mL) is withdrawn (Fig. 1) from the ventral sinus (arthrodial membrane) of the first abdominal segment using a 2 mL sterile disposable syringe with 23 gauge needle containing equal volume of fixative anti-coagulant (10 % formalin in 0.45 M NaCl) and transferred to a microcentrifuge tube for total haemocyte count (THC) as well as for granular haemocyte (GH) and nongranular haemocyte (NGH) counts adopting the method of Ananda Raja *et al.* (2012 & 2017). Haemolymph mixture is incubated for 10 min at room temperature.

Total haemocyte count (THC)

After 10 min of incubation at room temperature, 20 µL of the fixed haemocyte suspension is mixed with the same volume of Rose Bengal solution (1.2 % Rose Bengal in 50 % ethanol) and incubated at ambient temperature (27-35ºC) for 20 min. before being used for haemocyte counts. The counting chamber of haemocytometer (improved Neubauer, Marienfeld, Germany) is loaded with diluted haemolymph after placing the special cover slip on the supporting ribs of the haemocytometer. The cells are allowed to settle for three minutes. Haemocytes are counted under 10 x magnification from five of the 25 big squares. Duplication in counting the cells is avoided by counting the cells touching the top and left lines of the small squares in addition to the cells inside the squares. Care is taken to avoid variation of more than 20 cells between any of the five big squares (vol. of one square = 0.2 x 0.2 x 0.1 mm^3) using Eclipse NiU, Nikon microscope with DS Fi3 camera and NIS BR software. In case of uneven distribution of cells, fresh dilution is done.

THC is calculated as:

$$\text{THC mL}^{-1} \text{ of haemolymph } = 5 \text{ X count X} \left(\frac{10^3 \text{ mm}^3}{0.1 \text{ mm}^3} \right) \text{X 1mL X dilution factor}$$

Granular and nongranular haemocyte counts

For granular and nongranular haemocyte counts (GH and NGH), smears are prepared on clean, grease free glass slides, from the fixed and Rose Bengal stained haemocyte suspension. The smears are completely dried before counterstaining with haematoxylin solution (50 g aluminium or potassium alum, 1 g haematoxylin crystals, 0.2 g sodium iodate, 1 g citric acid, 50 g chloral hydrate and distilled water to 1 L) for 7 to 10 min. The slides are then rinsed with tap water for 10 min, dehydrated in ascending grades of ethanol (10 dips each), cleared in xylene (3 times for 3 min each) and mounted using DPX mountant (Merck) with a cover glass. The proportions of GH that included both large-granular haemocyte (LGH) and small-granular/semi-granular haemocytes (SGH) in 200 total haemocytes are recorded and these proportions are used to calculate the total number of GH (*i.e.*, GH count/200 × THC). NGH or hyaline cells (HC) counts are also calculated in the same manner. For better differentiation of granular cells, Periodic Acid Schiff (PAS) staining McManus' method is followed. Smear from the fixed haemocyte suspension is prepared and air dried. The smear is oxidized in 0.5 % periodic acid solution for 5 min at room temperature (RT) and rinsed in distilled water. Then, the smear is kept in Schiff reagent for 10 min followed by washing in running distilled water for pink colour to develop. The smear is rinsed thrice in sulphurous acid for 2-3 min each followed by rinsing in distilled water and staining in Mayer's haematoxylin for 2-3 min. It is then washed in running distilled water for 10-15 min. The slide is dehydrated in ascending grades of alcohol, cleared in xylene and mounted with DPX mountant for observation under light microscope. The development of rose to purplish red with granules and blue with nuclei is observed clearly to make differential count of shrimp haemocytes. Agranular haemocytes or hyalinocytes or HC are the smallest (12.4 x 7.8 µm), ovoid in shape with no granules. Semi-granular haemocytes or small-granular haemocytes (SGH) are ovoid and slightly longer and wider (14.8 x 8.3 µm) than HC with 1-40 granules in the cytoplasm. The third cell type is large granular haemocytes (LGH) which are ovoid and spherical in shape with the same size (13.6 x 9.5 µm) as the SGH (Fig. 2-4).

Fig. 1. Haemolymph collection from the ventral sinus

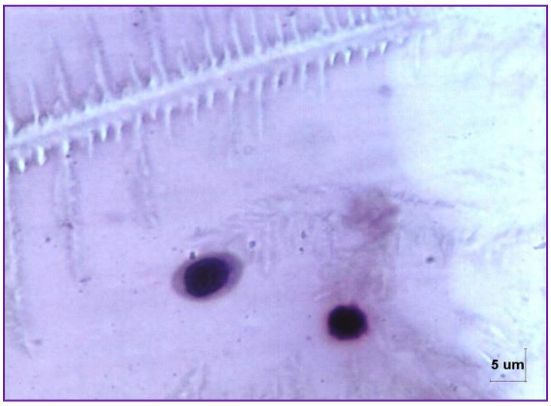


Fig. 2. Small and large nongranular haemocytes – No cytoplasmic granules; Nuclei blue PAS Scale bar : 5 µm

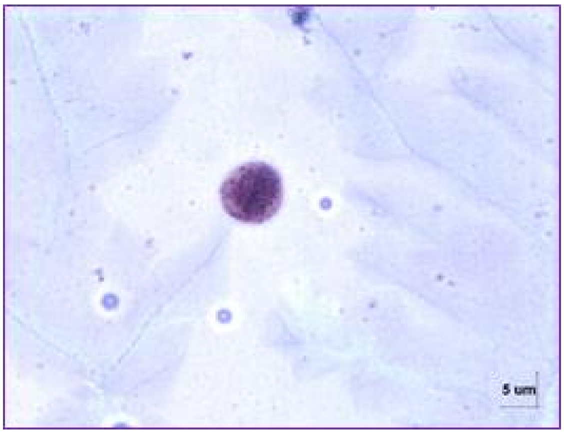


Fig. 3. Small granular haemocytes - Rose to purplish red granules in the cytoplasm;

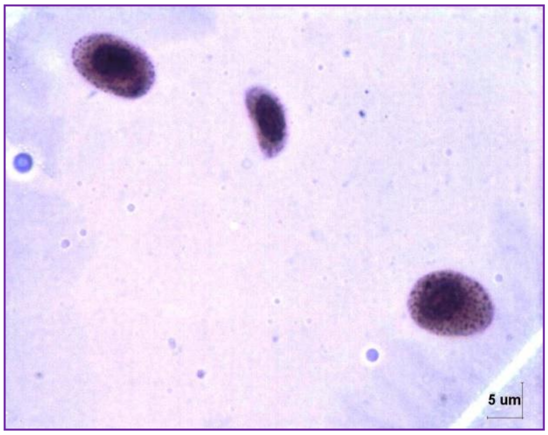


Fig. 4. Large granular haemocytes - Rose to purplish red granules in the cytoplasm;

Hematology of fish

Blood collection

Fish is handled following the ethical principles and restrained properly between fingers after being anaesthetized by bath treatment in tricaine methane sulphonate [MS-222] (100 mgL^{-1} buffered with 300 mgL^{-1} $NaHCO_3$). Blood for haematological parameters in heparinized vials is collected using a 2 mL sterile disposable syringe with 23 gauge needle either through caudal vein, gill arch and/or intracardiac puncture following the aquatic animal ethical principles.

Red blood cell (RBC) and white blood cell (WBC) count

The blood is diluted 1/1000 in Natt-Herrick's staining solution (gL^{-1}: NaCl-3.88; $NaSO_4$-2.50; $Na_2HPO_4.12H_2O$-2.91; KH_2PO_4-0.25; 37% formalin-7.50 mL; methyl violet-0.10; pH-7.3) to count RBC and 1/100 for WBC. Both are counted in a Neubauer's haemocytometer using a light microscope. The counting chamber of haemocytometer is charged with diluted blood after placing the special cover slip on the supporting ribs of the haemocytometer. The cells are allowed to settle for three minutes. RBCs are counted under high power (40x) from five of the 25 big squares. Duplication in counting the cells is avoided by counting the cells touching the top and left lines of the small squares in addition to the cells inside the squares. Care is taken to avoid variation of more than 20 cells between any of the five big squares. In case of uneven distribution of cells, fresh dilution is done. For counting the WBCs, the number of cells in each of the four large squares each containing 16 small squares are counted following the rules and precautions for RBC count. The following formulae are used for calculation of total number of RBC and WBC in fish blood.

$$Total\ RBC\ count\ in\ million\ per\ cmm = \frac{Number\ of\ RBCs\ counted \times Dilution\ factor \times 10 \times 5}{1000000}$$

$$Total\ WBC\ count\ in\ thousand\ per\ cmm = \frac{Number\ of\ WBCs\ counted \times Dilution\ factor \times 10}{4 \times 1000}$$

Differential white blood cell (WBC) count

Differential WBC counts are performed after staining the blood smears with the Leishman-Giemsa's stain (Methanol-100 mL; Leshman-150 mg; Giemsa-30 mg). 150 mg of Leishman powder is mixed in 50 mL of methanol and 30 mg of Giemsa is mixed in 50 mL of methanol, separately. Both the solutions are mixed well and incubated for 20 days at room temperature. On 21st day, the staining mixture is filtered in Whatman filter paper no. 4 and used. Fresh and air dried blood smear is stained with 1 mL of Leishman-Giemsa's stain for 30 seconds followed by dilution with 5 mL of distilled water. The staining is allowed for 20 minutes. The slide is washed in distilled water, air-dried and observed under oil immersion. The cells are identified based on cell morphology. Erythrocytes are identified as oval in shape with abundant smooth eosinophilic cytoplasm and a central, oval-shaped condensed nucleus. Polychromatophilic (hemocytoblast), immature erythrocytes (proerythrocyte) and smudge cells are also identified. Small and large thrombocytes are identified as oval, spindle or round in shape with clear, colourless cytoplasm and a central, oval-shaped condensed nucleus. Small lymphocytes are small round in shape with a rim of smooth light blue cytoplasm around the large oval-round condensed nucleus with higher N:C ratio as compared with thrombocytes. Large lymphocytes are categorized based on the presence of blebs or out-pocketing of the cell membrane. Neutrophils are large round cells with abundant clear cytoplasm and an eccentric, condensed nucleus that is either round or multi-lobed. The cytoplasm is often colourless or light grey and, in some cells, contained very fine azurophilic granules. Monocytes are recognized as large round in shape with abundant blue cytoplasm often containing vacuoles. The nucleus could be round, oval or lobed with loosely compact chromatin. Large round cells with clear to pale blue-grey cytoplasm filled with slender rod-shaped eosinophilic granules are named as eosinophilic granulocytes. The nucleus is either round or multi-lobed. Extremely rare basophils are identified with abundant granules and indistinguishable nucleus (Fig. 5).

Haemoglobin (Hb) estimation

Haemoglobin is estimated by the acid haematin conversion method using Sahli's haemoglobinometer. The graduated measuring tube was filled with 0.1N HCl up to graduation two by using a Pasteur pipette and placed in the haemometer. After mixing the blood sample, it is drawn up to 20 cmm mark in the Sahli's Hb pipette. If the level of the blood is above 20 cmm mark, it is brought to that level by gently tapping the pipette on a glass slide without applying cotton to the tip of the pipette. The excess blood sticking on to the side of the pipette is wiped off using cotton. The 20 cmm blood is transferred in to the acid in the measuring tube by rinsing the pipette by drawing the solution into it many times. The Hb is converted in to acid haematin within 5-10 minutes. After 10 minutes, 0.1N HCl is added drop by drop by mixing the solution using mixing rod. It is added slowly till the colour matched with the standard on either side to the haemometer. The level of solution in the tube is read and the Hb level is expressed in grams per 100 mL of the blood.

