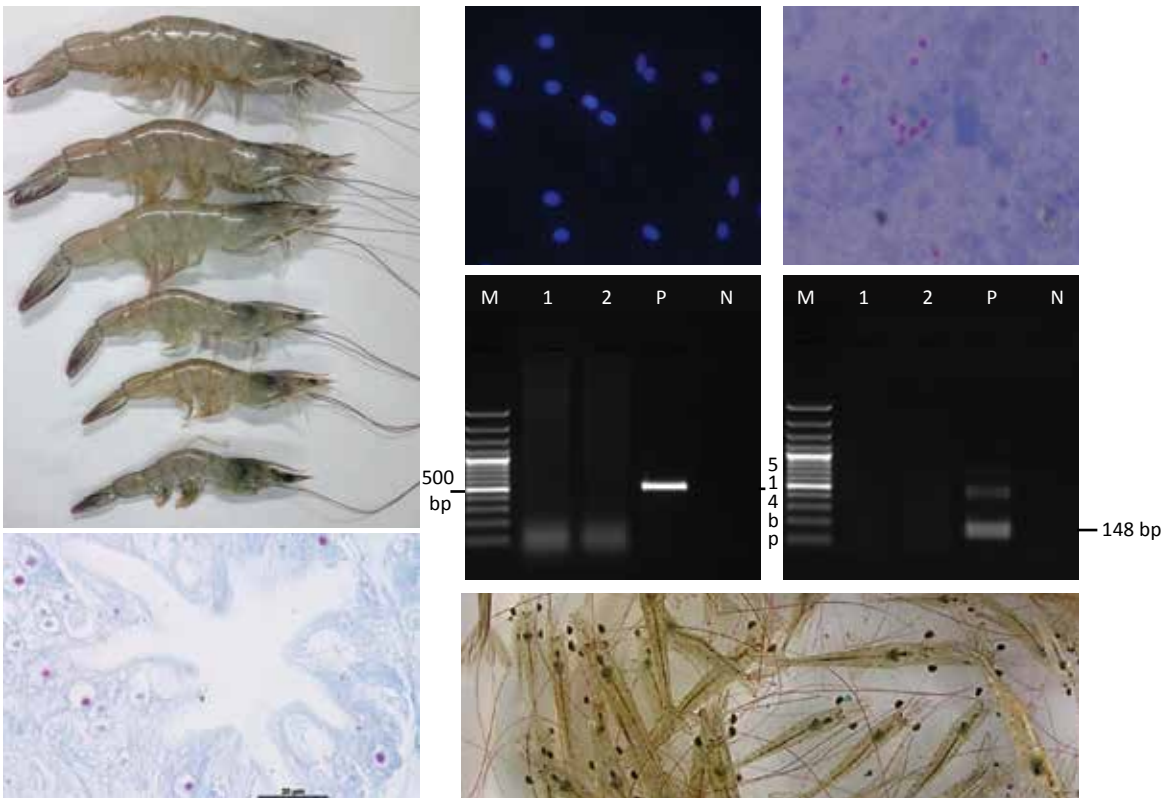
CIBA TM Series 2017 No. 9

Training Manual
On
Polymerase Chain Reaction (PCR) detection of *Enterocytozoon hepatopenaei* (EHP) in shrimp



Aquatic Animal Health and Environment Division
ICAR-Central Institute of Brackishwater Aquaculture

August 17-18, 2017

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On

Polymerase Chain Reaction (PCR) detection of *Enterocytozoon hepatopenaei* (EHP) in shrimp

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CONTENT

Brackishwater Aquaculture in India – an overview

Introduction

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

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

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

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

Origin and development

Brackishwater farming in India is an age-old traditional system confined mainly to the '*bheries*' (man made impoundments in coastal wetlands) of West Bengal, "*gheris*" in Odisha, "*pokkali*' (salt resistant deep water paddy) fields in Kerala, "*khar lands*" in Karnataka and "*khazans*" in Goa coasts. These systems have been sustaining production of 500–750 kg/ha/year with shrimp contributing 20–25% with no additional input, except that of trapping the naturally bred juvenile fish and shrimp seed during tidal influx. In this traditional method, low lying areas near the banks of saline water rivers and creeks are encircled by peripheral dyke and tidal water is allowed to enter the impoundment along with natural seeds of various species of shrimps, crabs and fish. Water is retained with periodical exchanges during lunar cycles and the animals are allowed to grow.

Realizing the importance of shrimp farming in Indian economy, Central Inland Fisheries Research Institute (CIFRI) under the Indian Council of Agricultural Research (ICAR) established first Experimental Brackishwater Fish Farm at Kakdwip, West Bengal in 1973. This was followed by inception of All-India Coordinated Research Project on Brackishwater Fish Farming in 1975 by the ICAR with centres in West Bengal, Odisha, Andhra Pradesh, Tamilnadu, Kerala and Goa. At the same time, shrimp seed production studies were initiated by the Central Marine Fisheries Research Institute (CMFRI) of ICAR. Commercial scale shrimp farming started gaining roots only after 1988–1989 and the semi-intensive farming technology

demonstrated production levels reaching 4–6 tons/ha. Area under shrimp farming increased substantially during 1990–1994 with remarkable growth rate till 1995 as the boom period of commercial-scale shrimp culture and the bust came in 1995-96, with the outbreak of viral disease. The fact that most of the farmers were new to commercial scale and high intensive shrimp farming, the general ignorance of good aquaculture practices and the lack of suitable extension services, led to a host of problems. Later with the advent of bio-secured closed culture technology using better management practices, semi-intensive and intensive shrimp farming again started to regain its lost glory during early years of the present century. Farmed shrimp production increased from 102940 tons in 2001-02 to record production of 144346 tons in 2006-2007 and operating at around 100000 tons over the years.

