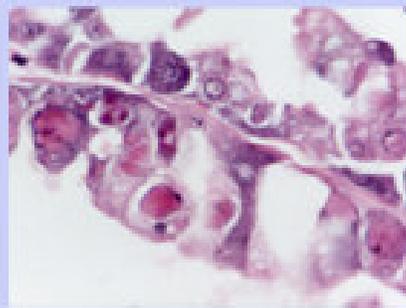
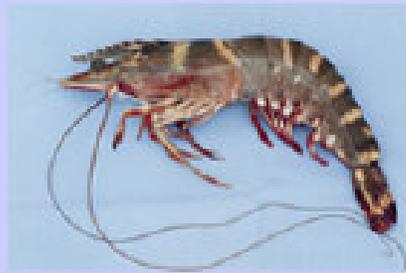


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Diagnosis and Management of Shrimp Diseases



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OVERVIEW OF STRATEGIES FOR PREVENTION AND CONTROL OF DISEASES IN SHRIMP AQUACULTURE

T.C. Santiago

The global aquaculture activity has been recognized as the fastest growing enterprise with an estimated annual growth rate of 11 %. The aquaculture activity in India has been contributing significantly in the various spheres of people's development, in terms of providing livelihood, food security, employment and trade. The aquaculture sector in our country is multifaceted. India is blessed with a long coastline of over 8000 km providing immense scope for brackishwater and marine aquaculture. The land base aquaculture systems include vast potential for freshwater and cold-water ecosystems. Although the aquaculture activity in India is not very new, modern practices with intensification on scientific bases using hatchery produced seeds, formulated feeds and pond and water management methods have been initiated over the last 20 years, and species cultured include diverse aquatic fauna such as finfish, shrimps, crabs, lobsters, prawns, oysters and mussels. However, this intensification of aquaculture has resulted in increased incidence of the disease problems in cultured stock. The White Spot Syndrome Virus (WSSV) alone is causing an annual loss to the tune of Rs.300 crores, since 1994 to the shrimp culture industry, according to conservative estimates. Similarly, the epizootic ulcerative syndrome in freshwater and brackishwater fish has been recognized as one of the major cause of decline in finfish production. The disease problems and related crop losses have become a major limiting factor in the growth of aquaculture activity. As a result of such continued setback, the scientific community and policy makers have initiated steps to overcome the disease problems in order to ensure sustainable development of aquaculture in the country. The objective of this article is to provide an overview of some important diseases that are responsible for production losses to commercially important species of aquaculture in the country. Further, a brief account on the preventive and control methods available and strategies for disease management are also provided.

DISEASES

Viral diseases constitute the most serious problems for shrimp culture due to high infectivity, pathogenicity and nonavailability of curative measures. Nearly 20 viruses are reported to affect penaeid shrimp throughout the world, among which, four viruses are important as far as their impact on the production is concerned (Table 1). The Monodon Baculo Virus (MBV) outbreak in Taiwan in 1988, followed by Yellow Head Virus (YHV) disease in

1992 in Thailand, Taura Syndrome Virus (TSV) in 1992 in Ecuador, White Spot Syndrome Virus (WSSV) in 1993 in China and Thailand, and the same virus in a number of other Asian countries including India have lead to production losses of cultured stock of shrimp. Among these the most devastating one is the WSSV in India. Although MBV has been also frequently found in our shrimp aquaculture systems, its impact on production is not reported to be as devastating as that caused by WSSV. TSV and YHV assume importance in view of the attempts of introduction of exotic shrimps for culture purposes in the country. The bacterial infections caused by *Vibrio* species are the second important cause of mortality of cultured shrimp both in hatcheries and grow-out systems.

The freshwater prawn, *M. rosenbergii* is reported to be relatively less susceptible to diseases than penaeid shrimps, possibly due to lower stocking densities. However, during the past several years, these species are also suffering mortality due to white tail disease, which resembles idiopathic muscle necrosis reported about 16 years back by Nash *et al* (1987). Recent reports suggest that an RNA virus of the family Nodaviridae causes the disease.

Fish Health Management Strategies

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

in harmonizing aquatic animal health management activities.

The ultimate goal of most aquaculture operations is to produce maximum possible biomass per culture unit area in a sustainable manner, regardless of the type of operation and the species cultured. However, the production depends upon a number of factors including environmental conditions, availability of good quality water, nutrition and disease and mortality of cultured stock. Incidence and severity of infectious disease outbreaks very often depend on the quality of environment. Hence the foremost important step in aquaculture health management is to provide the best quality environment within the culture unit.

Coastal aquaculture in general, and shrimp farming in particular, heavily relies upon wild brood stock for seed production. The health status of broodstock population has generally been neglected. The asymptomatic wild broodstock population plays a major role in the vertical transmission of the pathogen. There is a need to evaluate the health status of different stocks and to develop means of controlling the entry of the pathogen into the breeding and farmed populations.

Development of Specific Pathogen Free (SPF) stock is a must to produce disease free seeds. However these facilities need to be created and implemented adopting internationally accepted norms and with proper scientific evaluation.

Timely and correct diagnosis of the disease using the right diagnostic tool is one of the most important components in the aquatic health management. Present disease diagnostic capabilities depend largely upon the availability of sophisticated laboratories. Hence it is necessary to take procedures out of the laboratory and explore ways in which they can be better applied under farm/field conditions.

These are crucial component of an effective health management programme. Quarantine does not only mean that exotic species should be subjected to rigorous checks to avoid introduction of pathogens into a country or state, but it is also imperative that the broodstock/spawners/seeds arriving at a culture facility are screened for the presence of pathogens prior to their introduction to the system. Establishing effective quarantine guidelines and health certification procedures could help minimize the risk of introduction of harmful pathogens. Hence to provide a mechanism to facilitate trade in aquatic species, a proper health management mechanism such as quarantine and health certification is necessary for the trans-boundary movement of aquatic animals on the pre-border (exporter), border and post-border (importer), to minimize the risk of pathogen transfer and associated risk of disease outbreaks.

New generation approaches such as Surveillance techniques, Contingency planning and Import Risk Analysis (IRA) are gaining importance as critical tools in the health management

strategies of aquatic animals for quick and effective response to new disease outbreaks.

Trained manpower and capacity building are the important steps towards an effective extension system. A working extension system for awareness building and effective communication among farmers / aquaculturists, Govt. agencies and planners is pivotal for the successful implementation of any aquatic health management programme.

One of the most important factors dealing with a disease outbreak is information. Correct information is the key element in deciding upon the best means of dealing with a disease. To meet this objective a scientific and functional disease reporting system applicable at local (farm level), national and regional level and aquatic animal health information system at national and regional level is necessary. Policies and legislature governing resources (soil and water) allocation and quality assurance in aquaculture, related to the physicochemical components and biological components should be in place in all the countries practicing aquaculture. Functioning of a national level (each country) body with necessary responsibility and mandate to implement a 'national health management strategy' or 'health management regulation' on the basis of existing international standards, guidelines or recommendation from FAO, OIE and NACA and WTO must be there in issues related to aquaculture and aquatic animal health management for the region.

SHRIMP DISEASES: GENERAL ASPECTS

S. V. Alavandi and T.C. Santiago

Introduction

Coastal aquaculture, especially shrimp aquaculture has undergone a fast growth in recent times in India. While traditional type of shrimp farms were being improved, new extensive and semi-intensive farms were being established at rapid pace. Majority of the investors ventured into aquaculture by initially familiarising themselves with technical aspects of site selection, pond design, feeding techniques, intensive stocking etc. More often, the significant impact of disease was overlooked. However, concomitant with the rapid expansion and intensification of shrimp farming activities serious disease outbreaks were of frequent occurrence.

Attention to disease problems was paid only when widespread outbreak of disease alarmingly reduced the profit from shrimp farming projects. It has become essential for shrimp farmers to understand the biological and environmental factors that lead to disease development, the maladies that can cause considerable loss to cultured shrimp, the early detection of incidence of diseases and drawing up farming strategy that would minimise or prevent the onset of diseases.

What is disease and how diseases develop?

As any other living organisms, shrimp also have specific physiological functions for growth and development, which is greatly influenced by various factors of the environment in which they are living. Any impairment in the physiological functioning may lead to abnormal condition of an organism, and this phenomenon is known as disease. However, many experts consider that there are 3 factors, which interact with each other and result in the occurrence of disease. These factors are the host (shrimp), the environment and disease-causing organism (pathogen). Therefore, disease can be described as an expression of complex interaction of host, pathogen and environment (Fig.).

A decline in host's immunity is the main cause of disease. A lot of factors will impair shrimp health and the most important pre-disposing factors leading to diseases in shrimp culture are:

- I. Adverse environment
- II. High stocking density with limited water exchange facilities
- III. Nutritional deficiency/poor nourishment

- IV. Accumulation of unused feed
- V. Inadequate aeration
- VI. Sub-optimal or heavy algal blooms in the pond
- VII. Physical injury and
- VIII. Presence of virulent pathogens in high count.

In these, changes in the physical or chemical factors will be obvious, but the biological factors will be subtle and complicated. This can be explained by micro ecology. This refers to the interaction of biological factors and it explains the interaction between normal microorganisms and its environment.

Host

Like any other crustaceans, shrimp host's body is covered by exoskeleton, which is regularly replaced by a new one during moulting. The moulting process exerts energy requirement on the shrimp and renders the shrimp susceptible to disease agents or cannibalism. In addition, the shrimp's nutritional well being, size and immune response determine its degree of resistance to disease agents. Behavioural characteristic such as burrowing at the pond bottom also exposes the shrimp condition prevailing in the pond.

Environment

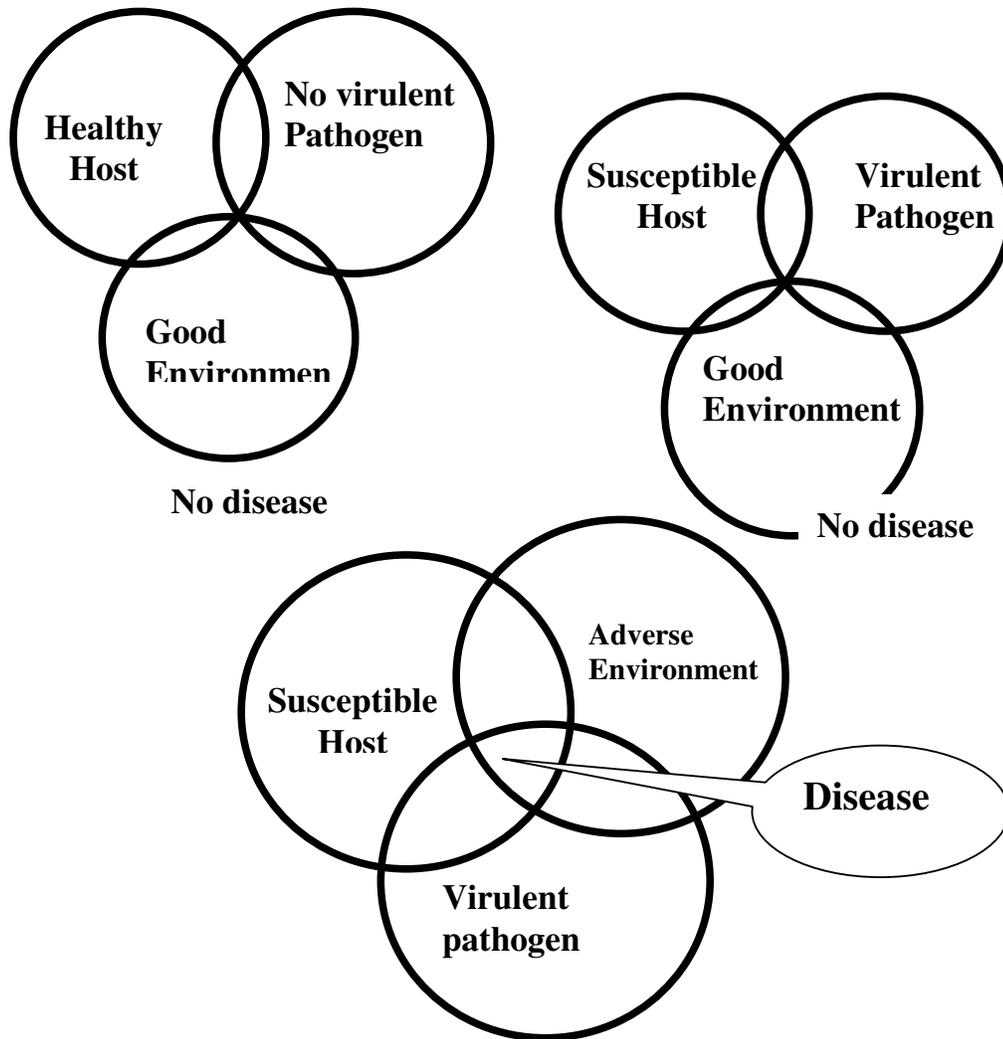
The term environment in aquaculture comprises the pond soil, rearing water and the various living organisms in it. The living organisms include not only shrimp but also other aquatic fauna and flora including pathogenic organisms. The survival and growth of the organisms is largely influenced by various physico-chemical parameters such pH, dissolved oxygen, temperature, light etc. Any abnormal change in these factors will adversely affect shrimp in the culture system. For example, high ammonia level, low dissolved oxygen etc. are stressful and may affect the survival of shrimp.

Pathogen

Various pathogenic organisms may be present in the aquaculture system. They may be the part of the natural flora and fauna of the rearing water or pond soil. Various disease causing organism of shrimp have been reported. Mere presence of these organisms may not cause any disease condition. However, when present in large numbers these may readily invade the injured tissues get established and multiply resulting in disease and death. Nevertheless, the

quantitative level of pathogen is influenced largely by prevailing culture condition such as availability of food source, temperature, dissolved oxygen, pH etc.

Fig. 1. Interaction between host pathogen and environment



VIRAL DISEASES WITH SPECIAL REFERENCE TO INDIAN SHRIMP FARMING

T.C.Santiago, K.K. Vijayan, S.V. Alavandi and N. Kalaimani

Viruses are ultramicroscopic, infective agents capable of multiplying in the host living cells causing improper cell function or cell destruction leading to the death of the host. Viral diseases constitute the most serious problems of shrimp culture due to the high infectivity, pathogenicity and total lack of curative measures. Worldwide, shrimp aquaculture has suffered substantial economic losses due to pathogenic viruses, and the Indian shrimp farming is no exception. So far, 15 viruses infecting cultured shrimps have been recorded across the shrimp farming countries of the world (Table). Till today, only five viruses have been recorded from Indian farms.

Monodon baculovirus (MBV)

Nature of infection:

Monodon baculovirus (MBV) is the first viral pathogen to be recorded from the cultured penaeids of India. Presently the virus is enzootic in Indian hatcheries and farms, infecting both *P. monodon* and *P. indicus*. MBV infections have been observed in the hepatopancreatic cells of all life stages of the prawn except egg, nauplius and protozoa 1 and 2 stages. Postlarvae and farmed shrimps of all sizes with severe MBV infections appear normal and healthy. The virus, widely distributed in the cultured populations is well tolerated by the shrimps, as long as rearing conditions are optimal. Hence, under good culture practices the impact of the MBV infection can be minimal. However, under adverse environmental conditions, MBV may predispose infected shrimp to infection by other pathogens, causing poor growth, secondary infections and mortality.

Pathogenesis and diagnosis:

MBV is a single-enveloped, rod shaped, occluded double stranded DNA virus belonging to the group baculovirus. The virus occurs freely or within proteinaceous polyhedral occlusion bodies in the nucleus, with virions measuring 75-300nm. The presence of MBV in the prawn can be detected by direct microscopic examination of impression smears of infected hepatopancreas (HP) or midgut tissue, stained with 0.05 to 0.1% of malachite green by demonstrating the usually multiple spherical intranuclear inclusion bodies. Histological preparations of the infected HP can be used for further confirmation due

to the presence of prominent eosinophilic single to multiple spherical bodies within the hypertrophied nuclei of the hepatopancreatic tubule or midgut epithelial cells. Transmission electron microscopy (TEM) can also be used to show the presence of MBV virions. DNA based rapid diagnostic tools, polymerase chain reaction (PCR) and DIG-labelled DNA probes are also available for the early diagnosis of MBV.

Prevention and control:

MBV infection may be prevented only through avoidance by quarantine methods, destruction of contaminated stocks, and disinfection of contaminated facilities. There is no treatment for MBV, however good farm management can minimize this disease.

Infectious Hepatopancreatic and Lymphoid organ Necrosis Disease (IHLN)

Nature of infection:

The first ever shrimp epizootic reported from India is the IHLN disease, from shrimp farms located along the Kandaleru Creek, Nellore, Andhra Pradesh during July 1994. This was a localized epizootic confined to the watershed areas of the Kandaleru Creek. The IHLN affected the crops of culture duration ranging from 60 to 100 days weighing 3-28 g. The onset of the disease was sudden and within 3-5 days post infection, more than 90 % of the stock in the farms was lost. The disease prevailed in a virulent form for about three months. Gross signs of the diseased shrimp were: light yellow or pinkish cephalothorax, reddish discoloration of the body and appendages, empty gut, lethargy, poor escape reflex, secondary bacterial infection and mortality. Dead shrimps were found scattered all over the pond bottom. Only *P. monodon* was affected, *P. indicus* was found refractory to the disease.

Pathogenesis and diagnosis:

The most prominent feature of the disease was the highly melanized and shrunken HP. Acute damages were observed in the HP, manifested by multi-focal necrosis of the tubule epithelium marked by hemocytic infiltration and encapsulation resulting in melanization. Densely stained, globular, basophilic bodies were observed in the HP cells and lymphoid organ (LO). The mortality pattern and external signs of infection (yellow cephalothorax) of the disease suggested resemblance to yellow head virus (YHD) in the *P. monodon*. However, the prominent necrotic changes in the HP and LO and absence of pathological changes in the gills, indicated that the disease clearly differed from YHD, however, the etiology of the disease was of viral nature. The peculiar host specificity to *P.*

monodon, presence of basophilic globular structures resembling viral inclusion bodies in the HP and LO, sudden and mass mortality, are the diagnostic features of IHLN disease.

Prevention and control:

None

Hepatopancreatic Parvo Virus (HPV)

Nature of infection:

The HPV has been observed in the hepatopancreas of cultured *P. monodon* and *P. indicus*. However, the infected shrimps did not show any external signs of the disease. Further, the virus was not associated with any mortality. Gross signs of HPV may not be specific, but in severe infections may include an atrophied HP, poor growth rate, anorexia and secondary infections by pathogenic Vibrios. Only few samples (3-5 samples) of shrimps collected during a 12 months period showed the presence of HPV, indicating the low incidence of this virus in Indian shrimp farms.

Pathogenesis and diagnosis:

Histopathologically, basophilic inclusion bodies of HPV can be seen in necrotic and atrophied hepatopancreatocytes. The HPV is a single stranded-DNA virus of 22-24 nm size. DIG-labeled HPV gene probes are also available for the sensitive diagnosis of HPV.