Packed cell volume (PCV) estimation

Haematocrit (HCT) or packed cell volume (PCV) is determined using micro haematocrit capillaries filled with blood and centrifuged at 8700x g for 5 min and expressed as percentage of total blood volume.

Calculation of erythrocyte indices

The erythrocyte indices are calculated based on the following specific formulae.

$$Mean\ Corpuscular\ Volume\ (MCV) in\ femtoliters\ (fl) = \frac{PCV\ \% \times 10}{RBC\ count\ in\ 10^6\ cmm}$$

$$Mean\ Corpuscular\ Haemoglobin\ (MCH) in\ picogram\ (pg) = \frac{Hb\ in\ g\ \% \times 10}{RBC\ count\ in\ 10^6\ cmm}$$

$$Mean\ Corpuscular\ Haemoglobin\ Concentration\ (MCHC)\ in\ gram\ per\ deciliter\ (gdL^{-1} = \frac{Hb\ in\ g\ \% \times 100}{PCV\ \%}$$

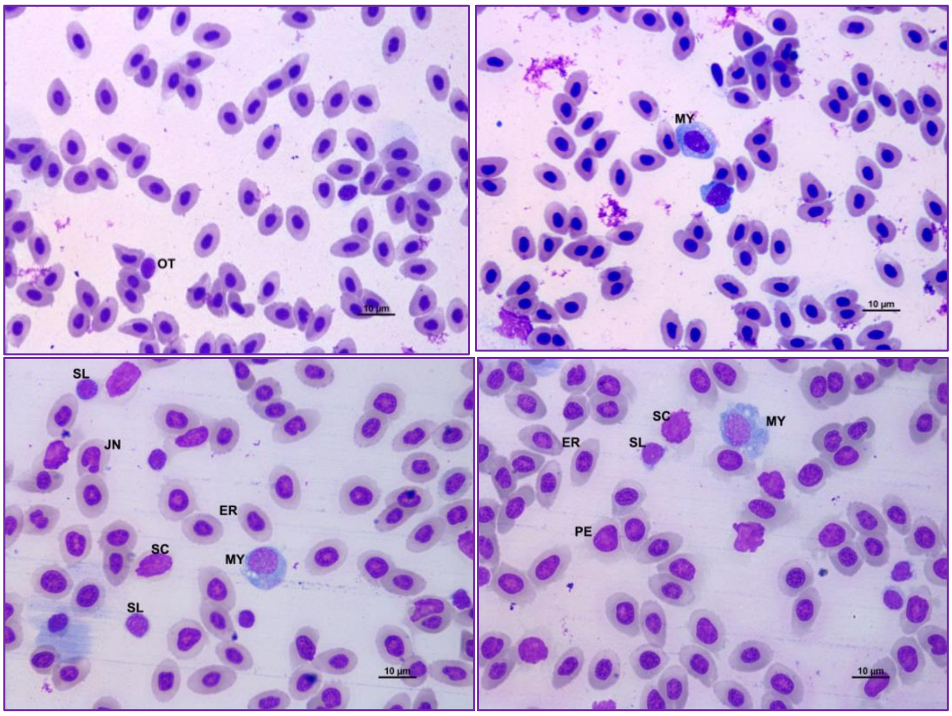


Fig. 5: Haematology of Asian Seabass (*Lates calcarifer*): Proerythrocyte (PE), Erythrocyte (ER), Smudge cell (SC), Oval thrombocyte (OT), Small lymphocyte (SL), Myelocyte (MY) and Juvenile neutrophil. Leishman Giemsa: Nucleus - brilliant violet; Cytoplasm of erythrocytes – pink; Cytoplasm of granulocytes – blue. Scale bar : 10 µm.

Histopathology of shrimp disease diagnosis

Pre-collection preparation

The diagnostic laboratory receiving the sample should be consulted to ascertain the best method of transportation (e.g., on ice, preserved in fixative, whole or tissue samples). The laboratory will also indicate if both clinically affected, as well as apparently healthy individuals are required for comparative purposes. The laboratory should be informed of exactly what is going to be sent (i.e., numbers, size classes or tissues) and the intended date of collection and delivery, as far in advance as possible. Screening can be also be planned ahead of time, based on predicted dates of shipping post-larvae (PL) or broodstock, which means the shipper has more time to notify the laboratory well in advance. In cases of disease outbreaks and significant mortalities, there may be less opportunity for advance warning for the laboratory. However, the laboratory should still be contacted prior to shipment or hand-delivery of any diseased samples. Some samples may require secured packaging or collection by designated personnel, if there are national or international certification requirements or risk of disease spread via transport of the sample to an area non-endemic for a suspected disease. Pre-collection discussions with the diagnostic laboratory can significantly speed up processing and diagnosis of a sample (days to weeks) since it allows preparation of the required diagnostic materials in advance of arrival of the sample(s) and ensures that emergency samples are scheduled in for rapid diagnosis.

Sampling

There are at least three purposes for which crustaceans are sampled with regard to the OIE listed crustacean diseases. They are either for surveillance, stock or facility certification and/or disease diagnosis. The number and type of samples to be collected for analysis varies greatly according to which of these purposes applies. For example, at 5% prevalence assuming 100% sensitivity and specificity of the technique, the random sample size should be at least 60 for a lot containing 2000 or more stock. In clinical disease episodes, carefully selected quality specimens with representative lesions and displaying abnormal behaviour should be obtained from live or moribund crustaceans. Shrimp should be collected by whatever means are available with a minimum of handling stress. Collection of dead specimens should be avoided. The samples should never be frozen. The recommended minimum numbers of specimens to be collected for diagnostic testing are 100 for the larval stages of most crustaceans; 50 for the PL stages; and 10 for juveniles and adults. It is important that carefully selected, quality specimens are far more valuable and cost-effective than dozens or hundreds of specimens collected at random to 'fill out' the sample. The crustaceans should be packed in seawater in double plastic bags with the airspace in the bag filled with oxygen. The bags should be sealed tightly with rubber bands or rubber rings and packed inside a foam box. A small amount of ice may be added to keep the water cool, especially if a long transport time is expected. This box is then taped securely and may be packaged inside a cardboard carton. The diagnostic laboratory should be consulted about packing requirements since some laboratories have specific packaging requirements for diseased organisms. Adequate aeration should be supplied to the container if the shrimp are to be left for a short period of time before actual fixation.

For diagnosis of the OIE listed diseases in subclinical crustacean carriers, it is highly recommended that the scheduling of sampling be planned so that the particular life-stage(s) are sampled at a time when the pathogen of concern is most likely to be detected. This is especially important when the available diagnostic methods are dependent on simple microscopy or histological methods and do not include molecular methods. For the baculoviruses such as Baculovirus penaei (BP) and Monodon Baculovirus (MBV), the larval and early PL stages are the most appropriate samples; for Taura syndrome virus (TSV), Infectious hepatopancreas and haematopoietic necrosis virus (IHHNV), White spot syndrome virus (WSSV), Infectious myonecrosis virus (IMNV), Necrotising hepatopancreatitis (NHP-B) and Yellowhead virus/Gill associated virus (YHV/GAV), juveniles and sub-adults provide the best samples; and for crayfish plague, juveniles and adults are suitable samples. All samples submitted for diagnosis should include as much supporting information as possible including:

- Gross observations and a history of environmental parameters.
- Approximate prevalence and pattern of mortality (acute or chronic/sporadic cumulative losses).
- History and origin of affected population.
- If the stock is not local, their origin(s) and date(s) of transfer should be included.
- Details of feed, consumption rates and any chemical treatments used.
- Clear labelling of the containers is important for identification and record keeping.

The above information provides valuable background details which can help focus attention on possible handling stress, changes in environment or infectious agents as the primary cause of any health problems.

Fixation

The term fixation means to immobilize. This is one of the crucial steps in histological procedure. The main objective of fixation is to preserve the cellular configuration of the tissue by preventing self-destruction of tissues through autolysis and bacterial degradation (putrefaction) besides denaturation of the proteins in the tissues. The tissue is fixed immediately after being taken out from the specimens to avoid rapidly setting post-mortem changes. For any preservation procedure, it is essential to remember that the main digestive organ of the shrimp (the hepatopancreas) is very important for disease diagnosis, but undergoes rapid autolysis (tissue digestion by digestive juices released from the dying hepatopancreatic cells) immediately after death. This means that the pre-death structure of the hepatopancreas is rapidly lost (turns to mush). Delays of even a few seconds in fixative penetration into this organ can result in the whole specimen being useless for diagnosis, thus, specimens must be immersed or injected with fixative while still alive. Fixation of shrimp tissues can be done in many ways. The whole specimen can be fixed live by immersion or injection of the fixatives into vital areas before immersion with proper fixative. Generally, minimum of 10 times the volume of fixatives should be used for one volume of tissue sample specimen. Larvae and early PL should be immersed directly in a minimum of 10 volumes of fixative to one volume of shrimp tissue. This 10:1 ratio is critical for effective preservation. Attempts to cut costs by using lower ratios of fixative to tissue can result in inadequate preservation of tissues for processing. For PL that are more than approximately 20 mm in length, a small and narrow incision should be made using a fine needle that breaks and slightly lifts the cuticle in the midline of the back, at the cuticular junction between the cephalothorax and first abdominal segment. This allows the fixative to penetrate the hepatopancreas quickly. For larger PL’s, juveniles and adults, the fixative should be injected directly into the shrimp using 27 gauge needles as follows:

- Shrimp should be placed briefly in ice water to sedate them.

- Using surgical rubber gloves and protective eyeglasses, the fixative (approximately 10 % of the shrimp's body weight) should be immediately injected at the sites such as hepatopancreas, region anterior to the hepatopancreas, anterior abdominal region and posterior abdominal region.
- Care should be taken to hold the shrimp so the angle of injection is pointed away from handler's body, since fixative can sometimes spurt back out of an injection site when the needle is removed and may injure the eyes.
- It is also best to brace the injection hand against the forearm of the hand holding the shrimp, to avoid over penetration of the needle into that hand.
- The hepatopancreas should receive a larger proportion of the injected fixative than the abdominal region.
- In larger shrimp it is better to inject the hepatopancreas at several points.
- All signs of life should cease and the colour should change at the injection sites.
- Immediately following injection, the cuticle should be slit with dissecting scissors along the side of the body from the sixth abdominal segment to the cuticle overlying the cephalothorax. From there, the cut should be made forward and upward until it reaches the base of the rostrum without damaging the underlying tissue.
- Shrimp over 12 g should be transversely dissected, at least once, posterior of the abdomen/cephalothorax junction and again mid abdominally. The tissues should then be immersed in a 10:1 volume ratio of fixative to tissue, at room temperature.
- The fixative can be changed after 24-72 h to 50-70 % ethanol, for long term storage.

Various fixatives have been used for the preservation of shrimp and other crustaceans with varying success. Among these are simple fixatives (eg. formalin, methanol, ethanol etc.) or compound fixatives in which mixtures of several fixing agents in liquid form are used. Most routine histological studies of shrimp employ Davidson's AFA (alcohol, formalin, acetic acid) fixative. However, Neutral buffered formalin (NBF) is also recommended. The fixatives can be prepared as follows.

Davidson's AFA (alcohol, formalin, acetic acid) fixative

Davidson's AFA fixative is recommended for most histological applications. The fixative is rapid, reduces autolytic changes in penaeid shrimp and its acidic content decalcifies the cuticle. The formulation for Davidson's AFA fixative for 1 litre is as follows.