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

The state wise area and production of shrimp is as follows:

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

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

Table 2: Area (ha) under farming, production and percent of total production during 2013-2014

State	Area under farming	Production (mt)	Percent of total production
West Bengal	48410	52581	19.4
Orissa	6302	14532	5.4
Andhra Pradesh	36123	159083	58.7
Tamil Nadu	7804	25815	9.5

State	Area under farming	Production (mt)	Percent of total production
Kerala	12917	5175	1.9
Karnataka	394	664	0.2
Goa	31	63	0.02
Maharashtra	1486	3513	1.3
Gujarat	2359	9393	3.5
Total	**115826**	**270189**	**-**

Source: MPEDA, 2014

Among Indian states, Andhra Pradesh is leading in *P. vannamei* farming contributing nearly 80% of Indian production. Table 3 depicts state wise area under culture and production of *P. vannamei* (Pacific white leg shrimp) in India. There is huge potential for mud crab farming in the country. Still there is no organized aquaculture of mud crab for supporting the export trade. Major reason is the non-availability or inconsistent availability of crab seeds for farming. Technology for seed production, culture and fattening of green mud crab *Scyllla serrata* has been developed by CIBA. Some farmers are practicing crab fattening in the coastal regions with considerable success. After the inception of crab hatchery by the Rajiv Gandhi Centre for Aquaculture (RGCA) in Nagapattinam district in Tamil Nadu, hatchery produced crab seeds are now available. Some progressive farmers have started crab grow-out farming with better performance of hatchery produced seeds compared to wild ones.

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

States		2009-10	2010-11	2011-12	2012-13	2013-14	2014-15
West Bengal	Area	0	0	0	0	130	326
	Production	0	0	0	0	479	395
Orissa	Area	0	0	25	46	485	2340
	Production	0		100	436	2907	11866
Andhra Pradesh	Area	264	2739	7128	20198	49764	37560
	Production	1655	16913	75385	133135	210639	276077
Tamil Nadu & Pondi-cherry	Area	0	34	397	1511	5087	5037.1
	Production	0	109	2863	8595	26281	32687.8
Kerala	Area	0	0	0	0	0	5.8
	Production	0	0	0	0	0	11.75
Karnataka	Area	0	0	72	154	157	124.76
	Production	0	0	232	484	517	623.2
Goa	Area	0	0	0	1	29	27.2
	Production	0	0	0	15	67	88.2

States		2009-10	2010-11	2011-12	2012-13	2013-14	2014-15
Maharashtra	Area	10	94	127	439	908	1274.51
	Production	30	508	941	1503	3291	4901.04
Gujarat	Area	9	64	88	366.71	707	3545.4
	Production	46	717	1195	3348.19	6326	26763
Total	**Area**	**283**	**2931**	**7837**	**22715.71**	**57267**	**50240.77**
	Production	**1731**	**18247**	**80717**	**147516.2**	**250507**	**353413.1**

Source: MPEDA (Kochi)

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

Farming systems

There are five different shrimp aquaculture practices mentioned in the literature, ranging from traditional to ultra-intensive techniques, but the most common techniques followed in India are traditional, extensive, semi-intensive and intensive. These three categories are divided, according to their stocking densities (shrimp/ m2), and the extent of management over grow-out parameters, i.e. level of inputs (Table 4).

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

Extensive shrimp aquaculture is primarily used in areas with limited infrastructure. Producers rely on the tides to provide most of the food for the shrimp and as a means of water exchange. Feed for shrimp is naturally occurring, in some cases fertilizers or manure is added to promote algal growth. Low stocking densities result in modest yields. Land and labour are the principal inputs, which keeps operational cost at a minimum. The extensive farming is commonly known as improved traditional farming. This system involves construction of peripheral canals/ ponds of size ranging from 1–5 ha. Shrimp seed at the rate of 15000 – 20000/ha are stocked. Water management is done by tidal effect. The

average yield is 1500 – 1700 kg/ha, including fin fishes. In most of the cases, the stock is left at the mercy of nature and the predators. Supplementary feeding is not generally practiced as the entire production system is dependent on utilization of natural productivities. However some farmers use oil cakes and rice bran as supplementary feed.

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

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

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

	Traditional	Extensive	Semi- intensive	Intensive
Pond size (ha)	0.1-50	1-10	0.2-2	0.1-1
Stocking	Natural	Natural + Artificial	Artificial	Artificial
Stocking density (seed/m^2)	Unregulated	2-6	6-20	20-50
Seed source	Wild	Wild + Hatchery	Hatchery + Wild	Hatchery
Annual Production (ton/ha/yr)	< 0.6	0.6-1.5	2-6	7-15
Feed source	Natural	Natural	Natural + Formulated	Formulated
Fertilisers	No	Yes	Yes	Yes
Water exchange	Tidal	Tidal + Pumping	Pumping	Pumping
Aeration	No	No	Yes	Yes

	Traditional	Extensive	Semi- intensive	Intensive
Diversity of crops	Polyculture	Monoculture, polyculture rarely	Monoculture	Monoculture
Disease problems	Rare	Rare	Moderate to Frequent	Frequent
Employment (persons/ha)	1-2	2-3	3-4	4-5

Human resource development through brackishwater aquaculture

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

Laws and regulations

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

Institutional setup

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

most of which also undertake aquaculture development activities. The MPEDA functioning under the Ministry of Commerce, besides its role in the export of aquatic products also contributes towards the promotion of coastal aquaculture. Many other organizations like Department of Science and Technology, Departments of Biotechnology, University Grants Commission also support or conduct R&D in the subject. Various NGOs and private organizations contribute substantially in this context.