Prevention and control:

Avoid the occurrence of the disease by quarantine methods and destruction of the infected stocks. There is no treatment for HPV.

White Spot Disease (WSD)

The nature of infection:

The first incidence of White Spot Disease (WSD) in India was noticed in December 1992 in *P. monodon* and *P. indicus* from a few seawater based farms near Tuticorin, Tamilnadu. Infected shrimps with prominent white spots on the cephalothorax region of exoskeleton succumbed to death. The incidence was a localised one and did not cause alarm due to the limited impact and localised nature. Since then for about one-and-half-years, there was a temporary reprieve from the disease. However, during November 1994, the disease staged a comeback in the shrimp farming belts of Andhra Pradesh and Tamilnadu. The virulence of the disease was such that the cumulative mortality reached 100% after the appearance of clinical signs in most of the infected farms, within a period of 3-10 days.

Disease affected the shrimps of all ages and sizes, extensive to intensive farming conditions and all range of salinities. The most important fact about the WSD is its wide range of hosts, i.e. it infects all cultured penaeids, crabs, lobsters and other crustaceans like copepods and amphipods. Acutely affected shrimps showed lethargy and anorexia. The moribund shrimp showed up on the water surface and gathered on the edges of the pond. By September 1995, the disease spread to the shrimp farms in Kerala, Karnataka, Goa, Maharashtra, Gujarat, Orissa and West Bengal. The impact was so severe that it forced the closure of many farms creating a total chaos in the Indian shrimp aquaculture industry. During the period 1994-1995 alone, the shrimp loss due to the disease was about 15000 tonnes valued at Rs 500 crores. Even now, the shrimp farms in the country are under the grip of this epizootic with changing virulence.

Pathogenesis and diagnosis:

The causative agent of the WSD was found to be a rod-shaped virus, the white spot virus (WSV). This non-occluded, enveloped, nuclear virus infects shrimp tissues of ectoderamal and mesodermal origin. Typical clinical sign in infected shrimp is the appearance of white spots or patches of 0.5 to 3mm in diameter on the inner surface of the exoskeleton. In many cases, moribund shrimps displayed reddish to pinkish coloration without any white spots. Histologically, the infection is characterized by eosinophilic to progressively more basophilic inclusion bodies in the hypertrophied nuclei of infected cells, due to the development and accumulation of intranuclear virions. Histopathological study demonstrates that WSV targets various tissues originating from mesoderm and ectoderm, particularly cuticular epidermis, gills, stomach, lymphoid organs, hematopoietic and antennal gland. The disease could be diagnosed by histology and confirmed by TEM. New generation DNA based diagnostic tools like gene probes and PCR are also available for the asymptomatic detection of WSD.

Though morphological features, histopathological response, and mode of infection are similar, the white spot disease has been named differently by various authors from different countries: rod-shaped nuclear virus of *Penaeus japonicus* (RV-PJ) and penaeid rod shaped DNA virus (PRDV) in Japan; systemic ectodermal and mesodermal baculovirus (SEMBV) and white spot syndrome virus (WSSV) in Thailand; hypodermal hematopoietic necrosis baculovirus (WSBV) in China and white spot baculovirus (WSBV) and white spot disease (WSD) in Taiwan. The name white spot disease (WSD) for the disease, and white spot virus (WSV) for the pathogen has been used for the Indian strain.

Prevention and control:

There is no treatment for WSD. Preventive measures include avoidance of the disease by quarantine methods, destruction of known contaminated stocks, and disinfection of the culture facility can help to remove the possibility of infection. Use of UV radiation and Ozone (physical disinfectants) and sodium hypochlorite, Benzalkonium chloride and povidone iodine (chemical disinfectants) at proper doses has been found useful in inactivating the WSV from the rearing systems.

Another interesting aspect of WSV infection in Indian shrimp farms is the changing virulence status. During the last two years, many farmers were able to make a reasonable harvest of 1-2 tonnes of 15-30g prawns, inspite of observing a few specimens with gross signs of WSV infection present in their ponds during the initial phase of culture and then later throughout the cultivation cycle. Similar observations were reported from other shrimp farming countries of Asia like Thailand. This stands out against the situation of massive and total mortality during the initial phase of the epizootic. It appears that either the shrimp is learning to live with the virus (viral accommodation) or the virus itself is changing its virulence to a less lethal level. However this phenomenon is not uniform across the country and the incidence of WSD mortality is still common. It is essential to resolve the scientific details of this phenomenon through research, which may be useful in the control of the white spot epizootic.

Table. Viruses affecting cultured and wild penaeid shrimp

Name	Approximate Virion size	Nucleic Acid	Probable Classification	Reference
Baculovirus Penaei (BP)	50-75X300 nm	dsDNA	Baculovirus	Couch, 1974
Baculoviral midgut gland necrosis (BMN)	75X300 nm	dsDNA	Baculovirus	Sano <i>et al</i> , 1981
Infectious hypdermal and hematopoietic necrosis virus (IHNV)	22 nm	ssDNA	Parvovirus	Lightner <i>et al</i> , 1983a
Monodon baculovirus (MBV)	75X300 nm	dsDNA	Baculovirus	Lightner <i>et al</i> , 1983b
Hepatopancratic parvovirus (HPV)	22-24 nm	ssDNA	Parvovirus	Lightner and Redman, 1985
Type C baculo virus (TCBV)	75X300 nm	dsDNA	Baculovirus	Brock and Lightner, 1990
Lymphoid parvo-like virus (LPV)	25-30 nm	ssDNA	Parvo-like virus	Owens <i>et al</i> , 1991
Iridovirus (IRIDO)	136 nm	dsDNA	Iridovirus	Lightner and Redman, 1993
Hemocyte- infecting nonoccluded baculovirus	90X640nm	dsDNA	Baculo-like virus	Owens, 1993
White spot syndrome virus (WSSV)	80-330 nm	dsDNA	Baculovirus	Wongteerasupaya <i>et al</i> , 1995; Lightner and Redaman, 1998
Reo like virus	SX70nm	dsRNA	Whispovirus	Redaman, 1998
Lymphoid organ vacuolization virus (LOVV)	30XSSnm	ssRNA	Reo-like virus	Tsing and Bonami, 1987
Rhabdovirus of penaeid shrimp (RPS)	75X125nm	ssRNA	Toga-like virus	Bonami <i>et al</i> , 1992
Yellow head virus (YHV)	44X173nm	ssRNA	Rhabdovirus	Nadala <i>et al</i> , 1992
Taura syndrome virus (TSV)	30-32nm	ssRNA	Picornavirus	Flegel <i>et al</i> , 1995 Lightner <i>et al</i> , 1995

BACTERIAL AND FUNGAL DISEASES OF SHRIMP

S.V. Alavandi

The bacteria causing diseases of penaeid shrimp constitute part of the natural microbial flora of seawater. Accumulation of un-utilized feed and metabolites of shrimp in the culture tanks/ ponds enrich the water with organic matter that supports the growth and multiplication of bacteria and other microorganisms. Bacterial infections of shrimp are primarily stress related. Adverse environmental conditions or mechanical injuries are important predisposing factors of bacterial infections and disease. The most common shrimp pathogenic bacteria belong to the genus *Vibrio*. Other Gram-negative bacteria such as *Aeromonas* spp., *Pseudomonas* spp., and *Flavobacterium* spp., are also occasionally implicated in shrimp diseases.

Bacterial Septicaemia (Vibrio disease)

Signs and Symptoms: This is one of the severe systemic diseases caused by bacteria. The affected shrimps are lethargic and show abnormal swimming behaviour. The periopods and pleopods may appear reddish due to expansion of chromatophores and the shrimps may show slight flexure of the abdominal musculature. In severely affected shrimps the gill covers appear flared up and eroded. In more severe cases extensively melanised black blisters can be seen on the carapace and abdomen.

Cause: Bacteria such as *Vibrio alginolyticus*, *V. anguillarum*, *V. parahaemolyticus*, *Vibrio* spp.

Diagnosis: The bacterial septicaemia or systemic vibriosis is diagnosed based on the gross signs and symptoms, and confirmed by isolation of pathogen from haemolymph by standard microbiological methods and histopathology.

Prevention: Maintain good water quality and reduce the organic load by increased water exchange.

Control: Increase water exchange with good quality seawater. Feed shrimps with antibiotic fortified feeds (only after ascertaining *in-vitro* sensitivity of the pathogen). *e.g.*, feeds containing oxytetracycline @ 1.5g /Kg, fed at 2-10% of body weight for 10-14 days along with proper water and pond management. Sufficient withdrawal period (about 25 - 30 days) should be allowed for the antibiotic to become inactive or harmless.

Luminescent Bacterial Disease

The luminescent bacterial disease is a serious problem in the hatcheries. Occasionally, the juveniles and adult shrimp may also be affected in the grow-out farms.

Signs and Symptoms: The infected larvae appear luminescent in darkness, and suffer heavy mortality.

Cause: Luminescent bacteria, viz., *Vibrio harveyi*.

Diagnosis: Gross signs and symptoms and microscopic demonstration of swarming bacteria within the haemocoel of moribund shrimp larvae would confirm luminescent bacterial disease. The luminescent bacteria can be readily isolated on Zobell's Marine Agar or a selective medium. Identity of the isolates could be confirmed based on their morphological and biochemical characteristics.

Prevention: Use ultraviolet irradiated and chlorinated (calcium hypochlorite 200ppm for 1 h.) water. Clean the debris collected at the bottom of the culture tanks daily.

Control: Exchange 80% of water daily with UV sterilised / sand filtered seawater.

Brown spot disease (Shell disease or Rust disease)

Signs and Symptoms: The affected animals show presence of brownish to black eroded areas on the body surface and appendages.

Cause: Bacteria such as *Vibrio* spp., *Aeromonas* spp., and *Flavobacterium* spp., with chitinolytic activity.

Diagnosis: Diagnosis of brown spot disease is achieved by simple observations on the gross signs and symptoms and confirmed by isolation of the bacteria from the site of infection on Zobell's Marine Agar and identification of the pathogen.

Prevention: Reduce organic load in water by increased water exchange. Avoid unnecessary handling and overcrowding to minimise chances of injury and infection.

Control: Induction of moulting by applying tea seed cake may be useful. Improve water quality by increasing water exchange. Although antibiotics may be useful their use in the culture system is not recommended.

Necrosis of appendages

Signs and symptoms: The tips of walking legs, swimmerets and uropods of affected shrimp undergo necrosis and become brownish and black. The setae, antennae and appendages may be broken and melanised.

Cause: The epibiotic bacteria such as *Vibrio* spp., *Pseudomonas* spp., *Aeromonas* spp. and *Flavobacterium* spp.

Diagnosis: Based on gross signs and symptoms.

Prevention: Maintain good water quality. Stock shrimp at optimum density. Avoid unnecessary handling of the shrimp, which may lead to injuries, leading to infection and necrosis.

Control: Induction of moulting by applying 0.5 - 1 ppm tea seed cake may be of help.

Vibriosis in larvae

Signs and Symptoms: The affected larvae show necrosis of appendages, expanded chromatophores, empty gut, absence of faecal strands and poor feeding. Cumulative mortalities may be very high reaching up to 80% within few days.

Cause: Bacteria, viz., *Vibrio alginolyticus*, *V.parahaemolyticus*, and *V.anguillarum*.

Diagnosis: Microscopic demonstration of motile bacteria in the body cavity of moribund shrimp larvae, and isolation and identification of pathogenic bacteria would help in the diagnosis of the disease.

Prevention: Maintain good water quality and reduce organic load in the water by increased water exchange.

Control: 10-15 ppm EDTA to the rearing water.

Filamentous Bacterial Disease

Signs and Symptoms: The affected shrimp larvae show fouling of gills, setae, appendages and body surface. Moulting of affected shrimps is impaired and may die due to hypoxia.

Cause: Filamentous bacteria, such as *Leucothrix mucor*.

Diagnosis: Diagnosis of filamentous bacterial disease could be achieved based on gross signs and symptoms and by microscopically demonstrating filamentous bacterial fouling of body surface and appendages of shrimp larvae.

Prevention: Maintain good water quality with optimal physico-chemical parameters.

Control: 0.25 - 1 ppm Copper sulphate bath treatment for 4-6 hrs.

FUNGAL DISEASES

Larval Mycoses

It is one of the most devastating diseases in shrimp hatcheries. However, larval mycoses have been successfully controlled during the recent years with better management practices.

Signs and symptoms: Affected larvae appear opaque followed by sudden mortality. The protozoal and mysis stages are highly susceptible. Within 1-2 day's, whole stock of shrimp larvae may suffer mortality.

Cause: Oomycetous fungi, *Lagenidium* spp, *Sirolopidium* spp, and *Haliphthoros* spp. These fungi are filamentous, non-septate and coenocytic. Upon infection, the fungal mycelium replaces the larval tissues and ramifies into various parts of the body. Vegetative propagation of these fungi is through production of bi-flagellate zoospores, which are released into the rearing medium. These zoospores further infect fresh shrimp larvae. These fungi can be isolated on peptone yeast extract glucose (PYG) agar or Saboraud's dextrose agar.

Diagnosis: Microscopic demonstration of presence of extensively branched non-septate, fungal hyphae within the body cavity of the shrimp larvae.

Prevention: Remove bottom sediments and dead larvae periodically. Disinfect the tanks and other equipment in the hatchery from time to time. Treat spawners with 5 ppm treflan bath for 1 h.

Control: When the disease is detected in early stages, Treflan (Trifluralin) 0.1-0.2 ppm bath for 1 day may help in reducing mass mortality.

Other fungi such as *Fusarium* spp. cause infections in nauplii, protozoa, juveniles and adults. Black gill disease is often caused by this fungus. The fungus can be identified by microscopic examination of its characteristic canoe shaped micro-conidia. Other oomycetous fungi such as *Saprolegnia* spp. and *Leptolegnia* spp. are also known to affect shell of shrimp and produce dark necrotic lesions causing gradual mortality.

PARASITIC AND NON-INFECTIOUS DISEASES

K. P. Jithendran

PARASITIC DISEASES

Among the disease causing organisms of shrimp, parasites, especially protozoan parasites form an important group. Although, several diseases caused by parasites have been noticed in shrimp, often, chronic conditions caused by protozoans play a crucial role in shrimp production. The protozoa, affecting shrimp can be grouped as parasites and commensals. Following are the major disease problems caused by the protozoa:

- Protozoan fouling
- Cotton shrimp disease
- Enterozoic cephaline gregarine infection
- Invasive protozoan infection

Protozoan Fouling

This is a serious disease problem commonly encountered both in hatchery and farm.

Signs and symptoms: Affected shrimps are restless and their locomotion and respiratory functions are hampered. Heavily infected, larger shrimp often have fuzzy-mat like appearance on the body surface, appendages and gills. Animals also show brownish discoloration due to algal filaments or debris entangled with the epibiont.

Causative organisms: Peritrichous ciliates such as *Zoothamnium*, *Epistylis*, *Vorticella*, *Acinata* etc.

Diagnosis: Based on gross signs and symptoms. Fresh smear preparation of the surface scraping or gill or appendages will reveal the morphology of the protozoan.

Prevention: Maintain good water quality, reduce the organic substance, silt and sediment on the pond bottom, maintaining optimum dissolved oxygen level (5-6 ppm) and frequent exchange of water.

Control: Formalin is the chemo therapeutant of choice. Treatment with formalin 15-25 ppm concentration (single treatment) for ponds or dip treatment of affected animals in 50-100 ppm for 30 min is useful. Good aeration during treatment is essential.

Cotton Shrimp Disease or Milk Shrimp Disease

Signs and symptoms: The pathogenic protozoan infect and replace striated muscle, causing it to become opaque and white. The muscle of such shrimp appears cooked. In severely affected shrimps, the exoskeleton appears bluish black, and white tumour-like swelling may be found on the gills and subcuticle. A few species infect gonads, heart, haemolymph vessel, hepatopancreas and produce enlarged gonads.

Causative organisms: Microsporeans such as *Agmasoma*, *Ameson* and *Pleistophora*.

Diagnosis: Based on gross signs and symptoms, the disease can be tentatively diagnosed. Microscopic examination of squash preparation or impression smears stained with Giemsa will reveal large number of microsporean spores. Spore characteristics may vary from species to species.

Prevention: Affected animals should be destroyed and buried away from the farm. Before stocking, the possible conditioning host / intermediate host should be eliminated.

Control: No treatment has been reported for Penaeids.

Enterozoic Cephaline Gregarine Infection

Signs and symptoms: Affected shrimp show loss of appetite, lethargy and weakness. Often, low levels of mortalities.

Causative organisms: Cephaline gregarines such as *Nematopsis* and *Cephalolobus*.

Diagnosis: Microscopic observation of the digestive system reveals the developmental stages of the parasites. Rectal portion show white, spherical gametocysts attached to the wall.

Prevention: Infection has been generally observed in culture system, which uses wild seeds. So the best preventive measure is avoidance of wild seed. Elimination of intermediate hosts from the culture system also prevents the disease occurrence.

Control: No treatment is reported.

Invasive Protozoan Infection

This has been noticed in a few cases in hatcheries. Often, heavy mortalities also have been recorded. Causative organisms include ciliate protozoa, *Paranophrys* and *Paraoronema*, leptomonad-like organisms. Control and preventive measures are not reported.

NON-INFECTIOUS DISEASES

Non-infectious disease are common in the grow-out farms, as influences of nutritional factors, environmental factors such as temperature extremes and oxygen depletion, toxicity from biotic and abiotic origins, become critical during the lengthy culture period.

Soft shell syndrome

Soft shell syndrome is a condition in which shrimp exoskeleton becomes soft. Cuticle of the affected shrimps is persistently soft, loose and papery for several weeks. Affected shrimps are weak and show poor escape reflex, and these animals are susceptible to cannibalism. Severely affected *P. indicus* often show undulating gut in the first three abdominal segments. Several factors are implicated as causative agent for this condition as: sudden fluctuation in water salinity, high soil pH, highly reducing conditions in soil, low organic matter in soil, low phosphate content and pesticide pollution in water, nutritional deficiency and insufficient water exchange. The disease may be prevented or controlled through environmental and dietary manipulations by providing favourable water and soil conditions in the pond and feeding adequately with balanced diets.

Black gill disease

A number of abiotic and biotic reasons have been attributed to the black gill in shrimps. Presence of excessive levels of toxic substances such as nitrite, ammonia, heavy metals, crude oils etc. in the culture water may lead to black gill disease. High organic load, heavy siltation and reducing conditions in rearing pond can also cause this disease in shrimps. Attack of certain bacterial, fungal and protozoan pathogens can also cause black gill condition in shrimp. Affected shrimps have gills with black to brown discoloration, in acute cases necrosis and atrophy of the gill lamellae may be apparent. The blackening is due to the deposition of melanin at sites of massive haemocyte accumulation, followed by dysfunction and destruction of whole gill processes.

Treatment of the black gill disease depends upon the cause of the disease. Preventive or corrective measure may be adopted to avoid or reduce the biotic / abiotic factors in the rearing pond to control the disease condition.