95 % ethyl alcohol	-	330 mL
100 % formalin (a saturated 37-39% aqueous solution of formaldehyde gas)	-	220 mL
Glacial acetic acid	-	115 mL
Tap water (for marine crustaceans, sea water may be substituted)	-	335 mL

The fixative should be stored in glass or plastic bottles with secure caps at room temperature. Fixation time is 24 - 72 h at room temperature and then the sample should be transferred to 50-70 % ethyl alcohol for storage.

Neutral buffered formalin (NBF)

The formulation for NBF fixative for 1 litre is as follows

Formalin	-	100 mL
Distilled water	-	900 mL
Sodium dihydrogen orthophosphate	-	4 g
Di-sodium hydrogen orthophosphate	-	6 g

Fixation time is 24 to indefinite at room temperature for storage.

Complete history of the specimens should be recorded at the time of collection. The gross observations on the condition of the shrimp, species, age, weight, source (wild, culture pond, tank number, stock number, etc.), and any other pertinent information that may be needed at a later time should be recorded. The label should stay with the specimens in the same container during fixation, storage and transport to the laboratory. Soft-lead pencil on water-resistant paper should be used to label the samples.

Transport and shipment of preserved samples

Because large volumes of alcohol should not be posted or shipped, the following methods are recommended to transport and shipment of preserved samples. The specimens should be removed from the 70% ethyl alcohol. The samples should be wrapped completely with white paper towels (do not use raw cotton). Towel-wrapped specimens should be placed in a sealable plastic bag and saturated with 70% ethyl alcohol. The sample should be kept in leak-proof and screw-cap plastic vials. If glass vials must be used, package should be done in such a way to prevent breakage. The bag should be sealed and labelled properly and kept within a second sealable bag. Multiple small sealable bags can again be placed within a sturdy, durable and crush-proof appropriately labelled container for shipment. There should be no free liquid in the bag. In most countries, small numbers of such specimens can be sent to diagnostic laboratories by airmail. However, some countries or transport companies (especially air couriers) have strict regulations regarding shipping any chemicals, including fixed samples for diagnostic examination. Confirmation should be made with the post office or carrier before collecting the samples to ensure they are processed and packed in an appropriate and acceptable manner. The containers should be labelled clearly with the name and telephone number of the contact person responsible for picking up the package at the airport or receiving it at the laboratory. If being shipped by air, it should be indicated as "HOLD AT AIRPORT AND CALL FOR PICK-UP". Where possible, shipping should be done early in the week to avoid arrival during the weekend which may lead to loss through improper storage of samples. The contact person should be informed as soon as the shipment has been sent. The name of the carrier, the flight number, the waybill number and the estimated time of arrival should be informed to the laboratory.

Processing for histopathology

Decalcification

A specimen may contain a mixture of hard and soft tissues. The soft tissues can be processed for histological examination without any special treatment. However, hard-calcified tissues such as cuticle may require special treatment like decalcification. This process will soften the calcified tissues by removing calcium ions from bony components, sufficiently to allow smooth sectioning. Tissues fixed in Davidson's fixative or NBF has to be placed in decalcifying solution for 24-72 h depending upon the nature and size of the tissues.

70 % ethyl alcohol	-	98 mL
Concentrated nitric acid	-	2 mL

After proper decalcification, the tissue should be washed in 70 % ethanol for 2-3 times and store in fresh 70 % ethanol.

Further processing of the fixed tissue involves dehydration through ascending grades of alcohol, clearing of tissue using a paraffin-miscible solvent such as xylene, chloroform or methyl benzoate and finally impregnation/infiltration with paraffin wax and embedding.

Washing

Washing is a process of removing excess fixative from the tissues. The most common washing fluid is running tap water or 70% alcohol. For routine histopathological procedure in shrimp tissues, washing is not required as done in case of Bouin's, Zenker's or Helly's fixed mammalian tissues.

Dehydration

The dehydration means removal of entire extractable water from a tissue by a chemical dehydrant diffusing through the tissue. Paraffin wax does not infiltrate in to the tissue in the presence of water or alcohol, hence dehydration and clearing are essential processes before going for embedding in paraffin wax at its melting point. The steps are as follows.

1. Ethyl alcohol 70% - 1 h.
2. Ethyl alcohol 90% - 1 h.
3. Absolute alcohol I - 1 h.
4. Absolute alcohol II - 1 h.

Clearing

The alcohol is not miscible with paraffin wax. Therefore after dehydration, it is essential to treat the dehydrated tissue with a wetting agent which must be miscible with both dehydrant as well as embedding media. When dehydrant alcohol is replaced by the wetting agent from the tissue, the latter is cleared and becoming transparent. The steps are as follows.

1. Absolute alcohol + Xylene (1:1) - 1 h.
2. Xylene I - 1 h.
3. Xylene II - 1 h.

Embedding

The entire process of embedding comprises of infiltration or impregnation and casting or blocking.

Infiltration/Impregnation

The process of adding supporting medium to the interstices of tissues is called as infiltration or impregnation. There are two methods of impregnation as follows.

a. Paraffin cold impregnation

Xylene and paraffin shavings (1:1) - 1 h. (OR)

b. Hot impregnation

The tissue is transferred in to a cavity blocks or other small tray containing molten paraffin kept at 58-60° C. Infiltration time depends on the size and nature of the tissues.

Casting/Blocking

The process of enclosing of the tissue in a solid mass of the embedding medium is called casting or blocking. Melted medium paraffin (melting point between 54-56 ºC) is poured into the mould (Leucard block) and the tissues are oriented in such a position that the cutting surface of the tissue faces down. The tissue blocks are removed from the moulds after complete solidification and they are ready for sectioning.

Labelling and storage

The tissue blocks should be labelled properly and stored at room temperature for future record.

Section cutting

The paraffin blocks are trimmed to a square or rectangular shape. The surface opposite to the cutting surface should be pressed firmly on the surface of the metal block holder or wooden block. The paraffin blocks molted in the plastic moulds are directly placed in the tissue carrier of the microtome. To get thin sections, the mounted paraffin blocks should be kept on the ice cubes or slabs for 5-10

minutes. After proper positioning and orientation of the tissue-paraffin blocks in the tissue carrier of the microtome, trimming should be done at 15-20 μ thickness followed by sectioning at 5-7μ thickness using new blades.

Spreading

The uniform ribbon of section should be removed with the help of camel hair brush and placed on an over exposed x-ray film. The ribbons of sections are floated on a thermostatically controlled tissue floatation water bath. The temperature of the water bath should be maintained between 40-45° C.

Mounting

Sections spread out uniformly are then taken on to a clean glass slides coated with Meyer's albumin-glycerin mixture and air dried. The composition of the Meyer's albumin-glycerin mixture is as given below.

Egg albumin (white of the fresh egg)	-	100 mL
Glycerin	-	100 mL
Thymol crystal as preservative	-	2 g

Mix the ingredients well and allow the mixture to stand for 2-3 days with occasional shaking. Filter and store the adhesive in a cool place.

Staining of sections

Before the tissue sections are subjected to staining, the sections should be deparaffinized thoroughly and dehydrated. Hematoxylin (basic stain for nucleus) and eosin (acidic stain for cytoplasm) staining can be employed for routine histological preparation and this is the best method for histological diagnosis of viral diseases. Steps involved in staining with hematoxylin and eosin are as follows.

a. **Harris haematoxylin**

Haematoxylin crystal	-	5.0 g
100 % alcohol	-	50.0 mL
Ammonium/potassium alum	-	100 g
Distilled water	-	1000 mL
Mercuric oxide	-	2.5 g
Glacial acetic acid (after cooling)	-	8 Ml

Dissolve the haematoxylin in absolute alcohol; add the alum, previously dissolved in hot distilled water. Heat the mixture to boiling points and add the mercuric oxide, cool rapidly and filter. This stain is ready for use when cool. Staining time 2-3 minutes

b. Eosin

Aqueous eosin or alcoholic eosin is prepared as 1 % solution and used.

Haematoxylin and eosin method of staining is the routinely used stain for tissue sections. The steps involved are as follows.

1. De-paraffinise in xylene I - 1 h.
2. De-paraffinise in xylene II - 1 h.
3. Absolute alcohol I - 10-15 min.
4. Absolute alcohol II - 10-15 min.
5. 90% Alcohol - 10-15 min.
6. 70% Alcohol - 10-15 min.
7. 50% Alcohol - 10-15 min.
8. Wash in tap water - 5-10 min.
9. Haematoxylin - 4-8 min.
10. Wash in running tap water - 5-10 min.
11. Bluing with NH_3 water (0.5% NH_4OH) - Adequately (or Acid alcohol)
12. Wash in running tap water - 1 min.
13. 50% Alcohol - 30 min.
14. 70% Alcohol - 10-15 min.
15. Counter stain with eosin 0.5% - 15-30 sec.
16. 90% Alcohol - 10-15 min.
17. Absolute alcohol - 10-15 min.
18. Alcohol + Xylene (1:1) - 10-15 min.
19. Xylene I - 30 min.
20. Xylene II - 30 min.

Mounting

The stained sections are mounted in DPX mountant (Distyrene plasticizer and xylene) without air bubbles and air dried.

Observation under light microscope

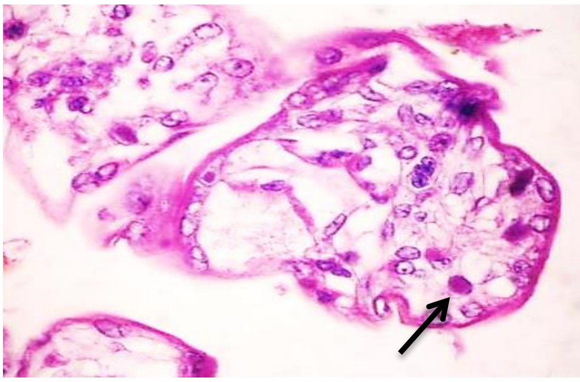
The slides are observed under light microscope for any histopathological changes and recorded. The intensive shrimp aquaculture has brought disease problems leading to great economic loss. Generally, diseases are broadly classified in to infectious and non-infectious. Former is caused either by virus, bacteria, fungi, parasites and/or rickettsia while later is due to environmental stresses, genetic factors and nutritional deficiencies. Diseases may be caused by a single or combinations of multifarious factors. The diseases caused by infectious agents are described hereunder with special reference to histopathology.