Research and Development

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

Way forward

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

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Diseases in brackishwater shrimp aquaculture

Aquaculture is a fast escalating industry, backing significantly national economy through export of aquaculture products and providing food security to the country. Brackishwater aquaculture development in India till 2009 was mostly focused on tiger shrimp, *Penaeus monodon* culture. However since 1995 tiger shrimp farming regressed mainly due to viral diseases. In 2009, the Coastal Aquaculture Authority of India (CAA), after the risk analysis conducted by ICAR-CIBA, permitted the entrepreneurs to introduce a new species, *P. vannamei* (Pacific white leg shrimp) in India with prescribed guidelines as an alternative culture species. Owing to SPF status, fast growth rate and culture feasibility in wide range of salinity, *P. vannamei* readily accepted by the farmers and consequently became the predominant cultured species. After the introduction of Pacific white shrimp, with respect to the shrimp production through aquaculture, Indian aquaculture sector has attained notable growth during the past five years. During 2016-17, seafood exports crossed 11,34,948 MT valued an all-time high of Rs 37, 870.90 crore (US$ 5.78 billion) as against 9,45,892 tons and 4.69 billion dollars in previous year. Shrimp exports increased by 16.21 per cent in terms of quantity and 20.33 per cent in value. Frozen shrimp is the chief export item, accounting for 38.28 per cent in quantity and 64.50 per cent of the total earnings. The overall export of shrimp during 2016-17 was at 4, 34,484 MT worth USD 3,726.36 million. The export of Vannamei shrimp, boosted from 2, 56,699 MT to 3, 29,766 MT in 2016-17, with growth rate of 28.46 per cent in quantity. However, the increasing trend in intensification and commercialization has exacerbated the epidemics of diseases and a major constraint for the sustainability of this industry. Aquatic animals are widely translocated across countries for enhancing aquaculture productions and species diversification. Such trans-boundary movement of live aquatic animals has the inherent risk of introduction of new diseases. Disease related losses in aquaculture have been a challenge to achieve higher productivity. Such setbacks have been experienced countries world over. The combined loss from shrimp diseases, at the global level from 11 shrimp farming countries for the period 1987–1994, was estimated at 3019 million USD.WSD alone is estimated to cause losses of over US$6 billion since its emergence in 1992. Since 2009, the newly emerging diseases early mortality syndrome (EMS), specifically known as Acute Hepatopancreatic Necrosis Disease (AHPND), caused economic loss excess of US$ 1 billion per year. These case histories clearly reflect the capacity of the lethal disease causing pathogens and the damage caused by the disease outbreaks to the aquaculture sector. Considering the limited therapeutic options available for the control of viral diseases, only timely disease detection using novel diagnostic tools for disease surveillance followed by active response to adopt and practise proper health management approaches would ameliorate the magnitude of the problem. The paper presents an overall view on diseases incidence in shrimp aquaculture.

EMERGING DISEASES OF ECONOMIC IMPORTANCE

HEPATOPANCREATIC MICROSPORIDIOSIS (HPM)

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

samples of frozen Artemia mass has been reported to test positive for EHP by PCR, but again, it is not known whether *Artemia* is susceptible to EHP infection or just a mechanical carrier. It is urgent that these possibilities be explored in order to improve control measures. Although EHP does not appear to cause mortality in *P. monodon* and *P. vannamei*; information from shrimp farmers indicates that it is associated with severe growth retardation in *P. vannamei*. Thus, farmers and hatchery operators monitor *P. vannamei* and *P. mondon* for EHP in broodstock, PL and rearing ponds. The best approach for maturation and hatchery facilities to avoid EHP is not to use wild, captured, live animals (e.g., live polychaetes, clams, oysters, etc.) as feeds for broodstock. Better would be pasteurization (heating at 70^{o}C for 10 minutes). Another alternative would be to use gamma irradiation with frozen feeds. Alternatively, polychaetes could be selected and tested for freedom from shrimp pathogens and then reared as broodstock feed in biosecure settings designed to maintain their freedom from shrimp pathogens (i.e. SPF polychaetes).

ACUTE HEPATOPANCREATIC NECROSIS DISEASE (AHPND)

Since 2009, an emerging threat, popularly known as early mortality syndrome (EMS) and recently termed as acute hepatopancreatic necrosis disease (AHPND) severely affected shrimp farming in many countries in the Southeast Asian region. Though the disease is reported to affect mainly Pacific white shrimps (*P. vannamei*), tiger shrimp (*P.monodon*) and Chinese shrimp (*P. chinenesis*) have also been reported to be susceptible. The disease is mainly characterized by mass mortalities (reaching up to 100% in many cases) during the first 20-35 days of culture (post-stocking in grow-out ponds). This new disease was first reported from China in 2009, followed by Vietnam in 2010, Malaysia in 2011, Thailand in 2012, and Mexico in 2013.Very recently during 2015, this disease has also been reported to be present in the Philippines. The disease has caused severe economic losses throughout the region. The early mortality syndrome in shrimp has been named based on unusually high mortality within the first 30 days of shrimp grow-out culture, due to a variety of pond management and pathogen related factors. Some of the farm level observations include onset of clinical signs such as soft shells, significantly emaciated pale to whitish hepatopancreas (HP) and partially full to empty guts and mortality starting as early as 10 days post stocking. HP is the main target in AHPND and therefore the pond level observations have to be further confirmed as AHPND by laboratory investigations involving characteristic pathology of HP such as necrosis of B, F, R cells and sloughing of cells into the lumen. Progressive degeneration and dysfunction of the HP tubule epithelial cells progressing from proximal to distal ends of HP tubules and its degenerative pathology suggested of a toxic etiology. Though histopathological investigations are the gold standard for the confirmation and presence of AHPND, recently detection of the causative agent of AHPND by molecular methods such as PCR has been developed. The causative agent of the disease is a special strain of *V. parahaemolyticus* (VpAHPND). A plasmid (pAP1) of about 69 kb present in this specific strain contains two genes that produce toxins (Pir A/B) and cause massive damage to the hepatopancreatic cells. Adoption of biosecurity measures to prevent this pathogen entering the culture system. The potential risk factors for EMS / AHPND include factors such as high stocking densities, older farms closer to the sea using higher salinity water, farms not employing reservoirs, farms overusing chemicals, inadequate aeration, and presence of toxic levels of H_2S etc.