Red disease

The juveniles and adult shrimps / broodstock affected with red disease have reddish discoloration in body, pleopods and gills. Definite causative agent is not known. One of the reasons believed to be the cause of disease is a microbial toxin in rancid or spoiled diets or in detritus of ponds rich in organic matter. Extreme conditions of pH or salinity in pond water may also cause the disease. Healthy management of ponds along with the use of good quality feed may help in the avoidance of red disease.

Cramped tail disease

Affected shrimps have rigid dorsal flexure of the abdomen, which cannot be straightened. These shrimps lie on their sides at the bottom of the pond and are susceptible to cannibalism. Exact cause for this disease is not known, but environmental and nutritional causes have been suggested. Maintenance of healthy conditions in the pond with proper feeding with balanced diet may be helpful in the prevention / control of this disease.

Gas-bubble disease

Super saturation of atmospheric gases and oxygen in the pond can result in the gas-bubble disease, which affects the shrimps of all sizes. Presence of gas bubble in the gills or under the cuticle is the characteristic of this disease. Gas bubble disease due to oxygen is not lethal, while that of nitrogen can be lethal. The threshold saturation level to cause the gas-bubble, in the case of nitrogen is 118 % while that of oxygen is 250 % of normal saturation. The severely affected or dead shrimp due to this disease may float near the water surface. Super saturation of the gases must be avoided to prevent the disease.

Muscle necrosis

Shrimps of all life stages are affected with muscle necrosis. Affected shrimps are characterised by the presence of white opaque areas in body musculature, usually in the lower abdomen or some times in the appendages. The condition is reversible in the early stages if the corrective measures are taken, but in severe cases sloughing of the affected areas occurs due to secondary bacterial infection leading to death. This disease is associated with poor environmental conditions such as low oxygen levels, and salinity or temperature shock. Overcrowding and poor handling also can cause muscle necrosis. Avoidance of overcrowding, proper handling and maintenance of favourable environmental factors may help to contain the disease.

INVESTIGATING SHRIMP DISEASES: OBSERVATION ON POND & SHRIMP AND SAMPLING

S.V. Alavandi, K.K. Vijayan T.C. Santiago and N. Kalaimani

Proper and accurate diagnosis of diseases forms the cardinal step in any disease control and prevention programme. To diagnose a shrimp disease problem, history of the farms, the soil and water conditions of the ponds, incidence of any disease problem in the adjoining areas, possibility of disseminating disease through birds or other carriers, are of great importance. These are the significant information related to the epidemiology of the disease. This section stresses the need to examine general information on the farming activity and the information regarding disease on site and some points on collection of samples for laboratory investigation.

1. Background information about the farming practices

A. Examination of ponds

This involves the various parameters of ponds such as methods followed in the preparation of ponds, depth, nature of the bottom, water treatment methods, nature of water inlet and outlet procedure etc. Apart from these, colour of water, algal blooms, turbidity of water and presence of bioluminescence during night are also important criteria, which determine the health of the shrimp.

B. Stocking parameters

These parameters include origin and source of seeds, health status of spawners and the larvae, survival rate of larvae within the hatchery and nursery, whether any antibiotic/disinfectant used for larval rearing, stocking density, time of stocking etc. These parameters are very much important in assuring the healthy or resistant nature of the larvae and in the diagnosis of any possible disease problem.

C. Management practices

These include data generated out of the close monitoring of the system for the growth, survival and occurrence of diseases. These also include the quality of the feed, feeding regime, consumption of feed by the shrimp, time and rate of water exchange and the use of chemicals, immunostimulants or bioremedial measures also to be recorded.

D. Environmental parameters

Diseases may be of infectious and non-infectious etiologies. Majority of the non-infectious diseases are due to nutritional deficiency or due to abnormal environmental

conditions. Hence, a close examination and recording of the various parameters of water and soil quality should be done periodically.

2. Field observation for signs and symptoms

A. Observation of behaviour of shrimp

Critical observation on the behaviour of shrimp will give an indication of the health of the animals. Significant behaviour includes escape reflex, swimming at the surface, moulting behaviour, feeding behaviour etc.

Healthy shrimp will have quick reflexes i.e., shrimp will respond instantaneously to any outside disturbances or artificial stimulation; Shrimp showing poor escape reflex may not be in a healthy condition.

Swimming at the surface is an indication of either inadequate oxygen level, or respiratory impairment. Disease conditions, such as fouling, white spot disease etc always show such behavioural changes.

Moulting behaviour is another important factor, which has to be observed. Regular and continued moulting indicates continuous growth. Abnormal moulting indicates a diseased condition. Chronic condition due to hepatopancreatic infection may cause abnormal moulting.

Feeding behaviour is also an important indicator of health of the shrimp. Diseased shrimp normally will show reduced appetite (e.g. white spot disease, protozoan fouling). However, it has been reported that juveniles and sub-adult affected with yellow head disease show an abrupt abnormal increase in feeding rate for several days.

B. Observation of external signs and symptoms

To assess the health status of a shrimp, following gross signs should be examined.

- i. *Colour and nature of exoskeleton*: Healthy shrimp will have pale blue coloured, bright, smooth and clear cuticle with proper hardness. Exoskeleton will show brownish discoloration and occasional mat-like appearance (muddy cuticle) in protozoan fouling. Moulded shrimp and shrimp with soft shell syndrome will show soft exoskeleton. However, the shrimp with soft shell syndrome will have a hard rostral spine.
- ii. Apart from these, visible blisters or brown or black eroded areas on the exoskeleton may indicate possible bacterial infection. Presence of white spots or patches on the carapace indicates the visual disease, white spot disease.
- iii. *Appendages*: Tips of walking legs, swimmerets and uropods may show necrosis and become brownish black indicating a possible bacterial infection. Often, physical injury

may be a predisposing factor for bacterial infection and resultant necrosis and melanisation.

iv. *Musculature*: White muscular opacity may indicate muscle necrosis due to environmental stress or a microsporean infection.

C. Examination of internal organs for pathological signs

i. *Gills*: Gills are normally clean, semi-transparent and colourless. External fouling due to epicommissals will cause dark yellow discoloration. Bacterial infection can cause blackening of gills. Vibriosis may cause yellow discoloration of branchiostegites.

ii. *Hepatopancreas*: Hepatopancreas of normal shrimp will be obvious, with proper size and shape. Colour of top half is brown and the bottom has a white membrane cover. Abnormal colour, enlargement or atrophy *etc.* are indication of bacterial or viral infection, or the presence of toxic substances or nutritional deficiency.

iii. *Haemolymph*: Haemolymph of normal shrimp have slight blue colour. It will easily coagulate in 1 min. after taking it out from shrimp. Some of the bacterial and viral infections cause the haemolymph non-coagulable, colourless or light reddish or muddy in nature.

3. Collection of samples

For accurate diagnosis of the disease, typical and representative sample of infected animals should be collected. Very often, in one pond itself there will be multiple infections. All the dead animals may not be a representative sample. Instead of dead animals, moribund animals will be suitable for analysing the symptoms, for pathological studies and for isolation of pathogens. Moribund shrimp may have secondary infection also, and more often, shrimp with disease in the initial stage may not exhibit the real symptoms. All these factors should be taken into account while collecting the required sample.

According to many experts, there are four methods to collect the samples of shrimp:

- i. Picking from the sides around the ponds
- ii. Catching from the middle of the pond
- iii. Using cast net
- iv. From the feed trays.

These samples will really reflect the actual disease status in pond. Samples of moribund shrimp, which are collected from the sides around the ponds, will be mostly at the terminal stage of infection. The samples collected from the middle may be in an intermediate

stage. Cast net will give a random sample and is preferable, while the samples from the feed tray will be usually healthy.

BACTERIOLOGICAL METHODS

S.V. Alavandi

The methods of microbiological examination of shrimp are essentially similar to those followed for the higher animals. The first step in shrimp diagnostic bacteriology is to isolate the pathogen from the diseased shrimp and then identify the same based on its cultural, morphological, physiological, biochemical and serological characteristics. The methods required for isolation and identification of shrimp pathogens are described here. However, for more details, the readers are advised to refer Austin (1988). A method for determination of total viable counts of bacteria in water samples is also included.

Aseptic techniques

Maintenance of aseptic conditions during all stages of microbiology work is the first step for successful microbiological investigation. It is essential to take suitable measures to ensure the recovery of bacteria of interest. There are several sources of contamination. The air may contain dust particles and aerosols (micro droplets of water). Other materials like glassware, buffers, culture media, and equipment used and even the careless personnel carrying out the work can be sources of contamination. Hence, it is very important to take suitable measures to get rid of the contaminating microorganisms. In order to achieve this, several methods have been evolved. The methods of sterilisation of different kinds of materials used in the laboratory are given in Table 1.

Pure culture technique

Growth of pure culture is necessary before any cultural, biochemical or sensitivity tests are run to identify and characterise the suspected bacterial pathogen. A number of methods are used for this purpose. These are:

- Streak plate technique (streaking onto solid media)
- Pour plate technique (Incorporation into molten semi-solid media)
- Dilution in liquid media

Among all these, the streak plate method is the most commonly method, which allows obtaining pure culture of specific bacterium from mixed bacterial populations. Bacteria from a mixed culture are streaked over the agar surface in a pattern that deposits them further and further apart. Towards the end of the pattern, the resulting colonies of different bacteria are separated from each other. The single individual colonies of different types are then picked up with the help of a bacteriological loop and streaked on another plate to obtain pure cultures. Purity of the culture should be confirmed by periodic streaking on plates and observing their cultural, morphological and biochemical characteristics.

Table 1. Sterilization methods

Materials sterilised	Methods of sterilisation
All types of glassware like pipettes, tubes, flasks, petri dishes etc.	Dry heat method Hot air oven 160°C for 2 hr or 180°C for 1 hr
Destruction of used, contaminated material, dead animals, tissues <i>etc.</i>	Incineration
Most bacteriological media, glassware / containers, (decontamination of used media), steel items, corks, rubber materials, filter pads, filter assembly, distilled water, buffers, solutions <i>etc.</i>	Moist heat Autoclaving 121°C for 15 min
Most tissue culture media, antibiotics, sera, solutions containing heat sensitive materials like amino acids, carbohydrates, biological materials <i>etc.</i>	Filtration Membrane filters of 0.22 µm / 0.45 µm pore size

Cultural and morphological characters of bacteria

For identification of bacteria, some general cultural and morphological characteristics like size, shape, pigmentation, opacity of the bacterial colonies on the solid media; cell shape (rods cocci, coccobacilli, comma), sporulation, *etc* are important and aid in preliminary grouping of the bacteria. Some of the cultural and morphological characteristics useful for distinguishing bacteria are given in Tables 2 and 3.

Table 2. Cultural characteristics of bacteria grown on solid media

Colony character	Description
Shape	Circular, irregular, radiated, rhizoidal <i>etc.</i>
Size	Size of colony in μm
Elevation from surface	Raised, low convex, convex, dome shaped, umbilicate
Surface	Smooth, contoured, rough, ridged, striated, dull, glistening
Edge	Entire, undulate, lobate, crenated, fimbriate, effuse, spreading
Colour	Different colours due to production of pigments
Opacity	Translucent, transparent, opaque

Table 3. Morphological characteristics of bacteria

Parameter	Description
Shape	Cocci, spherical, oval, short rods, long rods, filamentous, comma, spiral <i>etc</i>
Size	Length and breadth in μm
Arrangement	Single, pairs, chains, in fours (tetrads), in groups, grape like clusters, bundles, irregular
Irregular forms	Variation in shape & size, clubs, filaments, branched <i>etc.</i>
Flagella	Polar, monotrichous, amphitrichous, peritrichus <i>etc.</i>
Fimbriae	Polar, peritrichus (EM study)
Spores	Spherical, oval, elliptical, sub -terminal, single or multiple.
Capsule	Present or absent
Staining	Reaction to Gram stain

Isolation of Bacteria and Fungi from Infected Shrimp

- i. Inoculate the infected larvae/affected tissues / haemolymph on the culture plates with the help of sterile bacteriological loop and streak the inoculum to get isolated colonies. Commercially available dehydrated culture media may be used for culture and isolation of microorganisms in order to save time, expense, shelf space, uniformity of composition *etc.* The culture media routinely employed for isolation of bacteria from shrimps are Zobell's Marine Agar 2216 (ZMA), Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar and other selective media such as *Aeromonas* selective medium or *Pseudomonas* selective medium.

In place of ZMA, nutrient agar prepared in aged seawater would suffice. The ZMA favours the growth of all the heterotrophic bacteria occurring in the brackish and marine environments, whereas, the TCBS medium is selective for isolation of shrimp pathogens such as *Vibrio* spp. Mycological agar/ Sabouraud's dextrose agar is used for isolation of fungi. A selective medium for isolation of luminescent bacteria is used whenever required.

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

Gram Staining of Bacteria

Principle: Staining bacteria by Gram's method is widely used for classification of bacteria into Gram positive and Gram negative bacteria. The bacterial cell walls contain peptidoglycans, which is a thick layer in the Gram-positive bacteria. The pararosaniline dye such as crystal violet treated with iodine mordant remains trapped in the cell wall and hence, the cells are not de-stained upon treatment with alcohol.

Procedure:

- i. Prepare smears of bacteria on clean glass slide using sterile nichrome loop by mixing with a drop of sterile normal saline.
- ii. Fix the smears by air-drying or by gently passing the slide over the Bunsen flame.
- iii. Stain the smears with Crystal Violet solution for 1 minute.
- iv. Wash in tap water for few seconds.
- v. Flood the smears with Iodine solution for 30 seconds.
- vi. Wash in tap water for 15 seconds.
- vii. De-colorize with 95% ethyl alcohol for 30 seconds.
- viii. Wash with tap water.

- ix. Counter-stain with Safranin solution for 10 seconds.
- x. Wash in tap water. Blot dry and examine under oil immersion objective of the microscope.

Interpretation: Violet coloured bacteria: Gram positive; Red / pink coloured bacteria: Gram negative.

Record size, shape arrangement and other morphological characteristics.

Motility test (Hanging drop method)

- i. Place a very small drop of log phase broth culture of bacteria with the help of sterile inoculating loop (2 mm dia) at the centre of a cover glass.
- ii. Place small drops of water on the corners of the cover glass.
- iii. Invert the cover glass over the cavity of slide, so that the drop of culture is hanging at the centre of the cavity slide.
- iv. Observe the hanging drop of bacterial culture under the microscope for motility of bacteria.
- v. Darting or zig-zag motility indicates that the bacteria may have polar flagellation, while, slow motility or vibratory motility indicates peritrichous flagellation.

Oxidase test

Principle: Some bacteria possess cytochrome oxidase or indophenol oxidase, which catalyses transport of electrons from donor compounds to oxygen. In this test, the N N N'N' tetramethyl p-phenylene diamine dihydrochloride, a colourless dye serves an artificial electron acceptor. The oxidase enzyme produced by bacteria oxidises the dye producing coloured indophenol blue.

Procedure:

- i. Place a strip of whatman No.1 filter paper in a petri dish.
- ii. Add 2-3 drops of freshly prepared 1% solution of N,N,N',N'- tetramethyl paraphenylene diamine dihydrochloride.
- iii. Smear the test colony of bacteria on the filter paper using a sterile capillary.

Interpretation: Positive reaction is indicated by development of a deep purple colour of the smear.

Catalase test

Principle: Bacteria possess an enzyme called catalase which catalyses breakdown of toxic hydrogen peroxide (H_2O_2) formed during the cell's metabolism into water and oxygen. When a solution of H_2O_2 is added to bacterial cell suspension, the catalase enzyme is activated, resulting in the release of O_2 , which is observed as effervescence.

Procedure:

- i. Make a drop of heavy suspension of test culture of bacteria on a slide.
- ii. Place a drop of 10% hydrogen peroxide solution over the bacterial suspension.
- iii. Observe for small air bubbles.

Interpretation: Production of gas bubbles (effervescence) indicates a positive reaction.

Carbohydrate Fermentation test

Principle: When the bacteria are grown in basal media containing specific carbohydrates such as glucose, sucrose, lactose, mannitol *etc*, in the presence of a pH indicator, manifest into colour change depending on the metabolic pathway used by the bacteria.

Procedure:

- i. Inoculate the bacterial isolate in duplicate into phenol red broth base incorporated with sugars such as glucose, lactose, mannitol *etc*. in sugar fermentation tubes.
- ii. Overlay one tube with sterile mineral oil (*e.g.* liquid paraffin) about 1-2 cm. Incubate the tubes at 37°C.
- iii. Observe the tubes for colour change at 24, 48 and 72 h intervals.

Interpretation: Acid production in open tube indicates oxidative metabolism and acid production in the tube overlaid with mineral oil indicates fermentative metabolism of the bacteria.

Lysine decarboxylase, Ornithine decarboxylase and Arginine dihydrolase test

Principle: Some bacteria are able to produce enzymes that attack carboxyl group of amino acids. The reaction is anaerobic. Bacteria are inoculated into tubes containing the amino acid along with a control tube, which contains only the basal medium without the amino acid. The tube with amino acid is made anaerobic by overlaying with sterile mineral oil over the medium. If the test organism does not produce decarboxylase, both the control and test tubes turn yellow due to fermentation of small amount of glucose present in the medium yielding acidic products, lowering the pH of the medium. If the amino acid is decarboxylated, the tubes revert back to original purple colour, because of the alkaline amines produced during the reaction, which increase the pH of the medium.

Procedure:

- i. Inoculate the tubes of Moeller's decarboxylase medium containing appropriate amino acid (lysine / arginine / ornithine) along with a control tube without amino acid with bacterial isolates.
- ii. Overlay the tube containing amino acid with 2-3 cm mineral oil (liquid paraffin).
- iii. Incubate the tubes at 37°C and observe daily for 4 days for change of colour.

Interpretation: Purple colour (original colour of the medium, alkaline reaction) indicates decarboxylation of lysine and ornithine and positive reaction for arginine dihydrolase. Yellow colour indicates fermentation of glucose only and negative reaction for decarboxylase and dihydrolase.

O-Nitrophenyl- β -D-galactopyranoside (ONPG) hydrolysis test

Principle: The test demonstrates the ability of bacteria to ferment lactose. Two enzymes are involved in this activity. The permease permits the lactose molecule into the cell, while the β -galactosidase hydrolyses lactose to galactose and glucose. Some bacteria lack the ability to produce permease and possess the enzyme β -galactosidase. The ONPG, a compound similar to lactose molecule is hydrolysed by the enzyme β -galactosidase into galactose and o-nitro phenyl, which is a yellow compound.

Procedure:

Inoculate heavily, a tube containing ONPG broth with the bacterial culture

Incubate at 37°C for 1-2 h.

Examine the colour change of the broth from colourless to yellow.

Interpretation: Development of yellow colour indicates positive activity for β -galactosidase activity, (fermentation of lactose).

Nitrate reduction

Principle: Bacteria can assimilate inorganic nitrate into their proteins by virtue of one of the enzymes in a complex process called nitrate reductase, which converts nitrate to nitrite ($\text{NO}_3 \rightarrow \text{NO}_2$). NO_2 is detected by an inorganic assay using α -naphthylamine and sulfanilic acid.