Histopathological lesions in shrimp diseases

Viral diseases

White spot disease (WSD)

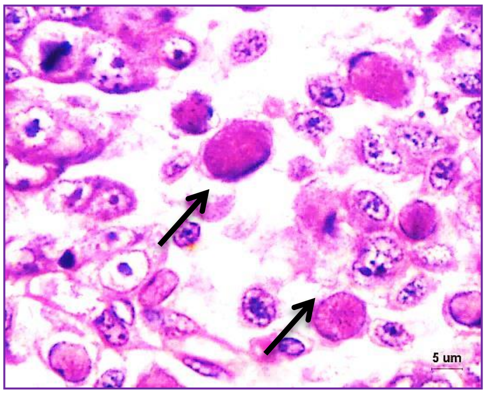
It is an acute, infectious disease of shrimp, caused by a large, ovoid, bacilliform, non-occluded enveloped dsDNA virus belonging to the family *Nimaviridae* (Nima: In Latin, means thread) and genus Whispovirus consisting of thread-like polar extension (Lo *et al.,* 2012). The virus is inactivated in <120 minutes at 50°C and <1 minute at 60°C and is viable for at least 30 days at 30°C in seawater under laboratory conditions and is viable in ponds for at least 3-4 days. The virus is viable and infective up to 19 and 35 days in pond sediment despite sun-drying and in water-logged conditions, respectively. It infects all life stages of decapod crustaceans of marine and brackishwater sources. The virus infects ectodermal and mesodermal tissues, especially the cuticular epithelium and subcuticular connective tissues. Both vertical and horizontal transmission is reported. Clinically, the disease is characterized by lethargy, inappetence, crowded at pond margin, red to pink discoloration of the body, loose cuticle, swelling of branchiostegites, broken antennae, damaged appendages, and the most conspicuous feature of small to large white spots on the inner side of the carapace which spread all over the body in advanced infection. Cumulative mortalities in infected populations may reach 100 % within 3 to 7 days of the onset of clinical signs. Histologically, degenerated cells are characterized by hypertrophied nuclei with marginated chromatin and eosinophilic to basophilic intranuclear inclusions. The histopathology of WSD is distinctive, and can provide a conclusive diagnosis. However, first time detection or detection in species not previously reported to be susceptible, require molecular assay or electron microscopy demonstration of a viral aetiology. Moribund shrimp with WSSV show systemic destruction of ectodermal and mesodermal tissues. Nuclei of infected cells are hypertrophied and when stained with haematoxylin and eosin show lightly to deeply basophilic central inclusions surrounded by marginated chromatin. These intranuclear inclusions can also be seen in squash mounts of gills or sub-cuticular tissue, or in tissue sections. The best tissues for examination are the subcuticular tissue of the stomach, cephalothorax or gill tissues.



Gill of affected shrimp showing inclusion bodies – H&E 100x

Infectious hepatopancreas and haematopoietic necrosis (IHHN)

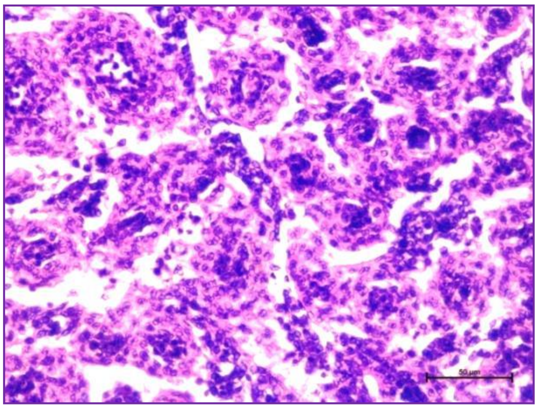
The disease is caused by infection with ssDNA, infectious hypodermal and haematopoietic necrosis virus (IHHNV) belonging to the family *Parvoviridae*. In *P. vannamei* it causes the chronic disease called runt deformity syndrome (RDS) characterized by lower overall crop production, shrimp with increased size variability, and cuticular deformity. Infected shrimp have been observed to rise to the water surface, remain motionless for a few moments then roll over and sink to the bottom. This behaviour may be repeated until mortality occurs. In juvenile *P. stylirostris,* more than 90% mortality reported within several weeks of onset of infection. Gross signs of infection include white to buff mottling of the cuticle, opacity of striated muscle and melanized foci within the hypodermis. In the later stages of infection *P. stylirostris* and *P. monodon* may appear bluish in color. Infected *P. vannamei* display deformed rostrums, cuticle and antennal flagella. IHHNV forms Cowdry Type A intranuclear inclusion bodies (CAIs) associated with widespread cytopathological changes including hypertrophy of the nucleus and margination of the chromatin in cells of ectodermal (epidermis, gills, fore and hind gut, antennal gland and neurons) and mesodermal origin (hematopoietic tissue, haemocytes, striated muscle, heart, lymphoid organ, antennal gland, gonads, and connective tissues). Infected nuclei are enlarged with a central eosinophilic inclusion sometimes separated from the marginated chromatin by an unstained ring when tissues are preserved with acetic acid containing fixatives. Since IHHNV intranuclear inclusion bodies can be confused with developing intranuclear inclusion bodies due to White spot disease, electron microscopy or *in situ* hybridization assays may be required for definitive diagnosis. Basophilic strands may be visible within the CAIs and cytoplasmic inclusion bodies may also be present.



Inclusion bodies in the haematopoietic tissue –H&E 100x

Infectious myonecrosis (IMN)

Infectious myonecrosis (IMN) is a recently identified viral disease caused by dsRNA infectious myonecrosis virus (IMNV), a putative totivirus belonging to a family *Totiviridae*. IMNV particles are icosahedral in shape and 40 nm in diameter. It causes mortalities in juvenile and sub adult pond-reared stocks of *P. vannamei* and the mortality range from 40 to 70 %. Outbreaks of the disease seems to be associated with certain types of environment and physical stresses (i.e. extremes in salinity and temperature, collection by cast net, etc.), and possibly with the use of low quality feeds. Experimental infection is observed in tiger shrimp, *P. monodon* and blue shrimp, *P. stylirostris* but mortalities do not occur as a consequence of experimental infection in this laboratory trial. IMN affected shrimp presents focal to extensive white necrotic areas in the striated (skeletal) muscle, especially of the distal abdominal segments and tail fan, which can become necrotic and reddened in some affected shrimp. By histopathology, shrimp with acute phase disease presents lesions with coagulative necrosis of skeletal muscle. In shrimp recovering from acute disease or those in the more chronic phase of the disease, the myonecrosis appears to progress from coagulative to liquefactive necrosis accompanied with haemocytic infiltration and fibrosis. Significant lymphoid organ spheroid formation is typically present, and ectopic lymphoid organ spheroids are often found in the hemocoel and loose connective tissues, especially in the heart lumen and adjacent to antennal gland tubules. In some histological preparations, perinuclear pale basophilic to dark basophilic inclusion bodies are evident in muscle cells, connective tissue cells, haemocytes, and in cells that comprise lymphoid organ spheroids.



Lymphoid depletion in affected shrimp – H&E 40x

Taura Syndrome (TS) or Red tail disease

It is caused by a non-enveloped, linear, ssRNA virus called Taura syndrome virus belonging to the family *Dicistroviridae*. This disease is widely distributed in the shrimp-farming regions of the Americas and South-East Asia. Both horizontal and vertical transmission is reported. During the preacute/acute phase of infection, shrimp appear pale red while their tail fans become bright red. They are soft shelled, lethargic and anorexic. Those with severe infections die during moult and cumulative mortalities may reach 80-95%. Recovering, chronically infected shrimp generally display multifocal, melanized cuticular lesions and may also have soft cuticles and red body coloration with normal feeding. Microscopically, Feulgen-negative inclusion bodies, which may first appear eosinophilic then change to basophilic, observed in the cytoplasm of cells in areas of necrosis. Diagnosis of TS in acute stages of the disease requires histological (H&E stain preparations) demonstration of multi-focal areas of necrosis in the cuticular epithelium of the general body surface, appendages, gills, hind-gut, esophagus and stomach. Sub-cuticular connective tissue and striated muscle fibres basal or adjacent to affected cuticular epithelium may also show signs of necrosis. Rarely, the antennal gland tubule epithelium is affected. Cuticular lesions may contain foci of cells with abnormally eosinophilic (pink-staining) cytoplasm and pyknotic (condensed nucleoplasm) or karyorrhectic (fragmented nucleoplasm) nuclei. Remnants of necrotic cells are often abundant within acute phase lesions and appear as roughly spherical bodies (1-20 µm diameter) that range in stain uptake from eosinophilic to lightly basophilic (blue-staining). Another feature of acute TS is the absence of haemocyte infiltration, or other signs of a host defence response. These features combine to give acute phase TS lesions a "peppered" appearance that is considered to be diagnostic for the disease, and can be considered confirmatory in susceptible species in enzootic waters. Confirmation by another technique is recommended for first time observations of these histopathological features, or their appearance in abnormal penaeid species or locations. In the transitional phase of TS, the number and severity of the cuticular lesions that characterize acute phase infections decrease and affected tissues become infiltrated by haemocytes. These may become melanized. If the acute cuticular lesions perforate the epicuticle, the affected surfaces may show evidence of colonization and invasion by Vibrio spp, or other secondary infections. In the chronic phase of TS, the only sign of infection is the presence of prominent lymphoid organ spheres (LOS), which correspond to aggregations of presumed hemocytes within the intertubular spaces of the lymphoid organ.

Yellow head disease (YHD)

Yellow-head disease is first in cultured *P. monodon* adults in central Thailand during 1991. Yellow head virus (genotype 1) is one of six known genotypes in the yellow head complex of viruses and is the only known agent of YHD. Gill-associated virus (GAV) is designated as genotype 2. GAV and four other known genotypes in the complex (genotypes 3–6) occur commonly in healthy *P. monodon* in East Africa, Asia and Australia and are rarely or never associated with disease. It is an ssRNA virus of genus *Okavirus*, family *Roniviridae* of the order Nidovirales. Contaminated water, cannibalism of weak or moribund shrimp, animate vector, net and other equipment transmit the disease. Vertical transmission has not been reported. Shrimp with YHD display yellow coloration of the dorsal cephalothorax caused by the underlying yellow hepatopancreas showing through the translucent carapace. Within the ponds, infected animals, usually between 5 and 15 g, begin consuming feed at an abnormally high rate for several days then cease feeding entirely. One day after cessation of feeding, moribund shrimp may be seen swimming slowly near the edges of the pond. By the third day, mass mortality occurs and the entire crop is typically lost. Histologically, moribund shrimp suffering YHD usually have extensive abnormalities in the lymphoid organ (Oka organ), hepatopancreatic interstitial cells (not tubule epithelial cells), heart, midgut muscle and connective tissue (but not epithelial cells), stomach sub-cuticulum and gill tissues. These include foci of necrotic cells which resemble degenerated tubules with occluded lumens and contain cells with hypertrophied pyknotic nuclei, large vacuoles and cytoplasmic basophilic Feulgen-positive inclusions. Similar inclusions may also be found in the interstitial tissues of the hepatopancreas, connective tissues underlying the midgut, cardiac tissues, haematopoietic tissues, haemocytes and gill tissues. Moribund shrimp show systemic necrosis of gill and stomach sub-cuticular cells, with intense basophilic cytoplasmic inclusions (H&E staining) due to phagocytosed nuclei and viral inclusions. In the lymphoid organ, high numbers of karyorrhexic and pyknotic basophilic inclusions are found in matrix cells of the normal tubules. On the other hand, similar inclusions are found only in lymphoid organ spheroids with Rhabdovirus of Penaeid Shrimp (RPS) described from Hawaii and Lymphoidal Parvolike Virus (LPV, LOV) described from Australia; Lymphoid Organ Vacuolisation Virus (LOVV) in P. vannamei in Hawaii and the Americas; and Taura Syndrome Virus (TSV) in *P. vannamei*, *P. stylirostris* and *P. setiferus* from central and south America. Gill Associated Virus (GAV) in Australian *P. monodon*; a Yellow-Head-Disease-Like Virus (YHDLV) in *P. japonicus* from Taiwan province of China produce similar histopathology to YHV.