IMPORTANT OIE LISTED DISEASES

WHITE SPOT SYNDROME (WSD)

White spot syndrome (WSD) is the most devastating diseases of penaeid shrimp and virus that has wide spread presence throughout the world. Originating from Taiwan in 1992, it spread to Japan during 1993 and subsequently very quickly to all other Asian countries. By 1995, it had already spread to North America and further by 1999 to South America. The quick spread of this virus to different regions and simultaneous investigation by different scientists speculated the same agent to be different ones and thereby called them in different names such as Systemic Ectodermal and Mesodermal Baculovirus (SEMBV), rod shaped nuclear virus of *Penaeus Japonicus* (RV-Pj), Hypodermal and Haematopoietic Necrosis Baculovirus (HHNBV), third *P. monodon* non-occluded

virus (PmNOB III), penaeid rod shaped DNA virus or white spot baculovirus. On subsequent investigation and data analysis when it was known that all these names are for the same agent, it was unanimously called as white spot syndrome virus (WSSV). WSSV is a rapidly replicating and highly virulent shrimp virus. It is one of the largest viruses designated as genus *Whispovirus* in the family *Nimaviridae*. WSSV is a rod-shaped, double-stranded, DNA virus, and the size of the enveloped viral particles have been reported to be 240 -380 nm long and 70 -159 nm in diameter. The typical clinical symptoms of WSSV infection are the formation of circular white spots on the carapace often with reddish discolouration. Outbreak of this disease wipes out the entire shrimp populations in the ponds within 3-8 days. Histopathology can be observed in the gill epithelium, antennal gland, haematopoietic tissue, nervous, connective tissue and Intestinal epithelium. Infected cells exhibit as prominent intra-nuclear basophilic hypertrophied nuclei with chromatin margination and cytoplasmic clearing. Rapid and specific diagnosis of the WSD can be accomplished using nested or quantitative polymerase chain reaction. WSSV is transmitted vertically from infected broodstock to larvae and horizontally by ingestion of infected organisms. Other than the penaeid shrimps which serve as host for this virus, a large number of other crustaceans serve as carrier and therefore it has been impossible to eradicate this virus from the culture system. While WSD transmission vertically is being prevented by screening out infected broodstock, its horizontal transmission in grow out farms is a serious challenge. At present there is no treatment available to prevent the unrestrained occurrence and spread of the disease. Better management practices (BMPs) have helped alleviate this problem to a great extent, by minimising risks of its transmission through carrier organisms such as mud crabs, *Artemia*, rotifer eggs, molluscs, polychaete worms, insect larvae and seabirds etc. However, concerns of WSD transmission through contaminated water and pond sediment remain unaddressed. Pond preparation practices have proved to be useful in eliminating the virus from the pond and reducing the risk of disease outbreaks.

White spots on carapace seen in shrimp infected with WSD

INFECTIOUS HYPODERMAL AND HAEMATOPOIETIC NECROSIS (IHHN)

IHHN was first reported in *P. stylirostris* from America in the year 1981. However, it was thought to have been introduced along with live *P. monodon* from Asia. Recent studies have revealed geographic variations in IHHNV isolates, and suggested that the Philippines was the source of the original infection in Hawaii, and subsequently in most shrimp farming areas of Latin America. Large-scale epizootics were responsible for multi-million dollar losses in *P. vannamei* culture in the Americas during the 1990s. IHHNV is caused by a small (20-22 nm) single-stranded DNA Brevidenso virus. Gross signs of disease are not specific to IHHN, but may include reduced feeding, elevated morbidity and mortality rates, fouling by epicommensals and bluish body coloration. Larvae, PL and broodstock rarely show symptoms. In *P.vannamei*, IHHNV can cause runt deformity syndrome (RDS), which typically results in cuticular deformities (particularly bent rostrums), slow growth, poor FCR and a greater size variation at harvest, contributing substantially to reduction in profits. IHHNV typically causes no problems for *P. monodon* since they have developed a tolerance to it over a long period of time, but they may suffer with RDS. *P. merguiensis* and *P. indicus* appear refractory to the IHHNV. However, these species may be life-long carriers of the virus and could transmit the virus to *P.vannamei*, which typically suffer from RDS due to IHHNV infection. IHHNV can be diagnosed using methods such as DNA probes in dot blot and *in situ*

hybridization and PCR techniques (including real-time PCR) as well as histological analysis of H&E-stained sections looking for intracellular, Cowdry type A inclusion bodies in ectodermal and mesodermal tissues such as cuticular epithelium, gills, foregut, hind gut, lymphoid organ and connective tissues. Transmission of IHHN is known to occur rapidly by cannibalism in shrimp. It can also be transmitted through waterborne route and cohabitation. Vertical transmission from broodstock to larvae is common. Strict hatchery biosecurity including checking of broodstock by PCR, or the use of SPF broodstock, washing and disinfecting of eggs and nauplii is essential in combating this disease.

YELLOW HEAD DISEASE (YHD)

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

TAURA SYNDROME (TS)

Taura Syndrome was first identified from farms around the Taura River in Ecuador in 1992 and the disease spread rapidly to the entire region of Latin and North America within three years. Subsequently, TS was also reported from Asia including Mainland China and Taiwan (from 1999), and in late 2003 in Thailand, probably through the regional and international transfer of live PL and broodstock of *P.vannamei*. Initial work suggested that TS was caused by a toxic pesticide. However, it is now known that a single or perhaps several very closely related strains (mutants) of the Taura syndrome virus (TSV) are responsible for the TS. TSV is a single stranded RNA virus of 32 nm size, non-enveloped icosahedrons and more prone to mutations causing more concern.TSV infections occur in juvenile shrimp (0.1-1.5 g body weight) within two to four weeks of stocking ponds and occur largely within the period of a single moult cycle. In the acute phase of the disease, during pre-moult stage, the shrimp are weak, soft-shelled, have empty gut and diffuse expanded chromatophores that appear red, particularly in the tail (hence the common name - red tail disease). Such animals will usually die during moulting (5-95%). Adult shrimp are known to be more resistant than juveniles. Shrimp that survive infection show signs of recovery and enter the chronic phase of the disease. Such shrimp show multiple, randomly distributed, irregular, pitted, melanised lesions of the cuticle. These gross lesions will persist, but may be lost during moulting, and the shrimp thereafter appear normal. TS can be diagnosed using standard histological and molecular methods of detection. Specific DNA

probes applied to *in situ* hybridization assays with paraffin sections provide the confirmatory diagnosis. Reverse transcriptase polymerase chain reaction (RT-PCR) assay is commonly used for larger sample sizes and non-lethal sampling for broodstock. Histological demonstration of enlarged lymphoid organs (LO) with multiple LO spheroids and multifocal areas of necrosis in the cuticular epithelium of the general body surface, appendages, gills, hindgut, and foregut (the oesophagus, anterior and posterior chambers of the stomach).The mechanism of transmission of TSV can be through contaminated PL and broodstock. Recently it has been shown that mechanical transfer through insect. The disease can be prevented by avoidance of reintroduction of the virus from wild shrimp and carriers and stocking with TSV-free PL produced from TSV-free broodstock.