Procedure:

- i. Inoculate test culture of bacteria to tubes containing about 2 ml of nutrient broth supplemented with 0.1% KNO_3 and 0.2% agar.
- ii. Incubate at 37°C for 24 h.
- iii. Add 1 ml each of α -naphthylamine solution and Sulfanilic acid solution.

Interpretation: Positive reaction (conversion of $\text{NO}_3 \rightarrow \text{NO}_2$) is indicated by development of pink colour.

Note: When there is no development of pink colouration, add a pinch of zinc dust. Absence of colouration indicates positive result for the reaction $\text{NO}_3 \rightarrow \text{NO}_2$.

Indole test

Principle: Indole is produced upon degradation of tryptophan by some bacteria. Production of indole is detected by formation of pink coloured compound when it reacts with an aldehyde such as p-dimethyl amino benzaldehyde.

Procedure:

- i. Grow bacteria in 1% peptone water broth for 24 h at 37°C.
- ii. Observe for turbidity indicative of growth.
- iii. Add 0.5 ml of Kovac's reagent to the broth culture of bacteria and shake gently.
- iv. Observe for development of pink colour.

Interpretation: Development of pink colour indicates a positive reaction.

Voges Proscauer's Test

Principle: The test detects acetoin or acetyl methyl carbinol, an intermediate product in the formation of butylene glycol during the metabolism of glucose. Acetoin is oxidised to diacetyl in the presence of oxygen by potassium or sodium hydroxide, which is a red coloured complex. Sensitivity of the test is further improved by addition of α -naphthol prior to addition of KOH.

Procedure:

- i. Grow pure culture of the bacteria in 5 ml of MRVP broth at 37°C for 48 h.
- ii. Transfer about 2.5 ml of culture to another tube.
- iii. Add 0.3 ml of alcoholic α -naphthol and 0.1 ml of 40% KOH solution, gently agitate the tube and allow to stand for 10-15'.
- iv. Observe for formation of red colour.

Interpretation: Development of red / crimson colour indicates that bacteria produce acetyl methyl carbinol.

Salt tolerance

Principle: Various species of *Vibrio* and related bacteria can be differentiated based on their ability to grow in the presence of different levels of sodium chloride.

Procedure:

Inoculate bacterial culture into nutrient broth tubes containing 0, 3, 6, 8 and 10% NaCl. Incubate at 37°C overnight and observe for growth, which is indicated by turbidity of the broth, compared to uninoculated control.

Sensitivity to O/129

Principle: *Vibrio* species are sensitive to 150 μ g of O/129 (2,4 di amino, 6-7 di isopropyl pteridine), while the other related genera like *Pseudomonas*, *Aeromonas*, *Plesiomonas*,

Alkaligenes, etc. are resistant. The test is done by using O/129 impregnated discs employing conventional disc diffusion method of Bauer *et al* (1966).

Preparation of O/129 discs: Prepare 7500 and 500 µg/ml (7.5 mg and 0.5 mg respectively) in sterile glass double distilled water. Spot 20 µl of these stock solutions onto sterile antibiotic discs to obtain discs containing 150 or 10 µg O/129. Dry the discs in a desiccator under aseptic conditions at room temperature. Store at 4°C till use.

Procedure: Test sensitivity of the bacterial isolates as the protocol given for antibiotic sensitivity testing.

Interpretation: Zone of inhibition of growth around the O/129 impregnated discs indicates susceptibility of bacterial isolate to O/129.

Antibiotic / Drug sensitivity testing

The antibiotic / drug sensitivity testing method employed is Kirby-Bauer's disc diffusion technique. Commercially available antibiotic discs are used for this purpose. The culture medium used for antibiotic sensitivity testing is Mueller-Hinton agar supplemented with 1% sodium chloride.

Preparation of the Inoculum:

Inoculate pure culture of bacteria into 5 ml Zobell's marine broth tubes with the help of sterile inoculation loop. Incubate for 2 to 8 h at 30°C till moderate growth is obtained.

Note: Obtain turbidity of broth culture (by diluting the culture using sterile sea water or sterile phosphate buffered saline, pH 7.4) equivalent to 0.5 ml of 1.175% BaCl₂.2 H₂O solution added to 99.5 ml of 0.36 N sulphuric acid.

Inoculation:

Dip a sterile swab into the inoculum and squeeze off the excess fluid by pressing the swab against the inside wall of the tube. Streak the entire agar plate thoroughly on the surface.

Application of antibiotic discs:

Apply discs onto the plates aseptically using sterile forceps. Press the discs firmly on the agar to enable smooth diffusion of antibiotic. Place the antibiotic discs at least 20 mm apart. Incubate the plates at 30°C.

Examine the plates after 24 h. Measure the zone of inhibition and record. See the zone interpretative chart given by the supplier of antibiotic discs and record as sensitive or resistant.

ESTIMATION OF TOTAL VIABLE BACTERIAL COUNT IN WATER SAMPLE

Estimation of total viable count (TVC) is used to determine the density of living bacteria in a sample. A simple serial dilution of the water sample followed by spread plate or pour plate method is employed to achieve this objective. The sample is diluted serially in sterile normal saline solution. The serial dilution helps to reduce the number of bacteria in the medium to manageable limit as the plates showing 30-300 colonies are considered countable. When a diluted sample is plated, the number of colonies produced can be used to calculate the original cell density in the sample using the formula as follows:

$$\text{Bacterial count (TVC)} = \frac{\text{No. of colonies observed}}{\text{ml of sample plated}} \times \frac{1}{\text{Dilution factor}}$$

Since it is virtually impossible to know if the colonies produced on plates originated with a single cell or a cluster of cells the term Colony forming unit (cfu)/ml is used instead of bacteria/ml.)

Example

Say the number of colonies counted on the plate = 55

Volume of diluted sample plated = 0.1 ml

Dilution factor in the tube from which the sample was taken for plating = 10^5

$$\text{Hence total no. of bacteria in the sample} = \frac{55}{0.1} \times \frac{1}{10^5} = 550 \times 10^5$$

$$= 5.5 \times 10^7 \text{ cfu / ml}$$

Culture Media, Reagents and Stains

Culture Media

- i. Zobell's Marine Agar
 - ii. *Aeromonas* selective medium
 - iii. *Pseudomonas* selective medium
 - iv. Zobell's Marine Broth
 - v. Nutrient broth.
 - vi. Thiosulfate Citrate Bile Salts Sucrose (TCBS) Agar
 - vii. Mycological agar
 - viii. Sabouraud's dextrose agar
 - ix. Marine Oxidation Fermentation medium (MOF)
 - x. Decarboxylase base (for testing decarboxylation of amino acids)
 - xi. MRVP medium
 - xii. Phenol red broth base
 - xiii. Amino acids: lysine, arginine and ornithine; NaCl, NaOH, and other required chemicals
- These items can be obtained as dehydrated powders from commercial sources.

Medium for isolation of bioluminescent bacteria:

Peptone:	5.0 g
Yeast extract:	3.0 g
Glycerol:	3.0 ml
Agar:	15.0 g
Distilled water:	250 ml
Aged sea water:	750 ml

Dissolve the ingredients and adjust pH to 7.8. Autoclave at 15 lb for 15 min, cool to 48°C, pour plates.

Peptone water:

Peptone:	1.0g
*NaCl:	0.5g
Distilled water:	100 ml

(*Increase the NaCl concentration to 1.0 to 1.5% when working with bacterial isolates of brackishwater or marine environment)

ONPG Broth:

1. ONPG Solution:

ONPG: 0.6 g

*Sodium phosphate buffer: 100 ml.

(*Dissolve 13.8 g Sodium phosphate (Na_2HPO_4) in 50 ml of warm distilled water in a volumetric flask. Add distilled water to make up to about 80 ml. Adjust the pH to 7.0 with 5N NaOH. Make the volume up to 100 ml. Filter sterilize the solution. Store in the refrigerator in a dark brown bottle)

2. Add 25 ml of ONPG solution to 75 ml peptone water. Dispense 0.5 ml volumes in sterile tubes.

Reagents

1. Reagents for nitrate reduction test:

Solution A : alpha-naphthylamine: 1 g
Distilled water: 20 ml
Dissolve, filter and add 180 ml of 5 N acetic acid.

Solution B : Sulfalinic acid: 0.5 g
5 N acetic acid: 150 ml.

2. Kovac's reagent for Indole test:

n-amyl or n-butyl alcohol: 150 ml
para dimethyl amino benzaldehyde: 10 g
Conc. HCl: 50 ml
Dissolve the aldehyde in alcohol and Slowly add acid.

3. Reagent for Voges Proscauer's test:

A : 5% alpha-naphthol in absolute alcohol
B : 40% KOH or NaOH.

Stains

For Gram's staining of bacteria

1. Solution A : Crystal violet: 2 g
Ethyl alcohol (95%) : 20 ml
Solution B : Ammonium Oxallate: 0.8 g
Distilled water: 90 ml
Mix both solutions and filter.

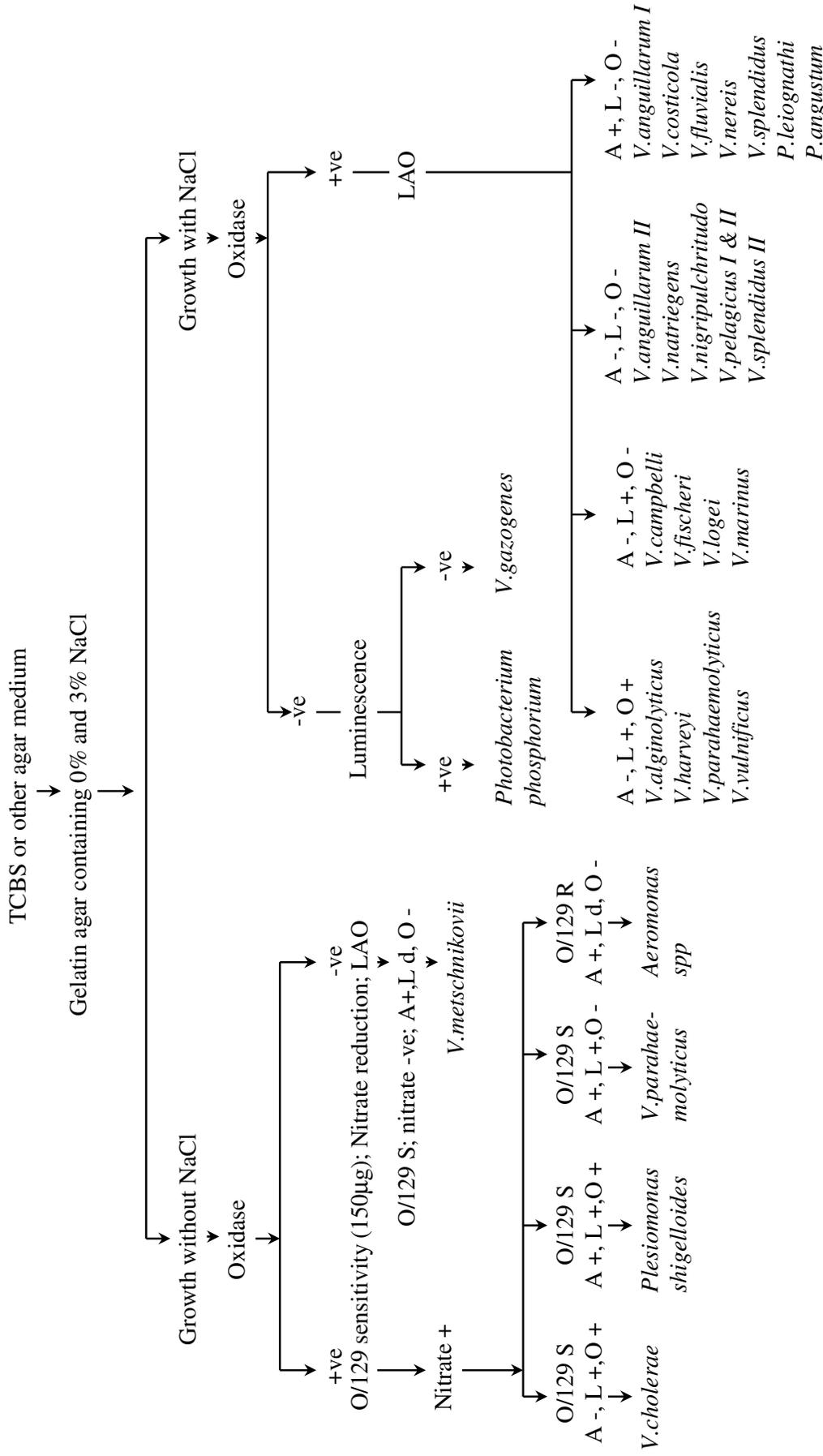
2. Gram's iodine:

Iodine: 1 g
Potassium Iodide: 2 g
Distilled water: 300 ml.

3. Safranin solution:

Safranin (2.5% solution in 95% ethyl alcohol): 10 ml.
Distilled water: 100 ml.

Scheme for preliminary identification of bacteria of family Vibrionaceae



A,L,O: arginine dihydrolase, lysine and ornithine decarboxylase; d: variable reaction ; S: sensitive; R: resistant

Source: Vibrios in Marine environment. West & Colwell eds. 1984.

Differential characteristics of *Vibrio*, *Aeromonas* and *Plesiomonas*

Characteristic	<i>V.cholerae</i>	<i>V.harveyi</i>	<i>V.campbelli</i>	<i>V.parahaemolyticus</i>	<i>V.alginolyticus</i>	<i>V.vulnificus</i>	<i>V.fluviialis</i>	<i>V.anguillarum</i>	<i>Aeromonas</i>	<i>Plesiomonas</i>
Lysine decarboxylase	P	P	P	P	P	P	N	N	D	P
Arginine dihydrolase	P	P	P	P	P	P	P	P	P	P
Ornithine decarboxylase	P	P	N	P	P	P	N	N	N	P
Gas from glucose	N	N	N	N	N	N	D	N	D	N
Acid from L-Arabinose	N	D	N	D	N	N	P	d	D	N
Acid from Inositol	N	N	N	N	N	N	N	N	N	P
Acid from Salicin	N	d	N	N	N	P	P	N	D	N
Acid from sucrose	P	d	N	N	P	N	P	P	P	N
Voges Proskauer test	d	N	d	N	P	N	N	P	D	N
ONPG hydrolysis	P	d	N	N	N	P	P	P	P	P
Growth at 43 C	P	d	N	P	P	P	d	N	D	P
Susceptibility to O/129: 10µg	S	d	S	R	R	S	R	S	R	d
Susceptibility to O/129: 150µg	S	S	S	S	S	S	S	S	R	S
Growth in 0% NaCl	P	N	N	N	N	N	d	d	P	P
Growth in 3% NaCl	P	P	P	P	P	P	P	P	P	P
Growth in 6%NaCl	N	P	P	P	P	P	P	d	N	N
Growth in 8%NaCl	N	d	N	P	P	N	d	N	N	N
Growth in 10% NaCl	N	d	N	N	P	N	d	N	N	N
Esculin Hydrolysis	N	N	N	N	N	N	d	N	D	N
Nitrate reduction	P	P	P	P	P	P	P	P	P	P
Growth in TCBS	Y	Y/G	G	G	Y	G	Y	Y	N	N

P:positive; N:negative; D: different reactions; d:delayed positive; Y:yellow; G:green; S:sensitive; R:resistant

HISTOLOGICAL TECHNIQUES AS DIAGNOSTIC TOOL IN SHRIMP DISEASES

K. K. Vijayan and S.V. Alavandi

Histology, the study of the microanatomy of specific tissue, has been successfully employed as a diagnostic tool in fish diseases, as is the case in medical and veterinary sciences. Knowledge about the pathological manifestations plays a very important role in the diagnosis of shrimp diseases. Many of the recognised diseases of penaeid shrimp, especially viral diseases, were first recognised and diagnosed by routine histological procedures. Even today, pathological manifestations based on histological sections stained with hematoxylin & eosin form the most important tool in shrimp disease diagnosis.

Collection and preparation of materials/samples

For histological studies, the tissue samples should be removed from living, anaesthetised animal by biopsy. For the study of diseased shrimp select those, which are moribund, discoloured and displaying abnormal behaviour, except in case of intentional random sampling for estimation of disease prevalence.

Fixation of samples

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 after being taken out from the specimens, fresh or dead, to avoid rapidly setting post-mortem changes. 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, 5-10 times the volume of fixatives should be used for each specimen. 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 Alcohol Formalin Acetic acid (AFA) as the fixative. However, Neutral Buffered Formalin (NBF) is also recommended. The fixatives can be prepared as follows.

Davidson's Alcohol Formalin Acetic Acid fixative (AFA)

95 % Ethyl alcohol	330 ml
Formalin	200 ml
Glacial Acetic acid	115 ml
Distilled Water	335 ml

- Fixation time 24 - 72 h at room temperature. Then transfer to 50-70 % ethyl alcohol for storage.

Neutral Buffered Formalin (NBF)

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

- Fixation time 24 h to indefinite.

Out of these two fixatives, Davidson's fixative is the best for shrimp histology. Larvae and early post larvae can be directly immersed in the fixative. Juveniles and adult shrimps should be injected with 1-10 ml (depending on the size of shrimp) of fixative into hepatopancreas, region anterior to hepatopancreas, anterior abdominal and posterior abdominal regions. A large share of fixatives should be injected into the cephalothoracic region and posterior abdominal region. The amount of fixative can vary approximately 5-10 % of body weight. After the injection, cut open the cuticle from sixth abdominal segment to the rostrum with a sharp scissors, without damaging the internal organs. The specimens should be immersed in 5-10 volumes of fixative (i.e. tissue of 1 ml volume require 10 ml fixative) for 24 h. For large animals, fixation can be done even up to 72 h. After that, the specimen is transferred to 50 % ethyl alcohol for storage.

Complete history of the specimens such as gross observation, species, age, weight, source etc. and any other pertinent information helpful in diagnosis should be recorded.

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.

Decalcifying solution

70 % ethyl alcohol	98ml
Conc. Nitric acid	2ml

After proper decalcification, wash the tissue in 70 % ethyl alcohol for 2-3 times and store in fresh 70 % ethyl alcohol.

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

Dehydration

In order to infiltrate with paraffin wax it is first necessary to remove all water from the fixed tissues by dehydration. Dehydration is a process of gradual or stepwise replacement of water by a graded dehydrating agents and it is usual to begin with 50-70 % ethyl alcohol, through progressive higher grades of alcohol to saturate the tissue with absolute alcohol to complete the dehydration, as shown below.

50 % alcohol	1h
70 % alcohol	1h
90 % alcohol	1h
100 % alcohol	30min. X 2

Clearing

As alcohol is not miscible with paraffin wax, it is first necessary to treat the tissue with an agent, which is miscible with both the substances. There are several such reagents in general use of which xylene (or chloroform, toluene, benzene, methyl benzoate, clove oil, cedar wood oil etc.) is the most favoured. The optimum time, for which the tissue should be kept in a clearing agent, is indicated by the shine or transparency of the tissue (1-2 h).