Spherical Baculovirosis (*Penaeus monodon*-type baculovirus; MBV)

MBV is associated with high mortalities in shrimp hatchery infecting all life stages except eggs and nauplii of *P. monodon*, caused by Type A baculo dsDNA virus first reported in laboratory reared *P. monodon* population in 1977, Taiwan during 1983 and in India during 1995. It infects other penaeid species also. But the virus does not infect *Litopenaeus vannamei*, *P. stylirostris* and *P. californiensis*. It is transmitted by ingestion of free virus and occlusion bodies, and by cannibalism. It is also believed to be transmitted vertically from broodstock to offspring, but not yet been proven. It is severe in PLs with over 90 % cumulative mortalities and senescent in adults. Clinically, sudden onset of mortality in the early life stages reduced feeding and growth rates, and an increase in gill and surface fouling. Severely infected prawns may display a whitish hepatopancreas and midgut since the virus is strictly enteric infecting epithelial cells of the hepatopancreas tubules and the anterior midgut. Persistent infection occurs commonly in penaeid hosts of MBV. Wet mount microscopical examination reveals large, single or multiple, roughly spherical, eosinophilic, polyhedral intranuclear occlusion bodies (OB's) in squash preparations of hepatopancreas and feces when stained with 0.05 % aqueous malachite green.

Tetrahedral Baculovirosis (Baculovirus penaei)

Tetrahedral Baculovirosis is caused by Baculovirus penaei (BP) belonging to a tentative species in the genus *Nucleopolyherdovirus*. All penaeid species and all life stages, except eggs and nauplii may be potential hosts. The larval stages (specifically protozoea and mysis) and early PL stages are the most easily infected in laboratory challenge studies and are the stages where the highest mortalities are likely to occur in penaeid shrimp hatcheries. High mortality rates are unusual as a consequence of BP infection in the juvenile or adult stages, but infection may cause poor growth and reduced survival in nursery or grow-out ponds at shrimp farms. Wet mounts of fresh tissue shows single or multiple tetrahedral occlusion bodies in epithelial cell nuclei in squash preparations of hepatopancreas or midgut examined by phase-contrast or bright-field microscopy. Occlusion bodies are tetrahedral or pyramidal in three-dimensional form, and range in size from less than 0.1 µm to nearly 20 µm from pyramidal base to peak, with a modal, vertical length of 8 µm. In some publications, the occlusion bodies of BP are referred to as PIBs (polyhedral inclusion bodies). Histopathology demonstrates pathognomonic (for BP) tetrahedral occlusion bodies in hepatopancreatocytes, gut epithelial cells, or gut lumen.

Hepatopancreatic parvovirus (HPV) disease

Hepatopancreatic parvo virus is an ssDNA virus. High levels of HPV infection occurs in early juvenile stages result into slow growth which finally stops to grow at approximately 6 cm in length. Horizontal transmission occurs by cannibalism and vertical transmission by infected broodstock. Microscopically, single prominent basophilic intranuclear inclusion bodies in the hypertrophied hepatopancreatic epithelial cells are seen.

Monodon slow growth syndrome (MSGS)

MSGS is a condition of *P. monodon* cultivation ponds characterized by abnormally slow growth and coefficients of size variation greater than 35%. It is assumed to be caused by Laem-Singh virus (LSNV). It is a positive-sense ssRNA virus measuring 25 to 30 nm, first identified in Laem Singh Province in Thailand during 2003. The virus is observed in the lymphoid organ, heart and other tissues of the affected shrimp. The major lesion observed is retinopathy. Retinopathy comprises abnormally enlarged haemolymphatic vessels, haemocytic infiltration and rupture of the membrane that separates the fasciculated zone from the overlying row of retinular cells. By transmission electron microscopy (TEM) and *In situ* hybridization (ISH), LSNV is detected in the fasciculated zone and in onion bodies of the organ of Bellonci in the affected shrimp. It suggests that retinopathy associated with LSNV may be linked causally to stunting of *P. monodon* in MSGS.

Loose shell syndrome (LSS)

LSS has been reported in India from farmed black tiger shrimp *P. monodon* since 1998 and is recognized as a major disease problem next to WSD causing significant economic loss. It is caused by a filterable infectious agent. It causes low-level progressive mortalities unlike the rapid mortalities associated with viral pathogens such as WSSV and

YHV. The clinical signs include a flaccid spongy abdomen due to muscular dystrophy, space between the exoskeleton and muscle, and shrunken hepatopancreas with reduced feed conversion efficiency and poor meat quality. Histologically, LSS-affected shrimp shows shrinkage of extensor and flexor muscles with occasional haemocytic infiltration, inflammation of hepatopancreatic tubules with enlargement of intertubular spaces and haemocytic infiltration, and low levels of lipid reserves in the R cells. In advanced stages, many tubules are in highly necrotic condition with a sloughed epithelium, reflecting the dysfunction of the digestive gland.

Bacterial diseases

Necrotising hepatopancreatitis (NHP)

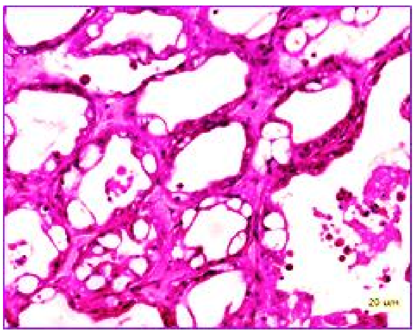
NHP is caused by a bacterium that is relatively small, highly pleomorphic, Gram negative, and an apparent obligate intracellular alpha-proteobacterium. The NHP bacterium has two morphologically different forms such as a small pleomorphic rod without flagella and a longer helical rod possessing eight flagella on the basal apex of the bacterrium, and an additional flagellum (or possibly two) on the crest of the helix. The NHP bacterium occupies a new genus in the alpha *Proteobacteria*, and is closely related to other bacterial endosymbionts of protozoans. NHP is also known as Texas necrotizing hepatopancreatitis (TNHP), Texas pond mortality syndrome (TPMS) and Peru necrotizing hepatopancreatitis (PNHP). NHP is characterised by an atrophied hepatopancreas showing moderate to extreme atrophy of the tubule mucosa and the presence of the bacterial forms through histological preparations. Principal histopathological changes due to NHP include hemocytic inflammation of the intertubular spaces in response to necrosis, cytolysis, and sloughing of hepatopancreas tubule epithelial cells. The hepatopancreas tubule epithelium is markedly atropied, resulting in the formation of large edematous (fluid filled or "watery") areas in the hepatopancreas. Tubule epithelial cells within granulomatous lesions are typically atrophied and reduced from simple columnar to cuboidal in morphology. They contain little or no stored lipid vacuoles and markedly reduced or no secretory vacuoles.

Bacterial white spot syndrome (BWSS)

The bacterium *Bacillus subtilis* has been suggested as the possible causative agent due to its association with the white spots but no causal relationship has been demonstrated, nor have infectivity studies been conducted. *Vibrio cholerae* is also often isolated in significant numbers and similar white spots have been described in farmed shrimp in Thailand as a result of exposure to high pH and alkalinity in ponds in the absence of the WSSV or bacterial colonisation of the spots, indicating that the bacterial involvement may be secondary. The lack of certainty as to the causative agent and the possibility of secondary involvement of bacteria need to be addressed through further research. Until the bacterial etiology is clearly demonstrated, bacteria cannot be definitively regarded as the causative agent. Histological examinations should be conducted to ensure that the soft-tissues associated with the cuticular lesions do not show signs of the WSD characteristic endodermal and mesodermal intranuclear inclusion bodies. In the case of BWSS, bacteria will be the primary microbial foreign particle and this should be in primary association with the cuticular lesions themselves.

Vibriosis

It is a serious problem in shrimp hatcheries, intensively raised farm and pond culture stocks especially before the juvenile stage. Higher salinity, increased ammonia level in the culture environment, low dissolved oxygen, rise in temperature and higher stocking density are found to be predisposing causes for Vibriosis. The identified and reported pathogenic *Vibrio* spp. for shrimp and fish are *Vibrio harveyi, V. alginolyticus, V. parahaemolyticus, V. anguillarum, V. mimicus, V. fluvialis, V. splendidus, V. penaeicida, V. campbellii, V. carchariae, V. cholerae, V. damsela, V. ordalii,* and *V. Vulnificus*. Characteristic luminescence is seen in severe *Vibrio* infection. Symptomatically the disease is associated with melanized cuticular lesions of the appendages and melanized nodules in the gills and other organs. Other major clinical signs such as disoriented shrimp swimming weakly, gathering along the edges of the pond, cloudiness of the musculature, red discoloration of the appendages and dorsal flexure at the third abdominal segment with slight rigidity. Fish eating birds gather and feed on the weakened shrimp. Haemolymph from moribund shrimp will fail to clot or will clot very slowly. Histopathological investigation of moribund shrimp reveals the presence of pathogens in different tissues and rounding and sloughing of tubular epithelial cells into the lumen.



Sloughing of tubular epithelial cells in affected shrimp – H&E 40x

Fouling/black gill disease

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

Milky hemolymph syndrome (MHS)

Black tiger shrimp *P. monodon,* European shore crab *Carcinus maenas* and spiny lobster *Panulirus* spp. can be affected by milky hemolymph syndrome (MHS). Four rickettsia-like bacteria (RLB) isolates of MHS originating from five geographical areas have been identified to date. Mesodermally derived tissues are the primary target tissues for RLB in MHS. In shrimp, the first and most severely affected organ appears to be the lymphoid organ (LO), followed by connective tissue elements of most other tissues (e.g. the capsule of the hepatopancreas, the serosal layers of the foregut, midgut, midgut caeca and heart), circulating hemocytes and fixed phagocytes in the hemocoel of the heart and gills. The hemolymph of severely affected shrimp visible in the hemocoel within the LO, HP, gills, heart and other tissues stains bluish with H&E due to the presence of large amounts of RLB.

Fungal diseases

Larval mycosis

Larval mycosis is the disease of penaeid hatcheries. The fungus *Lagenidium* and *Sirolpidium* are the responsible for larval mycosis. *Lagenidium* infections often occur in nauplial and protozoeal stages while the *Sirolpidium* disease is usually in the late protozoeal to mysis stage. Juvenile or older shrimp are not susceptible to fatal infection by the phycomycetes fungi, presumably because of thicker exoskeleton. Diagnosis of larval mycosis is based on microscopic demonstration of characteristic fungal mycelia that fills the body cavity of dying or dead infected larvae. The fungus can be grown on standard fungal media such as Sabouraud dextrose agar.

Parasitic diseases

Protozoan infection

Endocommensal/Invasive protozoa

It affects muscle and causes **"milk or cotton shrimp disease"**. Shrimp are infected by ingestion of spores. 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. The spores extrude a filament that penetrates the gut wall and deposits an infective unit which enters nucleus of the gut cells where schizonts are developed. The schizonts divide and develop to form spores which are located in muscles and other organs of the shrimp. The infected shrimp is infective to fish species and vice versa but not to the shrimp itself. The major genus under the order, Microsporidia are *Pleistophora* sp. (>8 spores/envelope), *Thelohania* sp. and *Agmasoma* sp. (8 spores/envelope). *Enterocytozoon hepatopenaei* (EHP) is a microsporidian parasite that was first characterized and named from the giant or black tiger shrimp *Penaeus monodon* from Thailand in 2009. Hepatopancreatic microsporidiosis (HPM) was discovered in slow growing shrimp and the EHP is confined to the shrimp hepatopancreas (HP). Although EHP does not appear to cause mortality, but it causes slow and stunted growth with or without white faecal syndrome. Microscopic pathological lesions include the presence of basophilic, cytoplasmic inclusions (microsporidian sporocysts) containing clusters of elliptical to somewhat ovoid spores of 1.1 ± 0.21μm by 0.6–0.7 ± 0.1μm in the hepatopancreas tubule epithelial cells. Sometimes free spores released from lysed cells may be seen in the tubule lumens. Order, Haplosporida affects the digestive glands of shrimp but the incidence is rare. The gross signs of disease included shrinkage of the hepatopancreas, flaccid bodies, cuticular melanization and retarded growth. Order, Gregarina affects the digestive tracts and other tissues of shrimp. Shrimp are infected by ingestion of spores. The developed sporozoites attach to the gut wall, grows in to trophozoites and form gametocysts. Gametocysts undergo multiple divisions to produce gymnospores which are released outside and be 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 shrimp. The major genus involved are *Nematopsis* sp., *Cephalobus* sp. and *Paraophioidina* sp. Several protozoa invade the body and feed on tissues of weakened and diseased shrimp are called body invaders. They are *Parauronema* sp., *Leptomonas* sp and *Paranophrys* sp. and amoeba.