INFECTIOUS MYONECROSIS (IMN)

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

Shrimp infected with IMNV displaying whitish necrotic areas in the striated muscle, of the distal abdominal segments.

NECROTIZING HEPATOPANCREATITIS (NHP)

NHPisalsoknownasTexasnecrotizinghepatopancreatitis (TNHP), Texas pond mortality syndrome (TPMS), and Peru necrotizing hepatopancreatitis (PNHP). NHP has been reported as an important disease since its first diagnosis in 1985. It has been reported to cause

mass mortalities to the tune of 20-90 percent of *P. vannamei* in highly saline commercial grow-out ponds nearly every year since then. By 1993, NHP spread to Ecuador, Guatemala, Honduras, Mexico and Peru and by 1995, coincided with warm waters with high salinity associated with El Nino, and caused severe mortalities (60-80 percent mortality) of *P.vannamei* and *P.stylirostris* throughout Ecuador. NHP has not yet been reported in Asia, but could cause significant damage were it to be transferred here with untested shrimp introduction. Necrotizing hepatopancreatitis is caused by obligate intracellular Rickettsia-like bacterium, a member of the order a-Proteobacteria (Gram-negative, pleomorphic, rod-shaped or helical-shaped bacterium). Affected shrimp are lethargic, anorexic with empty gut and show epibiotic fouling. Exoskeleton becomes soft and show abdominal muscle atrophy. Affected ponds have increased FCR and growth of affected shrimp is retarded. The hepatopancreas becomes watery with white or black streaks. Mortality rates reach up to 90% within 30 days of the appearance of clinical signs. NHP can be diagnosed by demonstration of lipid droplets and melanisation of hepatopancreas by microscopic examination of wet mount of preparations. It may be confirmed by histopathological examination showing atrophy and the presence of granuloma in the hepatopancreas and haemocyte aggregations around the hepatopancreatic tubules. Intra-cytoplasmic Rickettsia-like bacteria may be prominently seen in the cytoplasm. Molecular diagnostic tools such as *in situ* hybridization, dot blot hybridisation, and PCR for specific a-Proteobacterial DNA are also available. NHP could be transmitted horizontally with infected PLs. Maintaining optimal environmental parameters using BMPs will be useful in preventing NHP.

OTHER SIGNIFICANT DISEASES

VIBRIOSIS

Vibriosis is ubiquitous and all marine crustaceans, including shrimp, are susceptible. Epizootics occur in all life stages, but are more common in hatcheries. Major epizootics of vibriosis have been reported for *P. japonicas, P.monodon* and *P. vannamei*. *Vibrio* species are part of the natural microflora of wild and cultured shrimp and become opportunistic pathogens when natural defence mechanisms are suppressed. They are usually associated with multiple aetiological agents. However, some *Vibrio* species, or strains of certain species, have been identified as primary pathogens. Pathogenic strains of *V. harveyi, V. vulnificus* and *V. parahaemolyticus* have caused massive epidemics. Mortalities due to vibriosis occur when shrimp are stressed by factors such as poor water quality, crowding, high water temperature and low water exchange. High mortalities usually occur in post-larvae and young juvenile shrimp. Shrimp suffering vibriosis may display localised lesions of the cuticle typical of bacterial shell disease, localised infections from puncture wounds, loss of limbs, cloudy musculature, localised infection of the gut or hepatopancreas and/or general septicaemia.

NEW DISEASES OF UNKNOWN ETIOLOGY

RUNNING MORTALITY SYNDROME (RMS)

Since 2011, a new syndrome has brutally affected the shrimp industry and causing substantial mortality. The disease has been termed as Running Mortality Syndrome (RMS) by the farming community. The affected ponds show different mortality patterns with unusual symptoms, no relation to any known diseases and a slow mortality rate (e.g. <1%/day), but the cumulative loss over phase will be high.

Shrimp mortalities seen in RMS condition

Some farmers have lost up to four crops, with mortality percentage reaching 70% in most of the cases. Generally mortalities start after a month or 40 DOC, but few shrimp continue to survive and can grow to fully harvestable size. Investigations conducted in ICAR-CIBA revealed no association of RMS with known shrimp pathogens. Further, bacteriological examination of haemolymph samples of RMS affected

shrimp indicated predominance of *Vibrio* spp., such as *V. parahaemolyticus* and *V. azureus*. Histopathological examinations showed karyomegaly and increased inter hepatopancreatic tubular space with haemolymph infiltration, muscle necrosis, loosened LO tubule cells and constricted lumen. Bioassay experiments carried out by feeding RMS affected shrimp tissue to healthy 13-14 g shrimp did not elicit any disease in the experimental shrimp. Co-habitation experiment with healthy shrimp and the infected animals also failed to induce RMS. Relatively few studies done on Running Mortality syndrome, and still the causative agents or aetiology of RMS are unknown.

WHITE MUSCLE SYNDROME (WMS)

In recent years, shrimp farmers have been suffering from several cases of white muscle with muscle necrosis in the *P. vannamei* grow-out cultures associated with low mortalities. The white muscle syndrome affected shrimps show focal to extensive necrotic areas in striated muscle tissues, displaying a white, opaque appearance.

White, opaque appearance of shrimp with WMS

Similar lesions have been described with Infectious myonecrosis (IMN), penaeid white tail disease (PWTD) and non-infectious aetiology with sudden changes in water quality parameters such as temperature, salinity and dissolved oxygen. White muscle in shrimp can also be caused by the advanced infection of microsporidians belonging to the genera Ameson and Agmasoma, or dietary deficiency of selenium. Furthermore, histological analysis from white muscle syndrome affected samples with macroscopic lesions revealed a loss of sarcomeric structure accompanied by coagulative muscle necrosis along with haemocytic infiltration. Though histological lesions found in the suspected sample undistinguishable from those reported in *P. vannamei* for IMNV and PWTD there is a small difference in the histopathological change (i.e) no cytoplasmic inclusion bodies were observed in skeletal muscle of infected samples. The suspected samples found negative for IMNV and PvNV and suggested that the aetiological agent of this disease could be either a new infectious agent or a different strain of IMNV.