Absolute alcohol + Xylene (1 : 1)	1h
Xylene	1h X 2

Infiltration / Impregnation

The aim of impregnation is to make the tissue firm for the purpose of sectioning with microtome.

Paraffin cold impregnation

Xylene and paraffin shavings (1: 1) 1h

Hot impregnation

Transfer the tissue in the 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.

Embedding

The method of embedding or reinforcement of tissue is done using paraffin wax (or celloidin, gelatin etc.). After proper paraffin infiltration, the tissues can be transferred to appropriate blocks (depending on the size of the tissues) containing molten paraffin. We use a histoembedder (Leica, Germany) in our lab for impregnation in molten paraffin wax, dispensing molten wax for block preparation. Extreme care should be taken to get the correct orientation of the tissue and to avoid air bubbles. Allow the paraffin to solidify and remove the paraffin block containing tissue.

Labelling and storage

Labels with concise information on a small paper in lead pencil, is generally inserted on one side of the block during casting. Store the blocks in thick ziplock polybags or wooden boxes with cloth lining or alternatively in a mixture of equal volume of 70 % alcohol and glycerine in well stoppered bottles.

Sectioning

Sections of the tissues can be taken using a microtome. Before sectioning, the tissue-embedded paraffin blocks should be trimmed to suitable size. Care should be taken to see the proper orientation of the tissue. Fix the trimmed block on to a holder and take the sections in the form of a ribbon of appropriate thickness. Sections of 5 - 7 µm thickness are good for routine histopathological studies. Two main features govern satisfactory sectioning of tissues, a clean and sharp knife and reduction of the temperature of the block by keeping in freezer for few hours, which increase its hardness.

Spreading

The resulting ribbons containing tissue sections can be cut into smaller pieces, put on a clean glass slide, which is coated with egg albumin. One slide can hold one or more ribbons according to the size of the tissue or the width of the ribbon. Proper spreading of the ribbon can be done in two ways

1. Small pieces of ribbon can be put in a water bath containing warm water (or a tissue floating bath). When the ribbon gets spread due to the high temperature of water, put a clean albumin coated slide underneath the ribbon and just lift the slide in such a way that the ribbon sticks to the surface of the slide. Drain the water and keep the slide in a slanting position on a slide rack free from dust.
2. Cut the ribbon into small pieces; keep one or more ribbons over the slide coated with adhesive, put a few drops of water on the slide so as to float the ribbon on the water surface. Place the slide on a slide-warming table or pass it over a flame of spirit lamp. Two needles can be used to spread the ribbon to the maximum, drain the water and keep the slide in a slanting position.

Whichever the method we follow, proper care should be taken to avoid wrinkles in the section. Improper spreading will interfere with staining and also microscopic observation. After proper drying, slides can be kept in a dust-proof box for some time for adequate adhesion. These slides can also be stored indefinitely.

Staining

Before the tissue sections are subjected to staining, the sections should be deparaffinized thoroughly and dehydrated. Hematoxylin and eosin 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 H & E are as follows.

A. Harris' haematoxylin

Haematoxylin crystal	5.0 g
100% alcohol	50.0 ml
Ammonium / potassium alum	100 g
Dist. 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 min.

B. Eosin

1% aqueous eosin or alcoholic eosin

Procedure

De-paraffinise the slide in xylene	1h X 2
Absolute alcohol	15 min X 2
90% alcohol	15 min
70% alcohol	15 min
50% alcohol	15 min
Water	10 min X 2
Stain in hematoxylin	2-5 min
Wash in water	adequately
De-stain in acid alcohol (if needed)	adequately
Wash in tap water	1 min
50 % alcohol	30 min
70 % alcohol	15 min X 2
Stain in Eosin	1 min
90 % alcohol	15 min X 2
Absolute alcohol	20 min X 2
Alcohol + Xylene (1 : 1)	15 min
Xylene	30 min X 2

Mount in DPX and label

Observe under microscope

Histopathological characteristics of viral diseases of shrimp

Although numerous viral diseases reported from penaeid shrimps, only three are prevalent in India. Following are the account of histopathological characteristics of these important viral diseases.

Monodon baculovirus (MBV) infection

Shrimp infected with MBV often appear clinically normal. Hepatopancreas is the target organ of this virus. The pathological changes include focal to extensive necrosis of tubular epithelium of hepatopancreas. Single to multiple intra-nuclear, eosinophilic occlusion bodies can be detected in the epithelial cells. In case of severe infection, necrotic tubular lumen may be seen filled with the sloughed epithelial cell debris and viral occlusion. Due to the enlarged multiple occlusion bodies, the nuclei will appear hypertrophied with fragmentation and margination of chromatin material. Very often, hypertrophied nuclei may appear as 'signet ring' with peripherally displaced and compressed nuclei. Thus, hypertrophy

of the nuclei with subsequent cellular destruction and desquamation of epithelial cells will be the obvious histological changes.

Histopathology of white spot disease

Histopathologically, WSV is characterized by wide spread and severe nuclear hypertrophy, chromatin margination and eosinophilic (in the early stages the inclusion will be eosinophilic with a hyaline space between the inclusion and the nuclear wall and is known as Cowdry A-type inclusion) to large basophilic (in the later stage, the inclusions stain deeply basophilic and fills the entire hypertrophied nuclei) intra-nuclear inclusions. Similar but variable multifocal necrosis will be observed in all the tissues originated from ectoderm and mesoderm. The important target organs are: connective tissues, sub-cuticular epidermis, stomach, foregut and hindgut epithelium, heart, striated muscle, midgut, ovary walls, antennal gland and nervous tissues.

Histopathology of Infectious Hepatopancreas and Lymphoid organ necrosis (IHLN)

The causative agent has been not identified conclusively. However, preliminary studies indicate the involvement of a viral pathogen and the disease is presumptively diagnosed as IHLN based on the characteristic histopathological changes. Histologically the most prominent feature of the disease is the acute damage observed in the hepatopancreas. They include multifocal necrosis in the tubular epithelium marked by hemocytic infiltration and encapsulation resulting in melanization. Lymphoid organ also show marked necrosis associated with the degeneration of stromal matrix cells. In the case of secondary bacterial infection histological sections of lymphoid organ may show bacterial colonies in the necrotic area.

Biochemistry of Nucleic Acids with reference to polymerase chain reaction

T.C. Santiago, K.K. Vijayan, S.V. Alavandi and N. Kalaimani

Introduction

The primary aim of this lecture is to give a brief introduction to the basic structure and physical and chemical properties of nucleic acids so as to easily understand the in vitro amplification of DNA through Polymerase Chain Reaction (PCR). The knowledge on these basic principles will help to manipulate the reaction to suit ones need and effectively use the PCR technology in shrimp disease diagnosis. This will also help in understanding and solving the various problems one may face during the application of this technology. Therefore, I have avoided the cumbersome chemistry of the nucleic acids, but the most relevant principles that are required for the understanding the PCR are discussed and explained.

DNA was isolated way back in 1869

DNA was first isolated from pus cells and from salmon sperm by Friedrich Meiescher in 1869. Since it was isolated from nuclei it was called nuclein. DNA from different cells and viruses vary in their nucleotide sequence, nucleotide ratio and molecular weight. In fact nucleic acids are the major component of the cell. The genomic content of the cell varies from 0.01 pg in prokaryotes to 0.3 to 10 pg in higher plants and animals. However, the vast majority of the nucleic acid in cells is present as complexes with proteins. Prokaryotic DNA forms complexes with polyamines and proteins while eukaryotic DNA is associated with histones and various non-histone proteins. The amount of DNA in any given species of cell or organism is constant and can not be altered either by environmental or nutritional or metabolic conditions. The germ cells (sperm/egg) of higher animals possess only one half of the amount of DNA found in somatic cells of the same species.

Nucleotides are the building blocks of Nucleic acids

Just as the amino acids are building blocks of peptides (proteins), the nucleotides are the building blocks for nucleic acids. The monomeric units of DNA are called deoxyribonucleotides. Each of nucleotide contains three characteristic components. (a) a heterocyclic nitrogenous base, derivative of either a pyrimidine or purine. (b) a pentose sugar molecule and (c) a molecule of phosphate. There are four different deoxyribonucleotides

which serve as the major building blocks of DNA macromolecule. They are all similar except for the nitrogenous base. Each nucleotide is named after the base. The purine derivatives are adenine (A) and guanine (G) while the pyrimidine derivatives are cytosine (C) and thymine (T). Similarly, four different ribonucleotides are the building blocks for the RNA. They are the purine bases adenine and guanine. The pyrimidine bases are cytosine and uracil (U). The pentose sugar is different in DNA and RNA, DNA contains 2-deoxy ribose sugar, while RNA contains ribose sugar.

Nucleic Acids exist in different forms

Nucleic acids exist in two major types, namely DNA and RNA. Though DNA exists in one type they exist in different forms, as linear, circular, single stranded and double stranded forms. On the contrary mostly RNA exists as single stranded form. There are three types of RNA present in a living cell- messenger RNA (mRNA), Ribosomal RNA (rRNA) and transfer RNA (tRNA). Even though RNAs are single stranded, they form extensive secondary structures as in the case of tRNAs and rRNAs. Messenger RNAs of eukaryotic cells are unique that they contain long stretches of poly (A) sequences at the 3' ends (the carbon atoms are numbered by adding prime to the number, for ego 2nd carbon atom in the sugar is written as 2', 3rd as 3' etc). In general DNA contains the genetic information; however, in certain viruses RNA contains the genetic information.

DNA/RNA are formed by covalent links of Deoxy/Oxy ribonucleotides

A nucleic acid is polynucleotide - that is a polymer consisting of nucleotides. The pentose sugar is a cyclic five carbon ribose sugar in case of RNA and 2'deoxyribose sugar in the case of DNA. A purine or pyrimidine base is attached to the 1' carbon atom of the pentose sugar by an N-glycosidic bond. A phosphate is attached to the 5' carbon of the sugar by phosphoester bond. It is this phosphate which gives the strong negative charge for the nucleotides and the nucleic acids. This property is used in agarose gel electrophoresis of nucleic acids. The nucleotides in nucleic acids are covalently linked by a second phosphoester bond that joins the 5' phosphate of one nucleotide and the 3' OH group of adjacent nucleotide. This phosphate plus its bonds to the 3' and 5' carbon atoms is called a phosphodiester bonds.

DNA exhibits base equivalence

Edwin Chargaff and his colleagues using quantitative chromatographic separation methods analyzed the base composition of nucleic acids and proposed the Chargaffs rule for DNA. (1) The base composition of DNA varies from species to species. (2) DNA specimen isolated different tissues of the same species have the same base composition. (3) The base composition of DNA in a given species does not change with age, nutritional state or changes in environment. (4) The number of adenine residues is always equal to the number of thymine residues, A=T. Similarly Guanine residues is always equal to cytosine residues, G=C. (5) DNA extracted from closely related species have similar base composition whereas those of widely different species have widely different base composition.

Watson and Crick model of DNA

In the year 1953 Watson and Crick proposed a structure for the DNA based on the crystallographic structure. It was an epoch making proposal, which revolutionized the world, for which they got the Nobel Prize. The structure proposed by Watson and Crick explained the long known Chargaffs rule. The DNA exists in a double helix state. The two strands run in opposite direction (anti parallel). The polarity of the DNA strand is 5' to 3'. These two strands are held together by Hbonding between complementary N- bases. A bonds with T and G bonds with C. Phosphate and sugar form backbones on the outside and hydrophobic N-bases are inside, stacking on top of one another. The double helix is 20A in diameter. The helix makes a complete turn within 10 nucleotides at a distance of 34A. The raise per nucleotide is 3.4A. It is important to note that nucleic acids have polarity. This means that their ends (termini) are not the same. One end of the polynucleotide chain bears a 5' phosphate group whereas the other end bears a 3' hydroxyl group. By convention the sequence of the nucleic acid is written in the 5'-3' direction, the 5' terminus always being to the left.

DNA can exist in different forms

The geometry of the DNA double helix was deduced from X-ray diffraction studies using DNA fibers. Analysis of such patterns revealed the existence of three different conformations of the DNA. They are called as A-DNA, B-DNA and C-DNA. The Watson and Crick model is nothing but the B-DNA. A-DNA differs from the B structure in the following respects. The base pairs, although parallel to one another and spaced by the same amount (0.30nm) as in the

B form, are inclined by about 20 degrees to the planes perpendicular to the helix axis. The number of bases per turn is 12 and hence the structure is fatter, but longitudinally more compact. The two grooves that run around the outside helix are approximately the same size in A-DNA. But the grooves vary in their size in B-DNA, called major and minor grooves. The base pairs overlap in B-DNA such that the top view of the helix appears to be full of base pairs but A-DNA has a hole down the middle. C-DNA is a distorted B structure with a non-integral number of bases per full turn and the base pairs are somewhat more inclined to planes parallel to the axis. There is also another form of (synthetic) DNA the Z-DNA. It is left handed. The base pairs are inclined to 7 degrees and there are 12 base pairs per full turn. The biological significance of Z-DNA is still uncertain.

Secondary structure of DNA is drastically different from the primary structure

Linear nature of a DNA sequence is regarded as the primary structure. In the case of DNA, secondary structure consists of two independent, covalently linked chains coiled around a common axis and forms double helix. In the case of RNA molecule which is primarily single stranded, a number of intra-strand H-bonding can be found. This intra strand bending of the helix axis in different directions is called the secondary structure. They are termed differently as hairpin loops, hairpin bends, stem loops cruciform etc. Secondary structures of RNA are believed to play important biological roles such as recognition regions for certain enzymes.

DNA is stabilized by various forces

What holds the DNA double helix together? One of the factors is the hydrogen bonding. The Watson-Crick Model is thermodynamically correct. AT base pairing has two hydrogen bonding, while G:C pairing has three hydrogen bonding which is more stable than the former. Hydrogen bonding is not the only stabilizing factor. Hydrophobic interactions in base stacking interactions between aromatic rings inside the helix are the major stabilizing forces against repulsion by negatively charged phosphates. The presence of counter ions such as Mg^{2+} and K^{+} also play a role in stabilization of DNA double helix.

DNA is very stable compared to RNA

Compared to DNA, RNA is less stable. This is primarily due to the single stranded nature. The nature of the pentose sugar also plays a role in the stability. RNA can be easily

hydrolyzed by dilute alkali. The sugar moiety contains 2' hydroxyl group. Dilute sodium hydroxide produces a mixture of nucleosides, 2' and 3' phosphates. Cyclic 2', 3' monophosphates are the first products of the action of alkali on RNA. They are further hydrolyzed by alkali, which attacks either one of the two P-O-C linkages to yield a mixture of 2' and 3' nucleoside phosphates. Since DNA has no 2' OH group it can not be hydrolyzed by alkali.

DNA can be denatured by heat and acids but not by alkali

Gentle acid hydrolysis of DNA at pH 3.0 causes selective hydrolytic removal of all its purine bases without affecting the pyrimidine deoxyribose bonds or the phosphodiester bonds of the backbone. The resulting DNA derivative, which is devoid of purine bases is called apurinic acid. Selective removal of the pyrimidine bases, accomplished by some what different chemical conditions produces apyrimidinic acid. As mentioned above alkali cannot hydrolyze the DNA. Nucleases also hydrolyze the nucleic acids. Nucleic acids can be denatured by heating. This process is called melting. When nucleic acid is denatured the stacking is lost and hence the UV-absorbance increases. This increase is called hyperchromic shift. For total denaturation of the double stranded DNA, the hyper chromic effect is of the order of 30%. The temperature at which the solution contains 50% denatured and 50% double stranded DNA is called the melting temperature (T_m). The value of T_m is the function of the nature of the DNA, ions in the solutions and the ionic strength. Renaturation is not simply the reversal of denaturation. If the denatured DNA solutions are maintained 5-10 °C below the melting temperature, the complementary strands will slowly reassociate and the double helical structures will reform. The whole process is concentration dependent. Thermal denaturation is one of the properties exploited in polymerase chain reaction (PCR).

DNA replicates by semi-conservative mechanism

The most striking feature of the Watson-Crick model of DNA, *from* the genetic point of view is, that the two strands of double helical DNA are complementary. The replication of each to form new complementary strands results in formation of two daughter duplex DNA molecules, each of which contains one strand *from* the parental DNA. This process is called semi conservative replication. This model was conclusively proved by Meselson and Stahl in 1957 by ingenious experiment using bacteria. The same is true in other dividing cells.

DNA polymerase is the key enzyme in DNA replication

The enzymatic mechanism by which the DNA is replicated was elucidated by A. Kornberg and his colleagues in 1956. The enzyme involved in this process is DNA polymerase I. Later it was found out that, other enzymes, (Pol II and Pol III) were also involved in replication. Now it is shown that Pol III is the major enzyme concerned in the replication process, although Pol I participates. This also functions in repair of DNA. It also has the 3'-5' and 5'-3' exonuclease activity. The most striking and the characteristic property of DNA polymerase is that it requires the presence of some pre-existing DNA called primer, in the absence of which the purified enzyme will not be able to make any DNA at all.

Pre existing DNA primer and template is essential for DNA replication

Okazaki and his colleagues discovered that nascent DNA occurs in short pieces, called Okazaki fragments. These fragments are found in viral, bacterial and eukaryotic cells during DNA replication. Replication of DNA in short steps is a device that permits replication of both strands of DNA by DNA polymerase that replicates only in 5'.3' direction. These short pieces are quickly joined by covalent bonds. It was also shown that the purified DNA polymerase can not utilize the native DNA strand as a primer. Therefore, DNA replication is preceded by the formation of a short strand of RNA complementary to a section of double strand DNA. This priming RNA is generated by a DNA directed RNA polymerase. Once the priming RNA strand has been made, DNA polymerase begins to add nucleotides to form DNA from 5'.3' direction. This is the principle that is involved in the necessity of primers for the PCR.

RNA too can act as a template for DNA synthesis.

There are many viruses whose genetic material is made of RNA. These viruses replicate their genome via synthesis of DNA. This is mediated by an enzyme called reverse transcriptase (RT). This enzyme was first isolated by Temin and Baltimore. It is primarily an RNA dependent DNA polymerase. Such enzymes are purified from RNA tumor viruses. This enzyme is used in molecular biology to synthesize complementary DNA (cDNA) from mRNAs. They are also used to amplify RNA viral genes through RT-PCR. This technology is used for detecting the presence of RNA viruses.

Polymerase Chain Reaction (PCR) is the result of successful exploitation of the properties of DNA and its replication.