Ecto-commensal protozoa

They are found on the gill and body surface of the shrimp. They are *Zoothamnium* sp., *Epistylis* sp., *Acineta* sp., *Lagenophrys* sp., *Ephelota* sp., and *Apostome* ciliates. Special staining technique like silver impregnation staining is used for identification of *Apostome* ciliates. They cause **"protozoan fouling"** and **"Fuzzy mat-like appearance"** due to ciliate fouling. The affected shrimp show restless and difficulty in locomotion and respiration.

Metazoan parasites/Helminthiasis

Metazoan parasites in shrimp are categorized as Trematodes, Cestodes, and Nematodes. Immature forms and adult worms are found in the different parts of the body in shrimp.

Trematodes (flukes)

The cercarial forms of the flukes are infective to the shrimp. The cercaria penetrates 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. Eggs are hatched out and miracidia released which penetrate second invertebrate host, snail and develops in to sporocysts. Cercariae develop inside the sporocysts in second intermediate host and released in to water which become infective stage for shrimp. Eg. *Opecoeloides fimbriatus*, *Microphallidae* sp. and *Echinostomatidae* sp.

Cestodes (tape worms)

Shrimp ingest copepods or other crustaceans with larval form of tape worm which develop in to 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 shrimp. Eg. *Prochristianella penaei*, *Parachristianella* sp., *Renibulbus* sp., Pear shaped worms, and Cyclophyllidean group.

Nematodes (round worms)

Shrimp ingest copepods or other crustaceans with larvae which develop in to advanced larval stage in shrimp. The advanced larval stage enters the first intermediate host (toad fish) 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 shrimp. Eg. *Spirocumallanus pereirai*, *Leptolaimus* sp., *Ascaropsis* sp., and *Hysterothylactum reliquens*.

Other infestations

Single cell plant diatoms (on larvae), Over growth of algae of mixed variety, barnacle, leeches, colonial hydriod *Obelia bicuspidata*, insects eggs, Isopods -*Aega* sp. are occasionally observed among wild and poorly farmed shrimp populations. Bopyrid parasitic infestation caused by *Epipenaeon* spp. belonging to family *Bopyridae* which are lodged in the brachial cavity leads to impaired respiration and reproductive failures.

Conclusion

Experience and interest in disease diagnosis could get better results and can predict the disease outbreak well in advance. But more often difficult problems emerge to replace the solved problems of yesterday. The farm history and sample collection play vital role in diagnosis of diseases in aquaculture. With early diagnosis and adopting better management practices (BMPs), there are many reports of successful harvest even in the presence of dreadful disease causing pathogen like WSSV. Histopathology is an important tool for an initial understanding of the particular disease incidence. It becomes a confirmatory diagnosis for diseases along with molecular diagnostics.

Histopathology of fish disease diagnosis

Introduction

Brackishwater aquaculture plays a vital role in national food security, employment generation, significant seafood export earnings and proper utilization of coastal waste lands. The commercially important species for brackishwater aquaculture are penaeid shrimp (*Penaeus monodon* and *Litopenaeus vannamei*), crabs (*Scylla* spp.) and finfishes such as Asian seabass (*Lates calcarifer*), grey mullet (*Mugil cephalus*), milk fish (*Chanos chanos*) and pearl spot (*Etroplus suratensis*). In India brackishwater finfish production is still in its infancy with limited species under mono and polyculture systems. Seed production still remains a bottleneck in expanding mariculture activities to industrial level. However, limited culture of marine fish has taken up after the successful of breeding and hatchery technology for seed production of Asian seabass (*Lates calcarifer*) for the first time in India in 1997 (Thirunavukkarasu *et al*., 2001). This was followed by the improved hatchery technology by Central Institute of Brackishwater Aquaculture (Chennai, India) for seabass and Rajiv Gandhi Centre for Aquaculture, Sirkazhi (India) for multiple species like seabass, grouper etc. which enabled large scale fry production under controlled conditions. As in any animal production systems, infectious diseases are recognized as one of the major constraints for sustainable aquaculture. Broadly, infectious diseases of finfish are caused by virus, bacteria, fungi and parasites. Environmental stresses, genetic factors and nutritional deficiencies further make the animals more susceptible to these pathogens. A brief description of finfish diseases has been presented here with technical guidelines for health management. Environmental stresses, genetic factors, and nutritional deficiencies further make the animals more susceptible to these pathogens.

Diseases of finfish

The culture of commercially important brackishwater finfishes started with the success of captive breeding and standardization of larval rearing technology. Brackish water finfishes are susceptible to various pathogens of viral, bacterial and parasitic origin. Among these, VNN, Iridovirus infections, Thialpia lake virus, vibriosis and various parasitic infections are the major finfish diseases.

Viral diseases in fish

Viral nervous necrosis

Viral nervous necrosis (VNN) is a devastating disease of marine fish species cultured worldwide. It affects more than 50 wild and cultured marine fish species, especially the larval and juvenile stages which records high mortality. Based on characteristic lesions, VNN is also known as viral encephalopathy and retinopathy (VER).

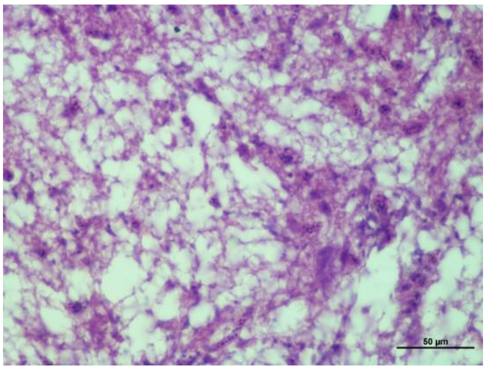
Etiology: The disease is caused by piscine nodavirus of the genus betanodavirus, family Nodaviridae. The virus contains single stranded, bipartite positive sense RNA genome.

Affected species: *Lates calcarifer, Mugil cephalus, Chanoschanos* and *Epinephelus tauvina* etc.

Mode of transmission: Nodaviruses are regarded as pathogens of marine fishes. However, natural development of disease has also been reported from low saline and freshwater environments. Translocation of species for stocking purpose from one location to another may be another way but it is yet to be validated. Latent infection among wild fishes also serves as source of infection. Apart from all these means of horizontal transmission, vertical transmission is highly suspected to take place from infected spawners to fry.

Symptoms and lesions: The major clinical signs of VNN are common behavioral changes such as lack of appetite, erratic, spiral or belly-up swimming and dark coloration of body. Clinically, the affected animals show spiral or looping swim pattern, swim bladder hyperinflation and later wasting. Vacuolation is seen in the grey matter of brain and retina of eye. Necrosis is observed in the spinal cord, brain and retina while intracytoplasmic inclusion in nervous cells. The severity of disease is more in juveniles with equal higher rate of mortality. In seabass, the earliest onset of clinical signs of the disease is during 16-21 day post hatch. Recently, the disease has been noticed in fishes irrespective of their age. Instances of asymptomatic/ sub-clinical infection among wild fishes may possibly act as potential carriers.

Diagnosis: Viral nervous necrosis can be diagnosed by demonstrating characteristic lesions in the brain and/or retina by light microscopy. Detection of virions by electron microscopy, viral antigens or antibodies by serological methods such as indirect fluorescent antibody test, and enzyme linked immunosorbent assay or detection of viral nucleotides by molecular techniques such as RT-PCR and nested PCR and by tissue culture are different ways for pathogen detection. The RT-PCR is the most sensitive test and has become the main diagnostic method for fish nodaviruses. The capsid protein gene is the target sequence for this test. More recently, nested PCR was reported to be 10-100 folds more sensitive than the RT- PCR and permitted diagnosis by using blood, sperm, as well as nervous and ovarian tissues.



Prevention and control: Against VNN, no vaccine or treatment method is available. Broodstock fish acts as the most important source of the virus to their larvae by vertical transmission. Therefore, after RT-PCR screening, virus-carrying broodstock should be eliminated. Fertilized eggs should be disinfected with disinfectants such as iodine or ozone. Strict hygiene within the hatchery should also be maintained by regularly disinfecting the hatchery facility and farm materials with chlorine. Separate rearing facility of larvae /juveniles from brooder should be maintained with each batch of larvae/ juveniles in the separate tanks supplied with sterilized (UV or ozone) seawater.

Iridovirus infection

Iridovirus infection causes significant mortality in cultured brackishwater fishes. It affects all the stages of fish, but the susceptibility of juveniles is generally higher than adults. The disease caused by double stranded DNA virus of genera *Lymphocystivirus* and *Ranavirus*. Affected fish become lethargic and exhibit severe anaemia, petechiae on the gills, and enlargement of the spleen. The major characteristic microscopic lesion is the presence of basophilic hypertrophied cells in large numbers in various organs like spleen, heart, kidney, intestine, and gills. Iridovirus infection is a significant cause of mortality in farmed red sea bream *(Pagrus major)* and more than 30 other species of cultured marine fish. The infection has also been detected in Asian seabass. It affects all the stages of fish but the susceptibility of juveniles is generally higher than adults.

Etiology: The disease is caused by double stranded DNA virus of genera Lymphocystivirus and Ranavirus which causes systemic disease in infected fish and are associated with high morbidity and mortality.

Mode of transmission: The principal mode of transmission of Iridovirus infection is horizontal via water. The vertical mode of transmission has not yet been established.

Diagnosis: Outbreaks of the disease have been mostly reported in the summer season at water temperatures of 25°C and above. Depending on host fish species, fish age, water temperature, and other culture conditions, mortality rates ranges between 0 and 100%. Affected fish become lethargic, exhibit severe anaemia, petechiae of the gills, and enlargement of the spleen.

The sample should be collected from moribund fish. Gill and visceral organs such as spleen, heart, kidney, liver and intestine should be collected. However, the spleen and/or kidney tissues are the most appropriate organ for pathogen detection by IFAT. The fish sample should be stored at 4°C for use within 24 hours or -80°C for longer periods. The disease is characterised by the appearance of abnormally enlarged cells stained deeply with Giemsa solution in the histopathological observations of the spleen, heart, kidney, intestine and gill of infected fish. These enlarged cells react to an anti -RSIV MAb by the antibody based antigen detection (Indirect fluorescent antibody test-IFAT) test. Electron microscopy confirms the presence of virions (200-240 nm in diameter) in these cells.

PCR is able to detect Iridovirus infection with high degree of sensitivity in short time. Recently real time PCR has been developed which shown improved rapidity, sensitivity, reproducibility, and the reduced risk of carry over contamination over normal PCR. An antibody based enzyme-linked immunosorbent assay (ELISA) has also been developed to detect iridovirus infection.