SIZE VARIATION/ GROWTH RETARDATION

More recently shrimp farmers have been reported several cases of size variation / growth retardation in *P. vannamei* grow-out cultures. It is reported that viruses, viz., IHHNV, lymphoid organ vacuolization virus (LOVV), monodon baculovirus (MBV), hepatopancreatic parvovirus (HPV) and Laem-Singhvirus (LSNV) are associated with slow growth and size variation in shrimp recorded.

Size variation observed in *P. vannamei* from grow-out culture

In India, multiple viral infections of IHHNV, MBV and HPV are reported to be associated with slow growth. In white faeces syndrome affected animals there is a decrease in feed consumption and growth rates were reduced as revealed by average daily weight gain (ADG) for the whole crop operation of less than 0.1 g/day compared to 0.2 g/day in normal ponds. Feed conversion ratios (FCR) range from 1.7 to 2.5 when compared to 1.5 or less for normal ponds. Recently

EHP found to be associated with size variation/growth retardation.

BLACK GILL DISEASE

Black gill disease is highly prevalent in the shrimp farms of Andhra Pradesh. More plankton in water, high stocking density, insufficient aeration and more mud in the pond bottom are the predisposing factors. The gill becomes black in colour and is generally colonized with different bacteria (*Flavobacterium*, *Cytophaga*, etc.) and parasite (e.g. *Zoothamnium* sp.). Increase in duration of aeration, water exchange and addition of lime according to pH may be the corrective measures.

Black gill condition in shrimp

In poor management/culture conditions the increase in population of the fouling organisms can cause impairment of physiological functions of the host. Usually, heterogenous mixture of filamentous and non-filamentous bacteria (*L. mucor* ,*Vibrio* sp. *Thiothrix* sp., *Flavobacterium* sp., *Flexibacter* sp., and *Cytophaga* sp.), blue green algae and protozoa (*Zoothamnium*) causes fouling and black gill. Disease occurs when there is abundant colonization on the gill lamellae, mouth parts and/or swimming appendages with respective physiological dysfunctions. Affected shrimps show slow growth rate associated with sporadic but persistent mortality.

WHITE FAECES SYNDROME (WFS)

White faeces syndrome reported since last decade, has recently been noted as serious problem for *P.vannamei* throughout the world. WFS usually occur after 60 days of culture (DOC) and it may be accompanied by very high mortalities. Ponds affected with WFS show white faecal strings floating on the pond surface while the shrimps show white/golden brown intestine, reduced feed consumption and growth retardation and often associated with loose shell. The disease can cause moderate to severe economic loss by reducing the shrimp survival by 20–30 percent when compared to normal ponds.

White faeces condition in affected pond

It has been estimated that the Thai production losses due to WFS in 2010 were 10–15%. The cause of white faeces syndrome and treatment is uncertain. However while investigating the aetiology of WFS; this disease has been associated with vibriosis, EHP, blue-green algae and loose shell syndrome. *Vibrio* spp. found in haemolymph and intestine will be significantly higher in diseased shrimp than in healthy shrimp. Six species of fungi (*Aspergillusf lavus, A. ochraceus, A. japonicus, Penicillium* spp., *Fusarium* spp., and *Cladosporium cladosporioides*) were found associated with from shrimp naturally infected with white faeces syndrome.

***P. vannamei* affected with White faeces syndrome**

Histopathological examination revealed diffused haemocyte encapsulation and dilated hepatopancreatic tubules accompanied by necrosis. The transformation, sloughing and aggregation of microvilli from the HP

tubule epithelial cells and the denuded epithelial cells subsequently undergo lysis, can lead to the phenomenon called white faeces syndrome (WFS) and transformed microvilli (ATM) in very severe cases they may retard shrimp growth and may predispose shrimp to opportunistic pathogens. Reduced stocking density, proper water exchange together with better management practices will be helpful in evading WFS.

ABDOMINAL SEGMENT DEFORMITY DISEASE (ASDD)

Abdominal segment deformity disease was first described in *P. vannamei* from Malaysia and Thailand. The disease is characterized by gross signs of deformed abdominal segments that were enlarged or twisted laterally and/or dorsoventrally and in few with opaque muscles. Due to unpleasant appearance it caused economic loss of approximately 10% in affected specimens. Despite the distortion, shrimp growth and survival were unaffected. The disease is not caused due to known shrimp pathogens. However, by transmission electron microscopy (TEM), 20–22 nm, non-enveloped viral-like particles were observed in in affected muscles, connective tissue, gills and neural tissues leading to suspected a viral cause. Subsequent work revealed that ASDD was associated with host genetic element that produced RNA transcripts called non-long terminal repeat (non-LTR) retrotransposons (NLRS). In addition, long term use of female broodstock after eyestalk ablation in the hatchery increased the intensity of RT-PCR amplicons for NLRS. The deformities visible in the larvae persisted upon further cultivation until shrimp harvest, but did not increase in prevalence and did not affect growth or survival. Thus, it was hypothesized that NLRS transcripts were upregulated by stress in repeatedly spawned female broodstock and that this caused ASDD in their offspring by an unknown mechanism. Until the situation is clarified, it is recommended that immediate control measures for ASDD include avoidance of long-term broodstock use and employment of PCR screening to exclude broodstock that give positive reactions for NLRS

***PENAEUS VANNAMEI* NODAVIRUS (PvNV) INFECTION**

The virus is reported to cause muscle necrosis. This was first reported in 2004 from *P. vannamei* cultured in Belize. Infection with this virus resulted up to 50% reduction in production in the affected farms. Based on molecular and ultrastructural characteristics, *Pv*NV is placed in the Nodaviridae family. The gross and histological signs were whitened abdominal muscles, coagulative muscle necrosis with haemocytic aggregation and basophilic inclusions similar to the signs of IMNV. Effect (s) on farmed shrimp has not been fully evaluated and is not clear at present. RT-PCR and In-situ Hybridization (ISH) methods are needed to detect *Pv*NV and differentiate it from IMNV.