DNA polymerase uses single stranded DNA as a template for the synthesis of a complementary new strand. These single stranded DNA templates can be produced by simply heating double stranded DNA to temperatures near boiling. DNA polymerase also requires a small section of double stranded DNA to initiate (prime) synthesis. Therefore the starting point for DNA synthesis can be specified by supplying an oligonucleotide primer (a small piece of DNA with 15-40 nucleotides) that anneals to the template-DNA at that point. This is the first important feature of the PCR- that DNA polymerase can be directed to synthesize a specific region of DNA. Both DNA strands can *serve* as templates for synthesis, provided an oligonucleotide primer is supplied for each strand. For a PCR, the primers are chosen to flank the region of DNA that is to be amplified so that the newly synthesized strands of DNA, starting at each primer, extend beyond the position of the primer on the opposite strand. Therefore, new primer binding sites are generated on each newly synthesized DNA strand. The reaction mixture is again heated to separate the original and newly synthesized strands, which are then available for further cycles of primer hybridization, DNA synthesis and strand separation. The net result of a PCR is the amplification of the DNA-molecules in geometric proportion. This is all possible due the DNA polymerase from a heat stable bacterium *Thermus aquaticus* (Taq). Using this technology it is now possible to synthesize DNA in the purest form, which is the basic requirement for any genetic manipulations. PCR technology now forms the most preferred, rapid, sensitive and specific diagnostic tool both in humans and animals.

Conclusion

The basic knowledge on the structure and function of nucleic acid is the basis of many revolutionary developments in biology. PCR is one such technology that has revolutionized molecular biology. The deeper understanding of the chemistry of nucleic acids will help not only to understand the novel technologies but also to develop new technologies in future.

**Molecular diagnosis in shrimp disease with special reference to PCR of
Indian white spot virus**

T.C. Santiago, K.K. Vijayan, S.V. Alavandi and N. Kalaimani

Introduction

Increasing disease problems mars the growth of promising aquaculture industry both nationally and internationally. Vulnerability of the aquaculture production system to disease is due to the co-existence and close interaction of host, pathogen and environment. A small shift of equilibrium between these three can trigger a disease outbreak leading to mortality resulting in crop failure. The latest viral disease problem in shrimp farming arena due to the white spot virus exposed the vulnerability of aqua-business.

Disease problems are inevitable, as aquaculture has to look forward to produce more animal protein, more jobs and more revenues for the people. To tackle the menace of disease problems, a scientific health management approach has to be developed emphasizing the conventional wisdom - 'prevention is better than cure'. An integral part of such a program is the use of diagnostic tests at the strategic point of production cycle to eliminate or control the disease causing pathogens.

Conventional diagnostic methods

The conventional diagnostic methods practiced in aquaculture are mostly adapted from the field of human health and veterinary sciences. Among the diagnostics mentioned (Table 1), visual examination, microscopic, histological examination and bacterial examination are the most widely used and still form the essential part of disease diagnosis.

But these methods often fail to deliver data in time to support a decision making in the health management to salvage the crop. This is mainly due to the time consuming and laborious methodologies and the inability of these tests to detect sub-clinical / latent / carrier state of infection.

DNA - based diagnostics

Developments in molecular biology enabled researchers to collect information on the genetic material that serves as the blueprint for all living organisms. The most recent development in diagnostics have utilized molecular biology to design new generation of diagnostics tools, the Polymerase Chain Reaction (PCR) and Gene Probes. These DNA-based diagnostic tools stand out among other conventional diagnostic methods with its speed, sensitivity and simplicity. Polymerase Chain Reaction and Gene probes capable of identifying

a number of viral, bacterial and parasitic pathogens are finding their way into the area of infectious disease diagnosis in aquatic species.

Table 1: Methods available for disease diagnosis and pathogen detection

Method	Tests and data obtained
History	History of disease at facility or region, facility design, source of seed, type of feed used, environmental conditions etc. Gross, clinical signs, lesions visible, behaviour, abnormal growth, feeding or food conversion efficiency, etc.
Direct microscopy	Bright-field, phase contrast, or dark field examination of stained or unstained tissue smears, whole-mounts, <i>etc.</i> of diseased or abnormal specimens
Histopathology	Routine histological or histochemical analysis of tissue sections
Electron microscopy	Ultrastructural examination of tissue sections, negatively stained virus preparations, or sample surfaces
Culture and biochemical characterization	Routine culture and isolation of bacteria and identification using biochemical reactions
Enhancement	Rearing samples of the appropriate life stages under controlled conditions to enhance expression of latent or low grade infections
Bioassay	Exposure to potential pathogens
Serological methods	Use of specific antibodies as diagnostic reagents in immunoblot, agglutination, ELISA, IFAT, or other tests.
Tissue culture	In vitro culture of pathogens in cell lines
DNA probes	Detection of unique portion of a pathogen's nucleic acid using a labelled DNA probe
PCR	Amplification of unique portion of a pathogen's genome to readily detectable concentrations using specific primer pairs

The key to the DNA based diagnostics is the generation of unique genetic information of the target pathogen through recombinant DNA technology. This is done by purifying the infectious agent of interest (Fig. 1) and isolating the nucleic acid. Isolated DNA is then subjected to restriction digestion and cloning. From the selected clones, desired DNA fragment has to be sequenced. Once the adequate genetic information (sequence information) is generated, the information can be used in PCR or gene probes.

Polymerase Chain Reaction (PCR)

PCR is relatively a simple technique by which a DNA or cDNA template is amplified many thousand or a million fold quickly and reliably in a short period of 3-4 hours. So far no other technique has equalled PCR in sensitivity, which is about one DNA target molecule.

A typical amplification reaction includes

- I. The sample of the target DNA
- II. Two oligonucleotide primers
- III. Four Deoxynucleotide triphosphates (dNTPs)
- IV. Reaction buffer
- V. Magnesium and optional additives
- VI. Taq-DNA polymerase
- VII. dd H₂O

The components of the reaction are mixed and placed in an automated instrument called thermocycler that takes the reaction to a series of different temperatures for varying amounts of time. This series of temperature and time is referred to as one cycle of amplification. In each cycle of amplification the quantity of target DNA doubles, and as few as 20 cycles would generate approximately a million times the amount of target DNA which was present initially.

The first step of PCR involves thermal denaturation of the double-stranded target DNA molecules. The next step is the annealing of oligonucleotide primers to the complementary target sequences by temperature reduction. Thereafter, primer directed DNA synthesis reaction will follow with the help of thermostable DNA taq polymerase, resulting in the doubling of the amount of target sequence in the sample. By repeating the cycle of denaturation, primer annealing and DNA synthesis (primer extension), the copy number of the target DNA is increased exponentially.

Once a product is obtained, it can be analyzed in a number of ways like agarose gel electrophoresis. The products will be readily visible by UV transillumination of an ethidium bromide stained gel.

Nested PCR

Nested PCR or two-step PCR is useful in reducing or eliminating unwanted products simultaneously increasing the sensitivity significantly. An aliquot of the first PCR product is then subjected to an additional round of amplification using primers complementary to the sequences internal to the first set of primers. Only the legitimate product is amplified in the second round. This approach of two-step amplification is often successful even if the designed product is initially below the level of detection by ethidium bromide staining.

Optimization of PCR

Designing of an ideal primer pair, optimisation of the concentration of Mg, primer and template DNA, buffer pH and cycling conditions are important for the success of a PCR. Ideally PCR primers should have 40 - 60 % G+C content, which generally range in length

from 15-30 bases. Negative controls are mandatory in each PCR run to rule out any false-positive results caused by contamination. However, every step should be taken to avoid the possible contamination during the setting up of a PCR.

Nucleic Acid Probes

Nucleic acid probes are segments of DNA or RNA that have been labelled with enzymes, antigenic substances, chemiluminescent substances or radioisotopes. Probes can be directed to either DNA or RNA targets. Probes can bind with complimentary sequences of pathogenic DNA during the detection process providing a signal (like colour change) that can be identified or measured. Nowadays, non-radioactive probes (eg: digoxigenin (DIG) labelled probes) are gaining importance due to their high level of sensitivity and safety as compared to the radioactive probes. The *in-situ* hybridization and dot blot hybridization are examples of gene probes, which are finding its use in aquatic disease diagnostics. However, PCR has advantages over the gene probes in its sensitivity to be used for direct detection in clinical specimens.

PCR - based diagnosis of Indian white spot virus

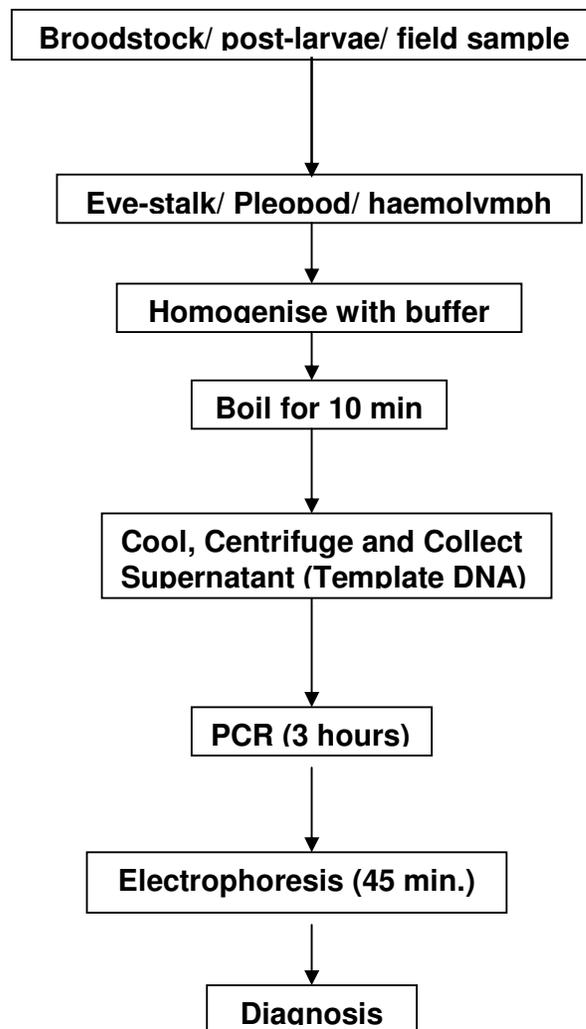
In the aquaculture of penaeid shrimps, White Spot Disease (WSD) caused by White Spot Virus (WSV) is the major cause of morbidity and mortality today in Asia, resulting in huge economic losses for shrimp farmers. Among all the recorded viral diseases in cultured penaeids, WSD is the most widespread. The rapid onset and lethality of WSV has put Asian shrimp farming as a whole at the breaking point. The WSV was first reported in 1992 from Taiwan, subsequently from most shrimp farming countries in Asia and recently from the western hemisphere.

White spot disease has diminished the prospects of shrimp farming in India. This is the most virulent virus known to affect cultured shrimps. Till date, no treatment is known to control the White Spot Disease. Hence, early diagnosis followed by suitable management practices is the only alternative in tackling this disease. Diagnosis of WSD can be done by methods such as histopathological techniques. A presumptive diagnosis can also be done by observing clinical symptoms such as the presence of white spots. These methods can detect the WSD only in the late stage of infection. The PCR is a powerful and sensitive diagnostic tools for identification of viral pathogens even at a very early stage (asymptomatic / carrier stage) of infection.

Purification of Virus, DNA extraction and sequencing

In the case of white spot virus, ectodermal or mesodermal tissues of the shrimp infected with WSV can be used for viral purification. The purity of the virus is checked using 2 % PTA stained TEM. Viral DNA isolation is done using proteinase K and CTAB treatment followed by phenol-chloroform extraction and ethanol precipitation. After checking the purity of the DNA using electrophoresis, sequence information of WSV is generated following cloning and sequencing of WSV genome.

Simplified scheme of diagnosis of the Indian white-spot disease by polymerase chain reaction in 4-6 h.



PCR Primers for Indian WSV

Two sets of PCR primers have been designed from the sequencing information of a fragment of Indian White Spot Virus, for the 1st step and Second step (nested) PCR amplification by scientists of CIBA, with products of 600 bp and 300 bp respectively.

PCR – Protocol

PCR and agarose gel electrophoresis are used in conjunction to determine the presence or absence of WSV virus in shrimp. The standard operation procedure consists of:

I. DNA – template preparation / nucleic acid extraction

Genomic DNA extracted from the infected animal tissue or DNA-template preparations using simple methods like boiling of the sample with a suitable buffer can be used as a template for WSV PCR. Samples used for template preparations can be stored frozen or preserved in 70-95 % ethanol. The template can be stored frozen at - 20 to -70 °C.

II. PCR preparation and reaction

1. Prepare a master mix of the following components aliquot into individual 25 µl PCR reaction vials.

dd-H ₂ O	12 µl X number of samples
10 X buffer	2.5 µl X number of samples
dNTP solution*	4 µl X number of samples

Prepare solution by adding 10 µM stock solutions of dNTPs into the ratio of
One part dATP: 1 part dGTP: 1 part dCTP: 1part dTTP: 4 parts ddH₂O

Primer A (µM)	1µl X number of samples
Primer B (µM)	1µl X number of samples
25 mM MgCl ₂	1.5µl

22 µl master mix per vial

To each PCR reaction vial containing 22 µl master mix, add template DNA (5ng/µl): 2µl

2. Program thermal cycler as follows:

- 1 Cycle of: 95 °C for 3 minutes
- 30 Cycles of: 95 °C for 0.5 minute
- 58 °C for 1minute

72 °C for 1minute
1 cycle of: 72 °C 5 minutes

3. Place the reaction tubes in thermal cycler, heat to 95 °C and hold at that temperature.
4. Add 1µl (1 µl of a 0.5 U solution) Taq polymerase to each sample. Resume program.

When program is complete, remove samples, add 5µl of loading buffer to each sample mix well and spin briefly.

Agarose gel electrophoresis

1. Assemble large gel electrophoresis box to run a maximum of 20 samples.
2. Prepare a 2.0 % agarose gel by adding 1.46 grams of agarose to 73 ml of 1 X TBE buffer*
(*1 X TBE buffer is prepared by diluting 5 X TBE. 5 X TBE is prepared by adding 54.0 grams Tris base and 27.5 grams boric acid to 980 ml of dH₂O. Add 20 ml of 0.5 M EDTA (pH 8.0). Filter solution through a 0.45-micron filter).

3. Cover gel with 1 X TBE and load samples. Load 5 µl of PCR marker into one lane of the gel.

Run the gel at 80 volts for about 1.5 hours, or until the loading dye lane runs off the anode side of the gel.

4. Place gel in a 0.5 µg/ml ethidium bromide dye solution for 15 to 20 minutes.
(A 0.5 µg/ml ethidium bromide solution is prepared by adding 10 µl of a 1% stock solution to 200 ml of 1 X TBE buffer).
5. Remove the gel from ethidium bromide solution and rinse TBE buffer. Place gel on a UV transilluminator with filter 312 nm to visualize PCR product and photograph.

Conclusion

Molecular diagnostic technique like PCR provides a fast, sensitive and specific tool for disease diagnostics in aquaculture. The selective amplification of a small segment of pathogenic DNA from the mass of unrelated host DNA sequences to the detectable level, and that too from an extremely small quantity of the starting material, makes PCR a revolutionary technology. The PCR has a wide and significant use in checking the entry of lethal pathogens like virus to the hatchery and growout system by helping to select healthy broodstock and seeds. This can also be used in the epizootiological study of the pathogen in an effort to draw disease control measures. In the coming days, DNA based diagnostic tools are going to play a major role in the newly evolving aquatic animal healthcare.

ANTIOXIDANTS IN MANAGEMENT OF OXIDATIVE STRESS AND HEALTH

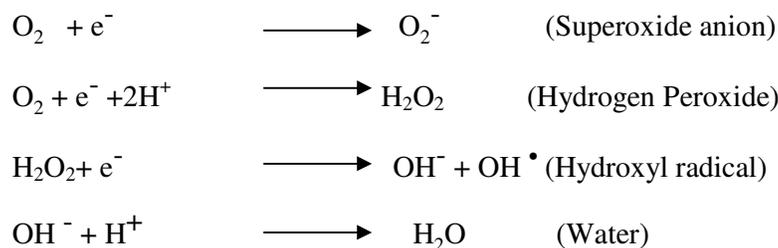
N. Kalaimani

Diseases in relation to oxidative stress

Oxidative stress has been implied, in the case of diseases that include immune injury, drug and toxin induced reactions, ischaemia and subsequent reperfusion injury, nutritional deficiencies, radiation injury, aging, hemolytic diseases, lung disorders, heart and cardiovascular system, kidney and gastro intestinal disorders and diseases affecting the brain, nervous system, neuromuscular disorders, cataract and retinal damages and a variety of skin diseases. In most diseases and as well as in several conditions of toxicity, increased oxidant and free radical formation is a consequence of the disease. Such pathological conditions have been implicated as resulting from damage caused by active oxygen/free radicals (oxy-rad) such as superoxide, hydrogen peroxide, hydroxyl radical, lipid radicals and nitrogen oxide.

Free Radicals, Reactive Oxygen Species in Cell and Tissue Damage

Molecular oxygen takes up electrons during respiration in the living cells in a sequential and orderly manner. Intermediates produced during this process are oxidants like superoxide and hydroxyl radical and hydrogen peroxide. (Saugstad, 1989).



Superoxide and hydroxyl free radicals together with H_2O_2 and singlet oxygen are called the Reactive Oxygen Species (ROS) or peroxidants, and are endogenous in origin. They interact with exogenous free radical inducers and with nitric oxide (NO). Oxygen radicals are formed (*albeit* in small amounts) during cellular respiration and may leak out of the mitochondria and interact with endoplasmic reticulum, plasma, membranes and other structures. A second source of ROS is the xanthine oxidase enzyme which also catalyses the univalent oxidation of purines with concomitant formation of superoxide radical, H_2O_2 and perhaps singlet oxygen.

Another source of ROS is the activated neutrophils, undergoing 'respiratory burst' as a sequel to the detection of foreign body in the system. Other sources of FR generation are by

the action of epinephrine, prostaglandin synthesis and calcium overload. Exogenous agents to produce active oxygen include photochemical smog and anticancer drugs. Added to this, transition metals, iron and copper contained in our body promote the generation of the most highly reactive class of active oxygen known as hydroxyl radical.

With the discovery of the biology of NO, the role of oxidants in cell damage has become better understood more than ever since most cells can produce not one but two radicals, O_2^- and NO. When NO and O_2^- coexist, these can react to give $ONOO^-$, a potent oxidant. It has been shown that $ONOO^-$ rather than O_2^- or NO is the most likely candidate for the actual cytotoxic molecule in reperfusion injury.

Oxidants are also encountered in the living cells formed by the nitroso derivatives of proteins or amino compounds. Nitrates and nitrites present in food and water can react to form nitroso proteins in the stomach and other parts of alimentary tract. The nitroso group is a free radical inducer. Formation of Nitrates and nitrites due to environmental pollution by residual feed may result in the formation of nitroso compounds which induce free radical formation which affects the health of the animal.

The metal chelating agents such as transferrin, lactoferrin and ceruloplasmin to bind harmful metal ions are also present to minimise the detrimental effects of oxy-rad. Cellular damage occurs in the condition in which the rate of oxygen radicals (oxy-rad) formation is increased and/or the activity of the defense system is impaired. Oxidatively damaged cell constituents can be removed and repaired by a restoration system that the cells possess. The pathways for the generation of reactive oxygen species and of the actions of some of the enzymes involved in antioxidant defenses in the cell is depicted in Figure 1.