Prevention and control: An effective and commercially available formalin-killed vaccine is available against Red Sea Bream Iridovirus infection (RSIVD). A number of general husbandry practices are used to reduce RSIVD associated losses. These includes introducing pathogen free fish, implementing hygienic practices on farms and avoiding practices that can decrease water quality and/or increase stress, such as overcrowding and overfeeding.

Tilapia lake virus (TiLV)

Mortalities attributed to infection with TiLV have been observed in wild tilapia Sarotherodon (Tilapia) galilaeus, farmed tilapia *Oreochromis niloticus* and commercial hybrid tilapia (Bacharach et al., 2016; Ferguson et al., 2014; Eyngor et al., 2014). Experimental infections with TilV by injection and co-habitation resulted in mortalities in the giant gourami (*Osphronemus goramy*) (Jaemwimol et al., 2018).

Routes of transmission (horizontal, vertical, indirect): Direct horizontal transmission is an important route of transmission. Detection of the virus in the gonads of breeders and detection of virus in fry at 2, 5- and 10-days post-hatching suggest possible vertical transmission of TiLV (Yamkasem et al., 2019). The biophysical characteristics of the virus are not well characterized, so it is difficult to determine the significance of indirect transmission by fomites. Host tissues and infected organs: The main organs where pathology is observed are the eyes, brain and liver (Eyngor et al., 2014).

Gross observations and macroscopic lesions: Gross lesions included ocular alterations, including opacity of the lens and in advanced cases ruptured lens. Other lesions included skin erosions, haemorrhages in the leptomeninges and congestion of the spleen (Eyngor *et al.*, 2014).

Microscopic lesions and tissue abnormality: Histologic lesions have been observed in the brain, eye and liver (Eyngor *et al.*, 2014). Lesions in the brain included oedema, focal haemorrhages in the leptomeninges, and capillary congestion in both the white and grey matter and neural degeneration. Foci of gliosis and occasional perivascular cuffs of lymphocytes have been detected. Ocular lesions included ruptured lenticular capsule and cataractous changes. Foci of hepatocellular swelling were observed. The spleen was hyperplastic, with proliferating lymphocytes. Melanomacrophage centres (MMCs) were increased in size and number in both the liver and the spleen. Transmission electron microscopy confirmed the presence of an orthomyxo-like virus within diseased hepatocytes and thus confirmed earlier reports of syncytial hepatitis (del-Pozo *et al.*, 2016).

Control methods: Restrictions on the movement of live tilapines from farms and fisheries where the virus is known to occur will limit the spread of the disease. Generic biosecurity measures to minimise fomite spread via equipment, vehicles or staff (i.e. cleaning and disinfection) should also be implemented. Common disinfectants are effective against TiLV provided usage conditions are adhered to (Jaemwimol et al., 2019). Appropriate disinfection protocols should be incorporated into biosecurity protocols.

Bacterial diseases in fish

Bacterial diseases occur as a result of the complex interactions between pathogen, fish and environmental stress. Environmental stresses can affect the homeostatic mechanism of fish thus reducing their resistance to disease-causing organisms. Fish reared in intensive culture conditions are exposed to extreme environmental fluctuations and they may be more sensitive to disease than that in wild populations.

***Aeromonas* sp.**

Aeromonas sp. is a common water-borne bacterium normally present in fishes. Whenever fishes are exposed to environmental stress or injury, it causes serious outbreaks of haemorrhagic disease with high mortality. Grossly, hemorrhages on the fin and tail and erosion of tail and fin can be clearly seen in severe cases. Temperature, pH, high CO_2, O_2 depletion, decomposition of products, free NH_3, over-crowding and low salinity are considered as predisposing causes for *Aeromonas* infection.

Vibriosis

It is caused by pathogenic *Vibrio* sp. like *Vibrio parahaemolyticus, V. anguillarum,* and *V. alginolyticus*. Generally, fingerlings are more susceptible than an adult. Most of the infected fish display clinical signs like darkened body colouration, loss of appetite and exihibit abnormal swimming behaviour with head floating near the surface of the water. Severely infected fish exhibit symptoms such as skin ulceration, hemorrhage at fin base and the body surface, rotted fins and tails and abdominal distension. The tissue surrounding the vent appears reddened and inflamed. Internally, the liver is pale and with petechiae, and the kidneys and spleen are congested. In most severely infected fish, the accumulation of reddish ascetic fluid in the peritoneal cavity noticed.

Members of the genus *Vibrio* and other related genera are the causative agents of vibriosis, a deadly haemorrhagic septicemia disease in a diverse range of marine, estuarine and freshwater fish species. The typical symptom of vibrio disease includes extensive cutaneous and systemic hemorrhages and localized ulcerations in mandible, eyes, isthmus, bases, rays of dorsal, pectoral, pelvic, anal and caudal fins and ecchymosis and petechiae on the body surface and frequently, ulceration of the skin and muscle tissue are noted. The affected fish are anorectic. Internally, there is congestion and haemorrhages in the liver, spleen and kidney, frequently accompanied by the presence of necrotic lesions. The gut and particularly the rectum may be distended and filled with a clear viscous fluid. The body is completely covered by a thick layer of mucus. Microscopically, extensive lamellar hyperplasty, honey-comb vacuolization and fatty degeneration in hepatocytes, and aggregation of melano-macrophage centres (MMC) in kidneys and spleen are observed. The degree of the lesions varies with the severity of the disease. Fingerlings die more rapidly than adults.

Treatment

It is advisable to know the quality of the water including pH and temperature which greatly affect treatment results. A few fishes are first treated and checked for how they react before treating the entire group. The total volume of water in the pond must be known to treat it accurately. To determine the volume of a pond, the surface unit area of water is multiplied by the average depth of the pond. Then, the approved and calculated therapeutics is evenly administered all over the pond.

Bacterial diseases are treated best by injection or use of food additives or antibiotics. Terramycin 2.5-3.0 g per 100 lb body weight (BW) per day for 10 to 12 days with a withdrawal period of 21 days, Sulfamerazine, Nitrofurans and Furazin 109 per 100 lb BW per day for 10 days, Erythromycin 4.5 g per 100 lb BW per day for two weeks are used as per the requirements. Potassium permanganate ($KMn0_4$) is used at the rate of 2-3 ppm as a wide-spectrum treatment in ponds against bacterial infections.

Fungal diseases in fish

Epizootic ulcerative syndrome

The disease is reported in freshwater as well as brackishwater fishes throughout India. It is caused by non-septate fungus *Aphanomyces invadans* and is invariably associated with secondary infection by gram negative bacteria and rhabdoviruses. The transmission of disease occurs through zoospores which are transmitted through water, direct contact between fishes, and transport of infected fishes into new area.

The disease is diagnosed by sudden high mortality rate in wild and farmed fish with different types of fishes gets affected and show abnormal behaviour. Hemorrhagic ulcers are seen on head which often extend to skull leading to exposure of brain. Confirmatory diagnosis of disease is done by detection of non-septate, branching fungal hyphae in the periphery of the lesion. Invasive fungus extends through muscle tissue to spinal cord, kidney, and peritoneum. No vaccine is available for the control of disease. Controlled feeding regime should be operated during the outbreak of disease as a means of preventive measures. The affected fish should be destroyed and all farm appliances should be disinfected. The affected pond should be dried followed by liming application before restocking.

Parasitic diseases of fish

The major groups of parasites in brackishwater fishes are (i) protozoans and (ii) metazoans. Based on the location on the host fish it can be also categorised either ecto or endoparasites.

Protozoan parasites

Protozoans are single celled microscopic organism with specialized structures for movement, food gathering and attachment. They are either external or internal parasites. The following are the major protozoan parasites in fishes.

Dinoflagellates

Amyloodinium is a common dinoflagellate found in fishes particularly young fishes. These are microscopic external parasites with flagella for movement. It is usually attached with gill filaments or body surface of the affected fish. Predisposing factors are high level of organic matter in water and higher stocking density of fish. The gill and skin of affected fish shows signs of necrosis and destruction. Darkening of body surface is observed. Affected fish often found on the surface or near the source of aeration. Mass mortality of fish is observed in severely affected pond, if not treated in time. Short formalin bath treatment (200 ppm) for 1hr and copper sulphate bath (0.5 ppm) for 3-5 days with aeration and daily water replenishment.

Ciliates

Ciliates are microscopic external parasites with cilia for movement. The most common ciliates are *Cryptocaryon* and *Trichodina. Cryptocaryon* is a pear shaped (0.3-0.5 mm in size) parasite. The parasites are found on the external body surface of fish. High stocking density and low water temperature serve as predisposing factor for infection. It affects mainly gills and body surface. The affected part has increased mucus production. The white spots are observed over the body surface. Wound often has the complication of secondary bacterial infection. Affected fish are found to rub body against submerged objects. Respiratory distress with mass mortality is seen in untreated cases. Copper sulphate bath with 25 ppm or/and formalin bath with 0.5 ppm for 5-7 days with aeration and daily replenishment of water. *Trichodina* has a circular body with cilia around the perimeter. It infects mainly gills, body surface and fins. High level of organic matter in water or poor water exchange serves as predisposing factors. Affected fish has pale gills with irritation on body and hence rubs body against objects. Excessive mucus production is observed on gills and body surface. This leads to respiratory distress by clogging of gills by mucus. 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.

Myxozoans

Myxozoans are microscopic internal parasites. The parasites either infect tissue of various organs or live freely in cavities like gall bladder, abdominal cavity, etc. Poor water quality, high stocking density, feeding with infected trash fish and lack of quarantine measures facilitate infection. Common examples of myxozoans are *Myxobolus, Myxidium, Kudoa, Ceratomyxa* etc. Clinical symptoms 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. No effective treatment is available. Preventive steps should be taken by having efficient water exchange, avoiding feeding of trash fish and by quarantine measures.

Microsporidian

Microsporidians are intracellular internal parasites. It forms spore and resembles myxozoans. Poor water quality and poor nutrition is the predisposing factor. No visible symptoms of infection are observed. Cysts are observed in various internal organs like intestinal wall, ovary, fat tissue etc. These cysts are brown or black in color and are of various size and shape called xenoma. No treatment is available. The disease can be prevented by good water exchange.

Metazoan parasites

Metazoans parasites of fishes include mainly helminths of different classes, arthropods dominated by parasitic crustaceans and some annelids such as leeches. Among helminths, trematodes or flatworms are of significance in cultured fishes though nematodes and cestodes are found rarely as massive infections. Trematodes constitute mainly external parasites with adhesive structures for attachment to the host, These are two types. Monogeneans and Digeneans. Monogeneans are mostly external parasites with specialized posterior attachment organs. It needs single host for completing its life cycle and multiply very fast in confined water bodies. They include skin and gill flukes. Digeneans as a group are mostly intestinal parasites and involves more than one host for completing their life cycle. These are of variable size based on the species. No treatment method is suggested, although anthelmintic drugs are used. 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. Nematodes or roundworms are large intestinal parasites, with un-segmented body and mostly 1-2 cm length. Infection is prevented by maintaining hygienic conditions. Parasitic crustaceans include mainly copepods such as *Caligus* sp., *Ergasilus* sp., *Lernanthropus* sp. etc. and are commonly found on the gills, skin and fins. *Caligus* sp. is oval shaped parasite has four pair of legs and a pair of suckers over the frontal edge of the body. Poor water quality serves as predisposing factors. Affected fish had sluggish behaviour, shows anorexia and become weak due to heavy infestation. Gills and skin have erosions and secondary bacterial infection leading to high mortality, if not treated.