COVERT MORALITY DISEASE (CMD)

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

WHITE GUT DISEASE (WGD)

This disease of *P. vannamei* is mostly caused by different species of *Vibrio* and is very much prevalent in Andhra Pradesh and Tamilnadu. Vibrios are normally present in water bodies. But sometimes stressed environmental factors such as sudden change of environment and salinity, low DO, mechanical injury, higher stocking density, etc. caused rapid multiplication of this organism in the gut and hepatopancreas. Vibriosis caused red discolouration and melanization of appendages (red disease), necrosis of tail, broken antennae, etc. Six

species of *Vibrio* viz. *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V.vulnificus* and *V. splendidus* are generally associated with the diseased shrimp. The diagnosis of the disease can be done by isolation of organism by plating haemolymph on the TCBS agar followed by identification of the species of *Vibrio* by biochemical tests or 16S rRNA gene sequencing.

MUSCLE CRAMP SYNDROME (MCS)

This disease is mainly caused by environmental stresses, mainly low DO and sudden rise of environmental temperature and is especially common when the stocking density is high. MCS is very much prevalent in Tamilnadu, Andhra Pradesh and West Bengal. The body of the shrimp bends and stiffness of the muscle observed. The rate of mortalities varies. The increase in duration of aeration circumvents the problem to some extent.

AQUATIC ANIMAL HEALTH MANAGEMENT STRATEGIES

The major objective of an effective health management protocol is to ensure not only improved production, but also to reduce the risk associated with diseases. However, even the stringent health management procedure may not be able to eliminate the risk of diseases or mortality completely. Main preventive measures at pond / farm level include proper pond preparation to eliminate pathogens and their carriers, treatment of water in reservoirs to inactivate free viruses and kill virus carriers, water filtration using fine filters to keep carriers out, closed systems to avoid contamination from source water, reduced water exchange to minimize the entry from source water and even changing the water source. Transmission of virus can be prevented by providing crab fencing, fencing, foot baths, wheel baths, and disinfection protocols. Improved husbandry practices have been successfully employed for the control of diseases. Shrimp ponds with a history of disease outbreaks have a greater likelihood of future disease outbreaks, and hence, special attention is required during pond preparation in such farms. Pond preparation is essential to reduce the risks of shrimp disease outbreaks. Removal of bottom sludge, especially in ponds with high stocking densities, ploughing of soil when wet, use of lime during pond preparation will help in minimizing disease risks. Farms with poor bottom soil quality such as presence of a black soil layer, will suffer crop failures. Hence, the sludge must be removed and disposed away from the pond site. Extra precaution should be taken while disposing sludge from farms affected by disease outbreak during the last crop. Sludge removal should pay attention to areas of the pond where there is a high accumulation of organic matter from pervious crop, such as feeding areas. Ponds must be ploughed to expose the black soil layer underneath bottom soil to sunlight and atmospheric oxygen. By this process, the organic waste (sludge) will be oxidized. Ploughing on wet soil is particularly recommended for ponds if the sludge cannot be removed properly by manual or mechanical methods. After ploughing, ponds must be dried for 2-3 weeks and even more when pond had a history of WSD outbreak since WSSV can be viable for three weeks despite sun-drying. In case a heavy tractor is used for ploughing, then plough the dry soil and then fill the pond with water to wet the soil and then again dry. Ploughed pond bottom leads to turbid water conditions during culture period. Therefore, compaction of the bottom using heavy rollers after the whole process of pond preparation, i.e., before water intake, helps avoid the turbid water condition. Liming during pond preparation optimizes pH and alkalinity conditions of soil and water. The type and amount of lime to be added depends mainly on the soil pH and also on pond water pH. Thus an effective health management programme comprises steps and control measures that are carried out on a daily-basis. Overall, health management procedure includes crop-planning, pond preparation, post-larvae/fry selection and stocking process, management of water quality, pond bottom, feed, health monitoring, farm record keeping, biosecurity measures, dealing with disease outbreaks, if any, and appropriate use of chemicals, if necessary.

SUMMARY

Aquaculture is now integral to the economies of many countries. Growing demand for seafood and limitations on production from capture fisheries will inevitably lead to the increased intensification in commercialization of shrimp aquaculture. Aquaculture sector has undergone spectacular transformation through expansion, intensification and diversification. This in turn increases

the number of diseases and leads to emergence of new diseases. However, as a consequence of disease problems and the resultant production losses have been the major limiting factor in aquaculture. The emergence and spread of infectious disease is usually the result of a series of linked events involving the interactions between the host, the environment and the presence of pathogens. Focusing efforts on producing high quality seed, better pond manage to reduce stress and risk of infection, following routine farm biosecurity, responsible trade practices, response to disease outbreak, and improved better management practices shall aid in preventing the epidemics of diseases. Further health management is a shared responsibility, and each stakeholder's contribution is essential to the health management process.

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An overview of Microsporidia-with special reference to EHP

Introduction

Microsporidia are obligate, unicellular organisms infecting wide range of eukaryotic hosts belonging to the phylum Microspora. All microsporidia are spore-forming, intracellular parasites that invade vertebrates and invertebrates. A characteristic feature of microsporidia is the polar tube or polar filament found in the spore used to infiltrate host cells. Development of the parasite generally occurs within the cytoplasm of the host-cell via nuclear proliferation, and spore formation (sporogony), though certain genera are known to undergo similar development within the host nucleoplasm. They are widely distributed in nature with over 1200 species characterized. The role of Microsporidia has been focussed on terrestrial hosts, ranging from infecting pest and beneficial insects to important parasites of humans. But, almost half of the identified microsporidian genera are known to infect aquatic hosts. Out of 187 genera described, approximately 91 genera infect fish, aquatic arthropods and non-arthropod invertebrates. Microsporidia include Agmasoma, Ameson, Nosema, Pleistophora, Tuzetia, Thelohania, Flabelliforma, Glugoides, Vavraia, Ordospora, Nadelspora and Enterospora have been reported to infect crustacean hosts.