Prevention and control of diseases using antioxidants:

Successful prevention or control of some of the diseases and toxicities using antioxidants and free radical scavengers has confirmed the role of free radicals and oxidants in toxicology and diseases. This is further confirmed from a variety of reports in which free radical scavengers and antioxidant enzymes are able to prevent or partially inhibit the pathological changes. Experimental production of colorectal cancer in mice was inhibited with Vitamin E (a free radical scavenger) in the diet. This was one of the earliest findings in this direction.

Many potent chemical carcinogens are metabolised into free radicals and potentiated the generation of Superoxide and Hydroperoxide. In studies made with naphthyl amines and

azodyes, it has been demonstrated that a correlation existed between carcinogenesis and the formation of free radicals and H₂O₂. Aflatoxin B₁ is also found to have the toxic function by the generation of free radicals and H₂O₂. AFB₁ induced killing of rat hepatocytes can be countered successfully by catalase and SOD (which destroy H₂O₂ and Superoxide radicals respectively), and mannitol, deferoxamine and other free radical scavengers. Most of the known chemical carcinogens are capable of forming free radicals in the host cells and also react with endogenous H₂O₂ to form superoxide and or hydroxyl radicals.

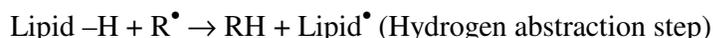
Mechanism of Damage to Cellular Architecture by Free Radicals

Reaction with lipids

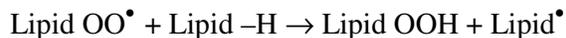
Free radicals attack at various levels in the cellular architecture and a few of these are described below. They damage cells through lipid peroxidation (LPO) on membrane producing structural and functional changes in the cell membrane. Membranes are dynamic fluid structures where lipids and proteins are held together and the fluidity is closely related to the presence of polyunsaturated fatty acids (PUFA) side chains. Oxidative deterioration of PUFA is triggered by an abstraction of hydrogen either by free radicals or ROS and the lipid peroxidation is different from the cyclooxygenase action. The reaction is outlined below:



Molecular rearrangement on the fatty acyl chain forms a conjugated diene which takes up a molecule of oxygen.

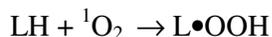


This free radical (Lipid OO[•]) can abstract hydrogen from another molecule of lipid to form lipid hydro peroxide.



And the chain reaction continues.

Lipid hydroperoxide can also be formed by the action of singlet oxygen on a lipid.



Lipid hydroperoxides are unstable and breakdown in biological systems giving rise to a variety of compounds including malondialdehyde (MDA) and 4-Hydroxy nonenal (HNE). Smaller components of LPO are 2-alkenals, and proteins and phospholipid bound aldehydes which may also be toxic. LPO products are measured by their reaction with thiobarbituric acid, and have been generally used as an index of oxidant and free radical damage.

Reaction with proteins

Free radicals and oxidants react with proteins mainly in two ways. By abstracting hydrogen from thiol groups, proteins are oxidised, leading to disulphide linkages and the resultant conformational and functional changes. Such reactions occur in the cells due to the simultaneous presence of molecular oxygen, transition metal ions of iron and copper and free radicals or free radical inducers. These are named 'Mixed Function Oxidation' and have been renamed 'Metal Catalysed Oxidation' by Stadtman and his group (Levine *et al.*, 1990). Oxidative stress can result from exogenous sources i.e., red-ox active xenobiotics or free radical generators. The possible fate of oxidised cellular proteins can be depicted as,

Proteins → Denatured/Hydrophobic proteins

This leads to cross-linking and formation of insoluble aggregates, fragmentation and increased susceptibility to proteolysis. Denaturation occurs due to variety of possibilities such as

- (a) The interconversion of aminoacids, exemplified by the oxidation of cysteine (SH) to cystine(-S-S-).
- (b) Other alterations in the aminoacid side chain reported include Carbonyl oxidation of the lysine residue forming γ glutamyl semialdehyde and
- (c) hydroxylation of phenylalanine to tyrosine.

These alterations change the primary structure of the proteins, the isoelectric point, folding and hydrophobicity. Hydrophobic patches on proteins contribute to protein aggregation. These authors have also observed good correlation between increases in hydrophobicity and proteolysis.

Reaction with DNA

Exposure to free radicals and oxidants and increased cellular generation of superoxide and hydroxide radicals and H_2O_2 lead to DNA strand breakage. The strand break may occur due to activation of some specific DNA-cleaving mechanism. Both purine and pyrimidine bases are modified by free radicals, especially the hydroxy radicals.

Exposure of *E.coli* cells exposed to H_2O_2 at concentrations in the range of 1-3mM is found to be mediated through superoxide and hydroxyl radicals, which leads to DNA damage. This can be blocked by iron chelating agents which prevent the Fenton-reaction within the cells. It has been shown that possibly some cancers may originate as a result of faulty repair of DNA damage produced by free radicals.

Lipid peroxidation, (LPO) in biological membranes causes alterations in fluidity, fall in membrane potential, increased permeability to H⁺ and other ions and eventual rupture leading to release of cell and organelle contents such as lysosomal hydrolytic enzymes. Earlier it was shown that the disrupted or damaged tissues undergo lipid peroxidation at a faster rate than their healthy counterparts. Hence the sequence of events may be conjured up as: Disease or Toxin → cell damage or death → increased LPO which will well explain the elevated lipid peroxidation products in disease and toxicology.

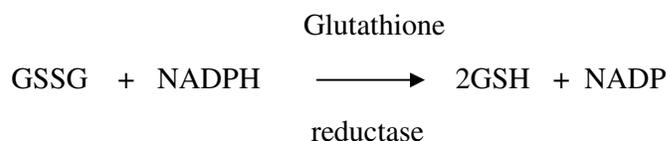
Cellular Defense by Antioxidant Enzymes and free Radical Scavengers

Avoidance of free radical formation

Potentially injurious effects of oxidants and free radicals on the living organism are prevented by a well-organised defense system and they function at four levels. The first and best effort is avoidance. This is achieved by cytochrome oxidase and other metallo enzymes. They help the cells in carrying out the tetravalent reduction of oxygen to water without releasing the toxic intermediates in a free state. Further, metal ions, which could participate in the oxidant producing reactions, are generally carried or sequestered by proteins like transferrin and ferritin, so as to minimise the amount of free iron in the cells. Similarly copper is bound to ceruloplasmin and the ionic form is not generally available in the free state. Albumin binds copper tightly and iron weakly. Haptoglobin/hemopexin binds free hemoglobin/heme. Hemoglobin and methemoglobin are powerful peroxides and can accelerate lipid peroxidation while haptoglobin inhibits the reaction by binding to haemoglobin. The antioxidant role of urate is due to its ability to tightly bind iron and copper ions.

Prevention of free radicals (FRs) acting on the cell

The second line of defense is prevention, by providing a continuous supply of GSH. The oxidised and reduced glutathiones are interconvertible and the cell maintains the bulk in the active (reduced) form. This reaction is catalysed by the enzyme glutathione reductase.



The liver is the major site of GSH synthesis in humans and animals. In the liver, it detoxifies endogenous metabolic peroxides through glutathione peroxidase and of exogenous substances such as drugs and other xenobiotics through glutathione-S-transferase. GSH

synthesised in the hepatic cells is either translocated to plasma or excreted into the bile through carrier mediated transport. During infection and inflammatory processes GSH is mobilised from the liver to the pathological site. Decrease of hepatic thiols is due to increased efflux of glutathione in shock induced inflammatory reaction.

Regulation of cellular levels of GSH can be broadly divided into four areas (1) Uptake of precursor aminoacids and intact GSH, (2) the regulation of the enzymes necessary for GSH synthesis, (3) alteration in the cellular redox system due to increased lipid peroxidation and cross-linking of glutathione with aldehyde, (4) direct oxidation of glutathione by ROS. Uptake of cysteine is the rate-limiting step for GSH synthesis.

GSH also forms conjugated products with ingested toxins and provide a detoxifying step, the GSH conjugates show less toxicity and are easily excreted in the faeces and urine. Glutathione-S-transferase (GSTs) is inducible and has been found to be induced in rat liver when AFB₁ is ingested.

Combating free radicals and their inactivation

The third line of defense is damage control which is achieved by providing free radical scavengers and antioxidant enzymes. The former arrests the initiation and propagation of the free radical chain reactions. They react rapidly with the free radicals, inactivate them and control the damage. While doing so the scavengers themselves are converted to radicals, which are many times less toxic than the original free radical. Vitamin E (α - tocopherol), Vitamin C (ascorbic acid) Vitamin A, β - carotene and reduced glutathione are free radical scavengers and are considered as antioxidants. These free radical scavengers interact as synergists and such interaction takes place at different levels, as detailed below :-

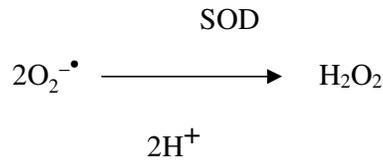
- a Antioxidant regeneration – e.g. Vitamin E is regenerated by Vitamin C
- b Protective mechanism – e.g. Vitamin E protects β -carotene from autooxidation
- c Compensatory mechanism – e.g. Vitamin E ameliorates selenium deficiency and *vice versa*
- d Complementary mechanism – e.g. β -Carotene may complement Vitamin E and prevent lipid peroxidation

AFB₁ induced mutagenesis in *Salmonella* is partially inhibited by the free radical scavengers, vitamin analogues, Ascorbic acid and vitamin E. Vitamin E was more potent

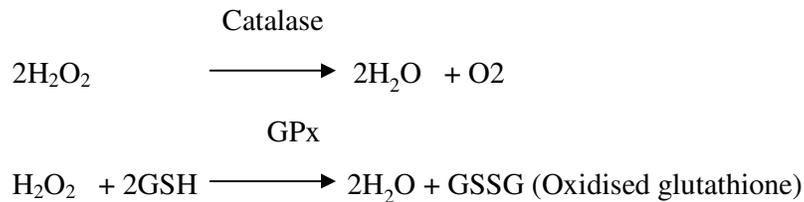
than vitamin C in AFB₁ induced mutagenesis. Dietary selenium supplementation was found to provide protection against AFB₁ induced neoplastic foci in rat liver.

Antioxidant enzyme activities

Antioxidant enzymes are universally distributed in all the cells and combat ROS and destroy them to prevent their interaction with cellular compounds. Superoxide dismutase (SOD) protects cells and tissues from inflammatory damage by inactivating the superoxide free radical.



Catalase and glutathione peroxidase (GP_X) act upon H₂O₂ and inactivate it. Both the enzymes have been detected in all human cells and organs with the highest activity in erythrocytes, liver and kidney.



GP_X is a selenium containing protein and can act not only on hydrogen peroxide but also on lipid hydroperoxide and arrest lipid peroxidation process.

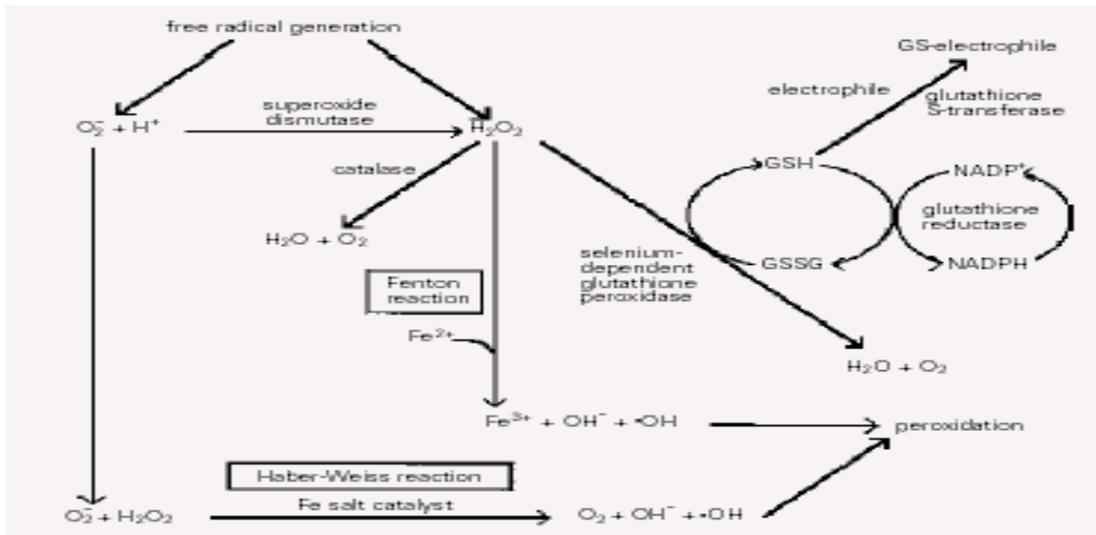


Figure 1 - Summary of the pathways for the generation of reactive oxygen species and of the actions of some of the enzymes involved in antioxidant defenses in the cell. Storey K B, 1996)

Repair of free radical induced damage

The fourth and final line of defense is to repair the damage which has occurred to the macromolecules in the cell structure, function and viability of the cells. Repairs are done presumably by the accelerated removal of damaged molecules. For e.g. lipid peroxidative product in the membrane are removed by the action of specific phospholipases, followed by replacement with new molecules. Damaged proteins are degraded into smaller fragments by proteases and damaged parts of the DNA are cleared and new segments formed at the site by DNA polymerase-I.

Inefficiency at any of the four levels of defense against free radicals and oxidants may lead to susceptibility to tissue damage, manifesting as infections, inflammatory or degenerative diseases. The process is schematically represented in figure 2.

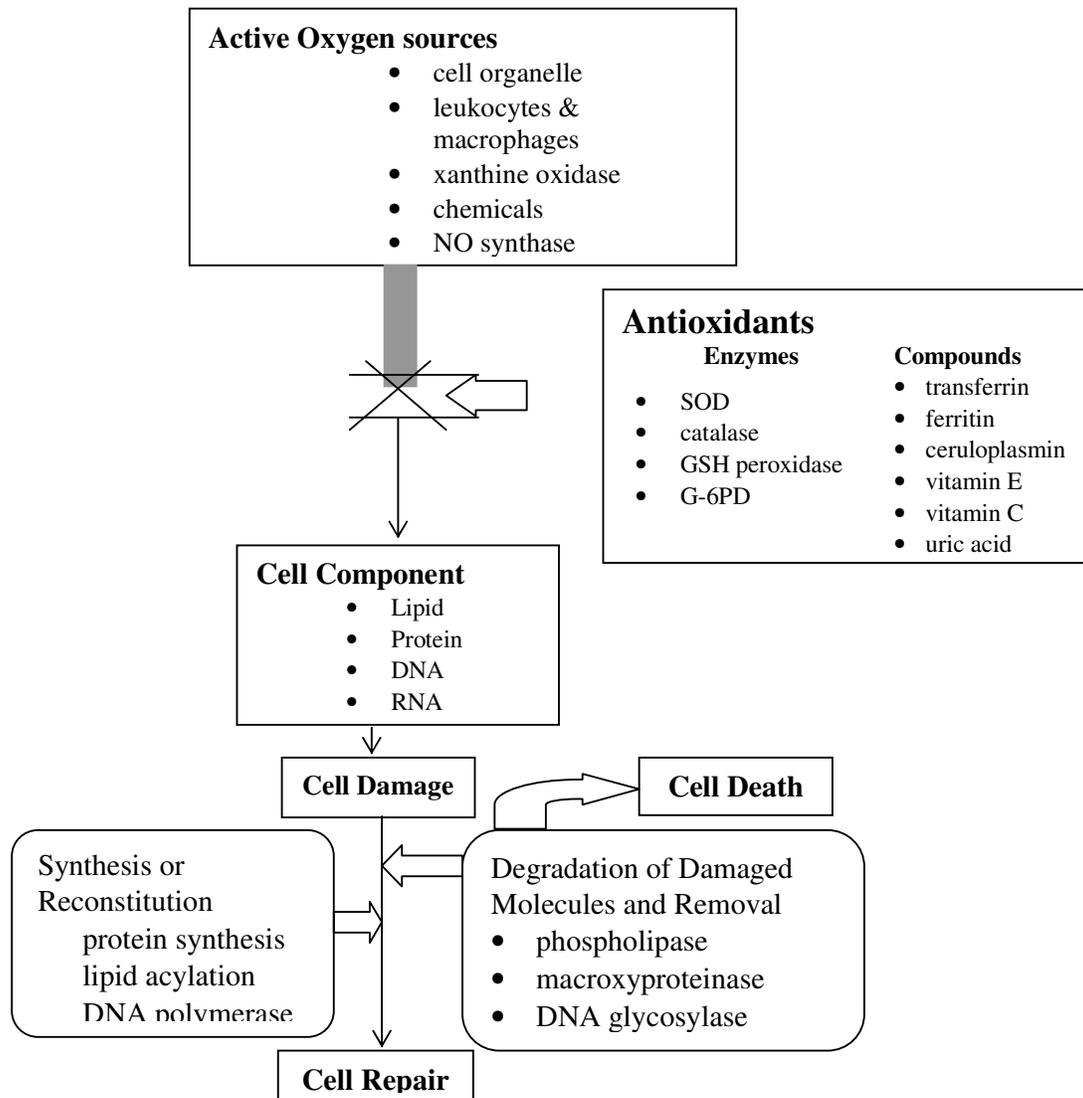


FIGURE 2. An overview of oxygen radical damage and repair (Nakazawa *et al.*, 1996)

Challenge tests with luminous bacteria and other causative agents frequently encountered in shrimp hatchery were conducted on the post larvae (pl2) of *P. monodon*. They were classified as low, moderate and high mortality producing group based on the observation of mortality rate during the course of the experiment. Oxidant stress studies were carried out in the three groups after pooling out the samples after the conclusion of the experiment. The parameters studied were the enzymes such as SOD, Catalase, Glutathione peroxidase, Glutathione reductase and antioxidants, GSH and Vit C and lipid peroxidation (LPO). Initially it was observed that the levels of antioxidant enzyme activities were high in the high mortality group and gradually reduced in moderates followed by low mortality group. This was due to the effect of combating the free radicals produced during the initial stage of infection.

SHRIMP HEALTH MANAGEMENT

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Introduction

Aquaculture has become the fastest growing activity contributing significantly to national economic development through export of fish and fishery produce and providing food security to the country. Indian aquaculture sector has achieved remarkable growth during the past 15 years, especially with respect to shrimp production through aquaculture. Disease problems in culture systems have become a significant constraint to production from aquaculture sector affecting economic development of the farming communities in many countries around the world including India. It has been estimated that economic losses due to disease and environment-related problems account to annual losses to aquaculture production to the tune of more than US\$3 thousand million per year in the Asian countries alone according to the estimates made in the year 1995 (Subasinghe, 1996). Despite good management practices adopted by aquaculturists, disease and mortality problems continue to confront shrimp aquaculture sector including emergence of new disease problems. In this context, it is essential to understand aspects of disease management in aquaculture systems both at macro-level, *i.e.*, global level, regional level, including national levels, and also at micro-level, *i.e.*, on farm / in hatcheries, and explore possibility of available avenues for disease prevention and control.