Health management strategies

Many brackishwater fishes can be cultured in a variety of aquaculture systems (open, semi-closed and closed) in freshwater, brackish and marine environments each of which present its own unique disease challenges. However, most of the losses due to microbial pathogens and parasites in aquaculture system can be prevented by adopting appropriate biosecurity protocols. These include routine health inspection, quarantine and treatment of wild-caught broodstock, egg disinfection, strict equipment sanitation, human traffic control, intake water treatment, discharge water treatment, clean feeds, restricting movement of stock, appropriate disposal of dead fishes and limiting interactions between wild and farmed fishes. Difficulties can arise once a disease is established and spread within a facility because eradication may be nearly impossible.

Following points to be considered for a better health management

- Animals should be obtained from disease free farms/sources after appropriate health testing and disease free certification; disinfect eggs or larvae where appropriate.
- Quarantine all animal introductions from rest of the farm for an appropriate period of time. The duration depends on species of aquatic animal and potential diseases.
- Seek advice on preventive treatments to all introduced animals while still in quarantine.
- Disinfection of eggs/larvae prior to use
- Remove sick and dying animals as soon as possible and treat in quarantine section or

dispose suitably so that there is no contamination of facility.

- Record all introductions and disposal of aquatic animals; source /destinations
- Record all outbreaks of diseases and death
- Measure and record growth rates
- Ensure the personnel are aware of the hazards of the chemicals used and trained on storage, use and disposal methods
- Comply with regulations on the use of chemicals in aquaculture
- Any unusual mortality with any of the symptoms
- Animals coming to the edge or water surface
- Unusual swimming patterns
- Reduced feeding and failure to thrive
- Unusual changes in physical appearance of the animal (redness, pale or black colouration, ulcers/mouldy growth on skin, pop-eye, erosions of fins and tails, fouling of gills etc.
- Isolate any animals showing signs of any diseases or ectoparasitic infestation
- Prevent movement of animal/vehicle/human from spreading disease
- Personnel should work on healthy animals first before they have contact with sick ones.
- Collect sick animals and attempt for diagnosis by laboratory examination.
- Advise destruction, disposal and disinfection methods for diseased and dead animals.
- Chemicals and therapeutics usage on advice

Prepare a contingency plan for any serious and infectious diseases like:

- A plan and ability to completely isolate each pond/tank to prevent transfer of diseases by water
- A capacity to disinfect an entire pond/tank/system
- A capacity and appropriate method to destroy, collect and dispose of large number of aquatic animals as per expert advice.

- Keeping records to allow tracing the origin and disposal of any affected group of animals.

Conclusions

Fish health management is a combination of prevention of onset of disease and measures to reduce losses from disease when it occurs. Health of the ecosystem that supports the fish supplies are critical. Effective management focuses on reducing stress, hygienic husbandry practices, environmental management, preventing introduction of pathogens by efficient biosecurity measures, and use of effective drugs and vaccines, if available. In view of continued reliance on wild brood stock and/or stocking enables vertical and horizontal pathogen transmission. Understanding the source and transmission of infectious pathogens can enable proactive management strategies. A fish health surveillance programme is a vital asset to keep the farm disease free and maximize efficacy of disease control strategies in the event of an outbreak. Thus it is again crucial to further our knowledge of the diseases in the context of host, pathogen and environment interactions as a basis for farm management strategies and prevention of diseases.

Recent advances in diagnostic histopathological techniques

Introduction

Advances have taken place in understanding the molecular biology of fish and shrimp pathogens and their hosts during last 15 years, and molecular biology has become a routine tool in the search for improved methods of diagnosis and control of fish and shrimp diseases and the epidemiology of bacterial, viral, and parasitical diseases. Detection of nucleic acid molecules has demonstrated its usefulness for highlighting hardly cultivable, non-cultivable, and even dead microorganisms, generating appropriate novel or replacement technologies. Molecular techniques can be used to solve that type of problems and increase sensitivity and specificity of pathogen detection. These techniques include probe hybridization, *in situ* hybridization, and microarray. Since molecular diagnostic techniques are faster and more sensitive than conventional diagnostic techniques, pathogens can be detected from asymptomatic fish, so disease outbreak could be prevented. Thus antibiotic treatment can be reduced therefore; creation of antibiotic resistant bacteria may be eliminated. A variety of labelling and detection systems exist for nucleic acid probes. The variety of labels and detection methods now available can provide a system suitable for any application, from dot blots to *in situ* hybridization. These include labelling with a variety of haptens such as biotin or digoxygenin and detection by antibody binding coupled with fluorescent, chemiluminescent or colorimetric detection methods.

In situ PCR

This technique has become a powerful molecular tool in research as well as clinical practice which has resulted in an increased understanding of infectious and neoplastic diseases and improvements in diagnosis of disease. *In situ* RT-PCR gives more detailed information by allowing highly sensitive detection of low abundance gene expression in a given cell while providing anatomical information. The usefulness of these techniques has been hampered by low detection sensitivity, poor reproducibility, and high backgrounds. Moreover, many of the methods used to visualize the results of PCR amplification within cells and tissues employ radioactive tracers, making performance of the techniques cumbersome and costly. Researchers have developed a method for specific fluorescent detection of gene expression using *in situ* RT-PCR. This method enables the researcher or clinician to detect low levels of gene expression within tissues with very low background interference while addressing many of the other existing drawbacks to using in situ RT-PCR.

In situ hybridization (ISH)

In situ hybridization (ISH) is another important tool to determine the location of pathogen nucleic acid in tissue sections of PCR positive animals. It can be done using digoxigenin-labeled (DIG-labeled) probes for the small subunit ribosomal RNA (SSU rRNA) or for the gene. The gene probe was labelled with digoxigenin-11-deoxyuridine triphosphate (dUTP) in a PCR reaction according to the manufacturer's instructions. Following PCR, the digoxigenin-labelled DNA probe was prepared stored at 20°C. The Davidson's fixed shrimp tissue were processed, embedded in paraffin, and sectioned (4 µm thick), deparaffinization, hydrated, proteinase K digestion, and post-fixation, sections were overlaid with hybridization solution containing the disease probe. Slides were placed on a heated surface at 90°C for 10 min and hybridized overnight in a humid chamber at 42°C. Final detection was performed with anti-digoxigenin antibody conjugated to alkaline phosphatase. The slides were counterstained with bismark brown and examined using a light microscope.

Fluorescence *In situ* hybridization (ISH)

Potential areas of application of this technique are detection and diagnosis of viruses and other infectious agents in specific cell types within tissues, detection, and characterization of tumor cells within a tissue, detection, and diagnosis of genetic mutations in inherited diseases. Fluorescence *in situ* hybridization, or FISH, is a method used to label cells or chromosomes according to the sequences of nucleic acids contained within them. The process works by taking fluorescently labelled pieces of DNA or RNA called probes that are around 20 nucleotides in length. The probes are incubated in the presence of cells under appropriate conditions to permit specific hybridization of probe to target nucleic acid. Cells types that contain ribosomes with complementary RNA sequences become labelled by the binding of the fluorescent probe in situ. These labelled cells can then be visualized by flow cytometric or fluorescence microscopy.

Transmission electron microscopy (TEM)

Electron microscopy has led to the discovery of many new viruses, for which it remained the principal diagnostic method until fairly recent times. Development of molecular techniques, which offer greater sensitivity and often the capacity to easily process large numbers of samples, has replaced EM in many areas of diagnostic virology. Hence the role of EM is evolving with less emphasis on diagnosis and more on research, although this is likely only to be undertaken in specialist centres. However, EM still offers tremendous advantages both in the speed of diagnosis and the potential for detecting, by a single test, any viral pathogen or even multiple pathogens present within a sample. TEM is an excellent tool for investigating the microscopic nature of defect. The limitations of transmission electron microscopy are that samples have to be mechanically and/or chemically thinned first to $\approx 0.15\ \mu m$ to allow the electron beam to traverse the sample, and these destructive preparation procedures usually provide only small areas suitable for examination. The sensitivity of cross-sectional transmission electron microscopy for dislocation observation is at best $\sim 10^5$-$10^6\ cm^{-2}$, which is similar to careful X-ray rocking curve measurements. *In-situ* TEM is a very powerful method for studying the defects and the strain-relaxation process. The distinguishing feature of transmission electron microscopy (TEM) is its ability to form images of atomic arrangements at localized regions within materials. It provides a view of the microstructure, that is, the variations in structure from one region to another, and the interfaces between them. TEM techniques are usually divided into two general categories, with a third one made up by combination of the two: Imaging techniques: conventional TEM (CTEM) imaging (in bright-field (BF) mode or dark-field (DF) mode); phase-contrast imaging (high-resolution TEM, HRTEM); STEM imaging (STEM BF, STEM DF, and *Z*-contrast imaging); electron tomography; electron holography (EH).

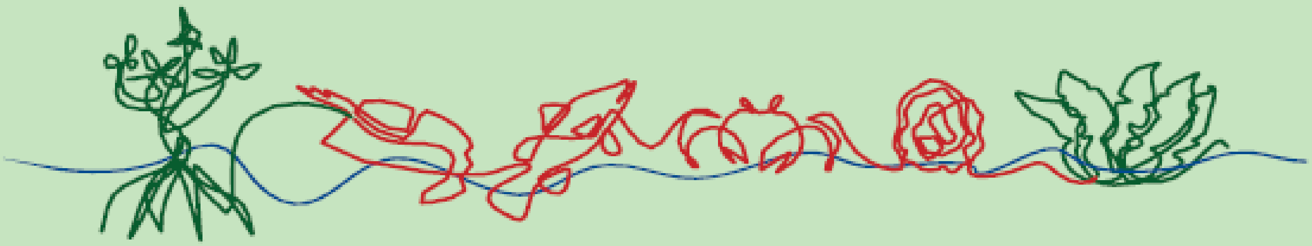
Small pieces (1-$2mm^3$) of HP tissue are rapidly fixed in 2.5% glutaraldehyde is ideal for transmission electron microscopy (TEM). Fixed tissues are processed based on the tissue type and post-fixed in osmium tetroxide. Semi-thin sections are made and the ideal area for sectioning is made. Thin sections cut with diamond knives are placed on copper grids, impregnated with uranyl acetate and lead citrate, and visualized for the details.

Scanning electron microscopy (SEM)

SEM constitutes a major tool used in many research. Actually, SEM acts at the interfaces between disease diagnosis and research in addressing challenging issues. Infected tissues were rapidly fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer at 4°C, dried using Hexamethyldisilazane (HMDS) as per the method described by Nikara et al., (2020) and Berger et al. (2016). Processed samples can be examined and imaged in Scanning Electron Microscope. The tissue surface changes can be visualized.

Conclusion

Molecular tools are increasingly relevant to fish and shrimp diseases. The sequencing of the complete genomes of pathogens is allowing great advances in studying the biology, and improving diagnosis and control of pathogens. Using nucleic acid as targets, and new methods of analyzing changes in this nucleic acid, can improve specificity, sensitivity, and speed of diagnosis and offer means of examining the relationships between genotype and phenotype of various pathogens. Progress in techniques aids epidemiological studies as well as identifying causes of disease outbreaks or the presence of pathogens. Therefore, all these can be a routine tool in the search for improved methods of diagnosis and control of fish and shrimp pathogens and the epidemiology of infectious fish and shrimp diseases. However, applications of these techniques on a routine basis in diagnostic laboratories are rare because of the economic feasibility. The time has now come for their application in the diagnosis of diseases in aquaculture.



"Brackishwater Aquaculture for Food, Employment and Prosperity"

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