Epizootics of shrimp

Disease problems in farmed shrimp are caused by infectious and non-infectious agents. Infectious diseases are caused by pathogenic organisms such as viruses, bacteria fungi and parasites. Viral diseases constitute the most serious problems for shrimp culture due to high infectivity, pathogenicity and nonavailability of curative measures. World shrimp culture has suffered economic loss due to pathogenic viruses. So far nearly 20 viruses have been recorded and four of them are of great concern. For example, Monodon baculo virus (MBV) outbreak in Taiwan Province of China in 1988, followed by a series of shrimp viral disease outbreaks due to Yellow-head virus (YHV) in 1992 in Thailand, Taura Syndrome virus (TSV) in 1992 in Ecuador, white spot syndrome virus (WSSV) in 1993 in China and Thailand, and the same virus in a number of other Asian countries including India

were responsible for production losses of cultured shrimp. Among these the most devastating one is the WSV in India as well as in Asia and TSV in the Americas.

Approaches to aquatic animal health management

Several factors may be involved in the occurrence of epizootics of cultured stock and are often complex and difficult to pinpoint. Therefore, disease management must be viewed with a holistic angle, considering the host, pathogen and environment and their inter-relationships. Health management in aquaculture is defined as *a process encompassing pre-border (exporter), border, and post-border (importer) activities, as well as relevant national and regional capacity-building requirements (infrastructure and specialized expertise) for addressing aquatic animal health related activities, and development and implementation of effective national and regional policies and regulatory frameworks to reduce the risk of disease spread through movements (intra- and international) of live aquatic animals*. Hence, treatment of disease should not consider the pathogen alone. Management of disease problems must comprise of broader ecosystem management with a view to control farm-level environmental deterioration and to take preventative measures against the introduction of pathogens to aquatic animals. The emphasis should be on better management for prevention, which is likely to be more cost effective than treatment, involving both on-farm management and the management of the environment. Steps must be initiated towards reducing the use of chemicals and drugs. Regulations with respect to water usage, environmental protective measures, inputs that go into the aquaculture systems, farm-wise and region-wise must be put in place by the Government for disease management of aquatic animals and sustainable development of aquaculture at large. In addition, research, training programs, extension, and information exchange would be more effective and responsive to farmers' needs if based on System Management Approach (SMA). The FAO's Code of Conduct for Responsible Fisheries would provide a good base for the national and international cooperation in harmonizing aquatic animal health management activities.

Aquatic animal Quarantine

Transport of shrimp (larvae, adult and broodstock) across different countries and within the country is a practice among aqua-farming countries to meet the specific needs. Lack of regulatory frameworks in most countries, till recently, to monitor movement of aquatic animals across the borders led to spread of pathogens and outbreak of infectious diseases. Although India is one of the leading countries in aquaculture production and export,

an aquatic animal quarantine policy is yet to be formulated. Quarantine procedures help in avoiding or greatly reducing introduction of unknown etiologic agents of disease. Further, it is important to note here that future exports of fishery produce would depend on an effective quarantine policy.

The Australian Quarantine policy is a sort of role model for those countries seeking to establish fish quarantine policy, which has been based on an exhaustive studies such as import risk analysis, pre-border, border, post-border quarantine protocols, community responsibility, inputs required for quarantine policy and capacity building to achieve the quarantine objective. The Canadian shellfish sanitation programme (CSSP) through food inspection agency (CFIA) regulates import and export, certification, depuration, and evaluates laboratories. In addition, the CSSP co-ordinates (i) disease reporting, provide advice on disease surveillance, and monitoring projects and aquatic animal disease information systems, (ii) assists prioritization of research needs in aquatic animal health, and (iii) undertake policy, co-ordination of functions related to aquatic animal disease management under the CCEAD (Consultative Committee on Exotic Animal Diseases).

One of the important aspects of quarantine is the disease surveillance and reporting system. Disease surveillance is a process by which information on the disease status, important diseases and pathogen of a farm, zone, country or region is gathered. This will give information required for export /import health certification and providing evidence for the presence or absence of a disease in that locale. A national surveillance programme is a structured plan for the detection of specified diseases and disease causing agents in a country. This requires input from personnel trained in the diagnosis of diseases in the field that reports these facts to the health management personnel. For success this procedure should be supported by accurate and rapid diagnostic procedures to identify the pathogen. The data should be entered into a database to help routine monitoring. These surveillance programmes greatly help in import risk analysis and zoning programmes of a country. Further, any new disease or outbreaks can be contained and eradicated in an area, if suitable surveillance practice is in place.

The design, structure and methodologies employed in a surveillance programme depend on the purpose, list of problems to be addressed, diseases and pathogens of concern, resources available and the manpower. This is of regional interest, where all participating countries follow a uniform code of reporting. FAO has developed suitable reporting system for Asiatic region. The participating countries should maintain clear records, which will help

in tracing the source material for taking corrective measures in containing the occurrence of diseases. In this regard, disease-reporting system developed for Asiatic region by NACA has to be taken seriously and implemented.

Import risk analysis: Prior to introduction of any exotic species, it is essential to conduct import risk analysis that is based on the best available scientific information. Risk assessment must include both qualitative and quantitative risk assessment, associated with a variety of aquatic animals and likely hazards associated with a specific import. The quarantine policy has to address a number of factors including safe-guarding humans, animals, plants and the environment. Import of aquatic animals involves a high degree of risk either to the native aquatic fauna or even to humans, due to possible introduction of new pathogens, which are not present in the importing country. Hence it is essential that the importing country conducts import risk analysis prior to importation of aquatic animals in to the country.

Epidemiological information: Countries have to make available to other countries, through the OIE, the information that is necessary to reduce spreading of important aquatic animal diseases and their aetiological agents and to assist in achieving better world-wide control of these diseases. In addition, information on the measures taken to prevent the spread of diseases, including possible quarantine measures and restrictions on the movement of aquatic animals, aquatic animal products, biological products and other miscellaneous objects that may be responsible for transmission of disease must be also made available to an importing country.

Diseases notifiable to the World organisation for animal health (OIE): It is the list of communicable diseases that are considered to be of socio-economic or public health importance within countries and that are significant in the international trade in aquatic animals and aquatic animal products. Reports of these diseases are normally submitted once a year. Taura Syndrome, white spot disease and yellow head disease are notifiable shrimp diseases listed by the OIE.

Aquatic Animal Quarantine Policy in India: India is yet to have a aquatic animal quarantine policy. However, an effort towards establishment of a quarantine policy has been initiated by the Indian Council of Agricultural Research involving National Bureau of Fish Genetic Resources, Lucknow, Central Institute of Brackishwater Aquaculture, Chennai, Central Marine Fisheries Research Institute, Kochi, Central Institute of Freshwater Aquaculture, Bhubaneswar and other fisheries Institutes in the country. In view of the recent decisionsken to introduce exotic shrimp like *P. vannamei*, the immediate need is to expedite steps to

evolve and put in place a national aquatic animal quarantine policy to help sustainable development of Fisheries and Aquaculture in the country.

Disease Diagnosis

Successful aquatic animal health management relies on the accurate and rapid diagnosis of various diseases. New assays from genetic engineering using nucleic acid probes have come as boon to the aquaculture industry. Techniques such as Polymerase Chain Reaction (PCR) can detect pathogens in the organism even in the sub-clinical stages of infection, when a very few copies of the pathogen would be present. Recently, the CIBA developed a simplified, cost effective, user-friendly and rapid PCR diagnostic kit for the detection of WSSV in shrimp, which is marketed by Bangalore Genei to cater to the needs of shrimp farmers. This diagnostic kit can diagnose WSSV infection in shrimp at any stage of life cycle within few hours. This cost-effective diagnostic kit has become very successful and popular in the aquaculture industry in the country. The development of PCR for the detection of WSSV is a major breakthrough in combating the shrimp viral epizootic in India.

Diagnostic kits based on similar molecular approaches using DNA probes, are also effective in detecting the presence of nucleic acid sequences of pathogens from infected tissues. However, these techniques are relatively time consuming than the PCR assay. Immunological assays, including fluorescent antibody techniques (FAT) and enzyme linked immunosorbent assays (ELISA) using monoclonal antibodies developed against WSSV and other pathogens are also presently available the diagnosis. However, the sensitivity and specificity of these assays needs to be compared with that of PCR, which is regarded as the most sensitive and rapid diagnostic technique available so far.

One of the latest diagnostic techniques is the development of peptide nucleic acid probes combining the use of a light-emitting polymer with peptide nucleic acid (PNA). This is so sensitive that the DNA amplification steps can be reduced and possibly eliminated. PNA is a synthetic analogue of DNA, which replaces the phosphate sugar backbone of DNA with a peptide backbone. For the purposes of the testing method, the PNA is tailor-made to come together with the DNA being sought, say, WSSV DNA. The PNA is bound to small fluorescent molecules. If the DNA has the complementary code of the PNA, the two strands come together. The resultant pairing will be negatively charged overall due to the negative charge of the DNA fragment. The negative PNA-DNA pairing will be attracted to the

positively charged conjugated polymer. When that happens, the little light attached to the PNA tail goes on, and the test is positive for the DNA being sought. If the light doesn't go on, the DNA is not present, meaning that the infectious agent is not present. Such diagnostic tests have been developed for the diagnosis of anthrax, tuberculosis and infectious diseases in humans. Similar approaches may be adopted for developing rapid diagnostic techniques for shrimp and fish pathogens.

Use of antibiotics for disease prevention and control

Diseases caused by various etiologic agents followed by mortality of cultured stock have become limiting factors in the production of aquaculture sector. The farm and hatchery personnel in their distress to save the stock often resort to the use of various remedial measures, including the antimicrobials for disease control. Antibiotics are also often employed as prophylactic agents in shrimp hatcheries. The frequency of use of antimicrobial agents is higher in the hatcheries and semi-intensive farms compared to the traditional systems of aquaculture. A wide range of broad-spectrum antibiotics, the tetracyclines, chloramphenicol, macrolides like erythromycin, amino-glycoside antibiotics such as streptomycin, kanamycin, gentamycin, nitrofurans like nitrofurazone, furazolidone, and even the third generation quinolones such as ciprofloxacin, norfloxacin and nalidixic acid are being used in aquaculture. Oxytetracycline and oxolinic acid are the most common antibiotics used in the shrimp hatcheries and farms. Chloramphenicol, although not approved by the FDA and banned by the Ministry of Commerce, Govt of India, has been extensively used in shrimp hatcheries as a prophylactic. The antimicrobials are usually administered to finfish and crustacea through feed or as bath. However, a strong scientific database is required for the use of antibiotics either as prophylactics or therapeutics for various life stages of cultured stock, and their pharmacological aspects in the prevailing agro-climatic regions.

Emergence of drug resistant strains: Widespread use and abuse of antibiotics in the hatcheries and farms has also lead to the development of multiple drug resistance among the bacterial flora. Antibiotic resistance in bacteria is often mediated by the drug resistant plasmids (R-factors) although, chromosomal mediated drug resistance is not uncommon. The drug resistant plasmids are transferable among the bacteria and hence the drug resistant genes can spread to pathogenic strains. Instances of drug resistance determinants from fish

pathogens to human pathogens have been also documented and hence can pose a serious problem in the treatment of infectious diseases in humans.

Antibiotic residues in tissues: The antibiotics used in aquaculture either for prophylactic or therapeutic purposes often accumulate in the tissues of aquatic animals. Presence of antimicrobial drug residues in the edible tissues of aquaculture products can cause allergies, toxic effects, changes in the intestinal microbial flora and acquisition of drug resistance by the bacteria of humans. The acceptable levels of drug residues in the fishery products are based on the maximum residue limit (MRL). Hence, from the context of food safety, usage of antimicrobials for prophylactic or therapeutic purposes in fish and shrimp prior to harvesting must be restricted or harvesting must wait till antibiotic residues are withdrawn from the tissues by natural metabolic processes. *e.g.*, MRL for oxytetracycline in the shrimp tissues must be <0.01 µg/g of tissue.

The antibiotics and other drugs used in the aquaculture environment can be taken up by flora and fauna in the vicinity of the site where they are applied. The medicated feeds un-ingested by the target group may be taken up by other fauna. The antibiotic / drug may leach into surrounding water bodies along with the effluents. The fauna prevalent near the aquaculture site and especially the filter feeding mollusks are thus vulnerable to secondary medication. In the poly-culture systems practiced in the traditional aquaculture sector, such secondary medication could easily affect non-target culture species resulting in residue problems.

Information on the fate of drugs in the environment is scanty. However, the antibiotics used in aquaculture go directly into the environment and persist for a few days to several months, depending on the environmental conditions. The drug residues in water undergo dilution and photo-degradation, while the residues in the sediments may persist longer especially under anaerobic conditions. Persistence in the environment largely depends upon the microbial activity, temperature, pH and availability of oxygen. Some antibiotics like oxytetracycline, oxolinic acid and flumequine may persist in sediments at least for six months after treatment.

Lack of regulations on the use of antibiotics in aquaculture could become as much a threat to the aquatic organisms as the pathogens themselves, and the environment and the humans at large. Some of the developed nations, the USA, Canada, Australia, Japan and the European countries have in place the regulatory policy for use of antibiotics and other drugs

in aquaculture. The drug approval process and regulations differ in each of these countries. *e.g.*, oxytetracycline is approved for use in aquaculture in the USA, Canada, European countries and Japan, whereas, its usage in Australia is not approved. Similarly, oxolinic acid is approved in Japan and European countries, while in USA, Canada and Australia its use in aquaculture is not approved. Chloramphenicol, despite its ban by the regulatory bodies in several countries including the FAO, has been widely used in our country until its recent ban in shrimp aquaculture by the MPEDA along with other antibiotics, such as furazolidone, neomycin, nalidixic acid and sulphamethoxazole.

In view of the marketing problems associated with antibiotic residues in the shrimp tissues, it is prudent for the aquaculture managers to refrain from using antibiotics until the scientific basis of their usage and withdrawal periods are available. The need of the hour is to put in place a regulatory policy and provide guidelines on the use of antibiotics in aquaculture and bring about enforcement on the aquafarming community to comply with the regulations in order to protect environmental problems associated with their use and sustainability of aquaculture industry.

Probiotics

Microbes play both direct and indirect roles in aquaculture. They not only cause diseases but also are beneficial. As a soil flora, they too influence the aquatic environment. Their beneficial role had been recognized more than fifty years ago and the beneficial bacteria had been defined as probiotics in animal husbandry. They have been used to raise healthy and disease resistant farm animals. They have been accepted as better, cheaper and more effective in promoting animal health than antibiotics, in farm animals. Of late researchers tried to look for beneficial bacteria for use in aquaculture. Large number of isolates have been isolated that are useful in imparting resistance to disease and improving soil and water quality. However, the results are not always encouraging. This may probably be due to the selection of inappropriate microorganisms. Before recommending an organism as a probiotic it is necessary that the bacteria have to be evaluated whether it is able to impart resistance and compete with the potential pathogenic bacteria. It should be able to colonize and prevent the establishment of the pathogenic bacteria. This involves the viability of the strain and its ability to live within the larvae and in the environment. The pathogenesis of the organism to the target animal has also to be tested in actual farm condition apart from the laboratory experiments. If these characteristics are positive it may be an efficient probiotics. The next step would be to determine the route of administering the strain either by bath or by mixing

with the feed. Finally economic evaluation has to be done to see whether it is worth the investment.

Immunostimulants

The Invertebrates encounter all the usual sorts of challenges to self-integrity. Their habitats are laden with infectious agents: viruses, bacteria, fungi, protists, and other animals. Shrimps have innate defense systems, including the phagocytic cells, production of toxic oxygen and nitrogen metabolites, and melanization pathways, use of RNA interference (RNAi), pattern-recognition receptors (PRRs), anti-microbial peptides (AMPs), which effectively help in combating infection. However, with the advancement in aquaculture technology, application of extraneous substances to boost immune system of shrimps has come into practice. These are usually chemical substances, which aid animals (shrimp) in defending themselves against disease outbreaks and are called immunostimulants. β -1, 3 / 1, 6- glucans have been reported to be most promising substances with immunostimulatory properties. A number of other substances, such as laminarin, chitosan, saponins, barley glucans, lactoferrin, zymosan, dextran, peptidoglycans (PG), lipopolysaccharides (LPS), inulin, levamisole, herbal extracts, *etc.* are also reported to be useful in enhancing shrimp immune system.

Phage Therapy

With the developments in aquaculture and associated disease outbreaks, use of antibiotics and therapeutants has also substantially increased over the years. Bacteria resistant to most or all available antibiotics are increasingly causing serious problems, raising widespread fears of returning to a pre-antibiotic era of untreatable infections and epidemics. As an alternative to the use of antimicrobial agents against the pathogenic bacteria, the possibilities of using a specific kind of virus that attack only pathogenic microorganisms (phage therapy) appears to be promising. Almost a century ago, Hankin had reported that the waters of the Ganges and Jamuna rivers in India had marked antibacterial action, which could pass through a very fine porcelain filter. He also had reported that boiling destroyed this activity. He particularly studied the effects on *Vibrio cholerae* and suggested that the substance responsible kept cholera epidemics from spreading by ingestion of the water of these rivers. This filterable substance was nothing but the viruses (called as bacteriophages) that specifically affected *Vibrio cholerae* and brought about their lysis and killing. This and several initial studies gave birth to a new concept to treatment of disease: “Phage therapy”. Phage therapy was tried

extensively and many successes were reported for a variety of diseases, including dysentery, typhoid and paratyphoid fevers, cholera, and pyogenic (pus-producing) and urinary-tract infections. Phages were poured directly into lesions, given orally or applied as aerosols or enemas. Similar approach could be incorporated in the therapy of bacterial diseases in the aquaculture systems. We need to create phage banks by isolating and characterising bacteriophages specific to bacterial pathogens that are encountered in the aquaculture systems and evaluate their efficacy. Phages of some fish pathogenic bacteria, such as *Aeromonas salmonicida*, *A. hydrophila*, *Edwardsiella tarda* and *Yersinia ruckeri*, have been reported. However, application of phage therapy in aquaculture requires a great deal of research.

Specific pathogen free (SPF)/high health stock

The concept of specific pathogen free / high health stock has been developed in order to tackle the growing disease problems in penaeid aquaculture. U.S. Marine shrimp farming program has adopted this concept. The specific pathogen free stock is the one which is developed as free from certain pathogens, such as viruses and protozoan parasites. For practical purposes, captive populations of selected *L. vannamei* maintained in the oceanic Institute nucleus- breeding center is referred to as SPF. However, the putative advantages of stock could be useful in conjunction with proper husbandry practices to tackle the disease problems. Most of the shrimp aquaculturists consider the SPF stock as panacea for disease control. There is no specific evidence to show the advantage of SPF stock in the control of major disease such as viral disease. According to Lightner, there is no cultured penaeid which shows any type of disease resistance to the most dreaded viral disease, the white spot virus syndrome (WSSV). However, the SPF *L. vannamei* is projected as a disease resistant stock to tide over the problem of WSSV. This approach of introduction of nonnative species has to be viewed with caution, especially in countries such as India, where native species such as *Fenneropenaeus indicus*, the Indian white shrimp is already existing and considered as candidate species along with *P. monodon*.