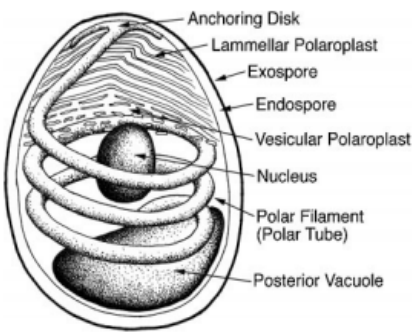


Fig. 1 Diagram of a microsporidian spore

(Keeling *et al*., 2002)

Recently, *Enterocytozoon hepatopenaei* (EHP) (measure about 1.7 ×1 µm), have been reported in shrimp farming nations of south-east Asia in cultured *Penaeus vannamei.* The parasite was first recorded from growth retarded tiger shrimp, *P. monodon* from Thailand and reported as an undesignated microsporidian. Subsequently, the parasite was identified and characterised from *P. monodon* from Thailand in 2009. EHP infects *P. monodon* and *P. vannamei* and is suspected to infect *P. Japonicus*. EHP infection in shrimp cannot be detected by visual inspection of animals and there are no specifically distinctive gross signs, except that it is suspected to be associated with growth retardation and white feces syndrome (WFS). The target organ for EHP is shrimp hepatopancreas; being the power house of the animal, infection in the digestive organ may cause impairment in metabolism and ultimately resulting in stunted growth.

Taxonomy

Phylum: Microspora (Sprague, 1977).

Class: Microsporea (Delphy, 1963).

Order: Microsporida (Balbiani, 1882).

Family: Enterocytozoonidae (Cali and Owen, 1990).

Genus: *Enterocytozoon* (Desportes *et al*., 1985).

The scientific classification of microsporidia had evolved over the years. Initially, phylum microspora are thought to be a protozoan (kingdom Protista) based on the traditional identification by observing the physical characteristics of the spore, life cycle and relationship with the host cell. However recent studies using genetic tools (namely ribosomal RNA sequencing) indicating that phylum microspora should be classified under Kingdom Fungi. At present more than 1200 species were identified in 187 genera. Out of that 20 genera infect fish, 50 genera infect aquatic arthropods and at least 21 genera infect aquatic non-arthropod invertebrates, protists, and hyperparasites of aquatic host.

The placement of the family Enterocytozoonidae within the Clade VI of the Terresporidia poses an intriguing taxonomic conundrum. The pathogens belong to the family Enterocytozoonidae infect host inhabit marine,

freshwater, and aquatic environments and span several trophic levels (including hyperparasitic copepods, decapods, fish, and humans). Besides similarity in their their ssrRNA gene, pathogens grouped in Clade VI share characteristic morphological traits (including the ability for intranuclear infection in host) and, generally, infect gut epithelial cells. *Enterocytozoon bieneusi* is a common pathogen in immunosuppressed patients in humans (infection in AIDS cases). *Enterocytozoon bieneusi* closely related with the marine crab pathogen *Enterospora canceri*, fish pathogens within the genus Nucleospora, and, recently, salmonid fish pathogen the parasite *Paranucleospora theridion* (=*Desmozoon lepeophtherii*) and with the marine shrimp pathogen *Enterocytozoon hepatopenaei*.

The name *Enterocytozoon hepatopenaei* was proposed with justification based on ultrastructural characters that conform to the unique features of the family Enterocytozoonidae but distinguish it from other species in the family, i.e., *E. beiniusi* that also develops in the host-cell cytoplasm and species in the genera Enterospora and Nucleospora that develop within the host-cell nucleus. Distinction from Enterospora and Nucleospora is clear based their intranuclear rather than cytoplasmic development. However, *E. hepatopenaei* had several features similar to *E. bieneusi*, considering the mean spore size marine habitat (in case of *E. hepatopenaei)* and 16% difference in the 18 SSU r RNA gene sequence, it was proposed with a new species name *E. hepatopenaeii* within the family Enterocytozoonidae..

Biology

In biology, germination of the microsporidian spore is one of the most interesting and dramatic series of subcellular events. Generally, spore germination begins with an environmental trigger that varied for different species depending on their habitat.

In vitro, spores may be germinated by a number of physical and chemical stimuli including alterations in pH, dehydration followed by rehydration, hyperosmotic conditions, the presence of anions or cations, or exposure to ultraviolet light or peroxides.

Germination of spore started with general swelling of the spore followed by specific swelling of the polaroplast and posterior vacuole and ultimately resulted in increase in osmotic pressure in the spore. The internal pressure of the spore and the breakdown in sporoplasmic membranes culminate in the rupturing of the anchoring disk and the discharge of the polar filament by eversion. The discharged polar tube can range in length from 50– 500 µm in length. The entire event of germination takes place in fewer than two seconds. If a potential host cell lies nearby, the discharging tube can strike this cell and pierce its membrane. Once the polar tube is fully discharged, the continued pressure within the spore forces the sporoplasm through the polar tube. If the polar tube has penetrated a host cell, the sporoplasm emerges from the tube directly into the host cytoplasm, thus infecting the host without its recognition the parasite as a foreign invader.

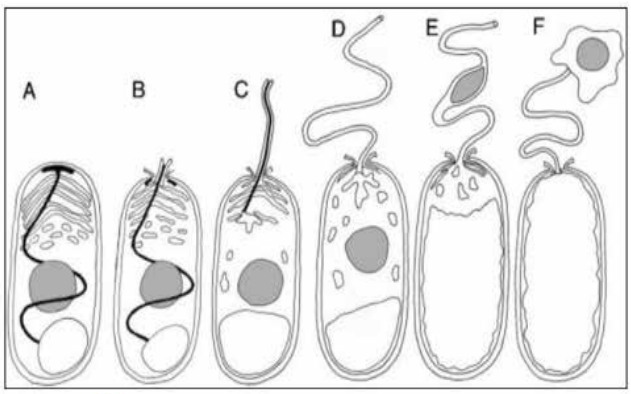
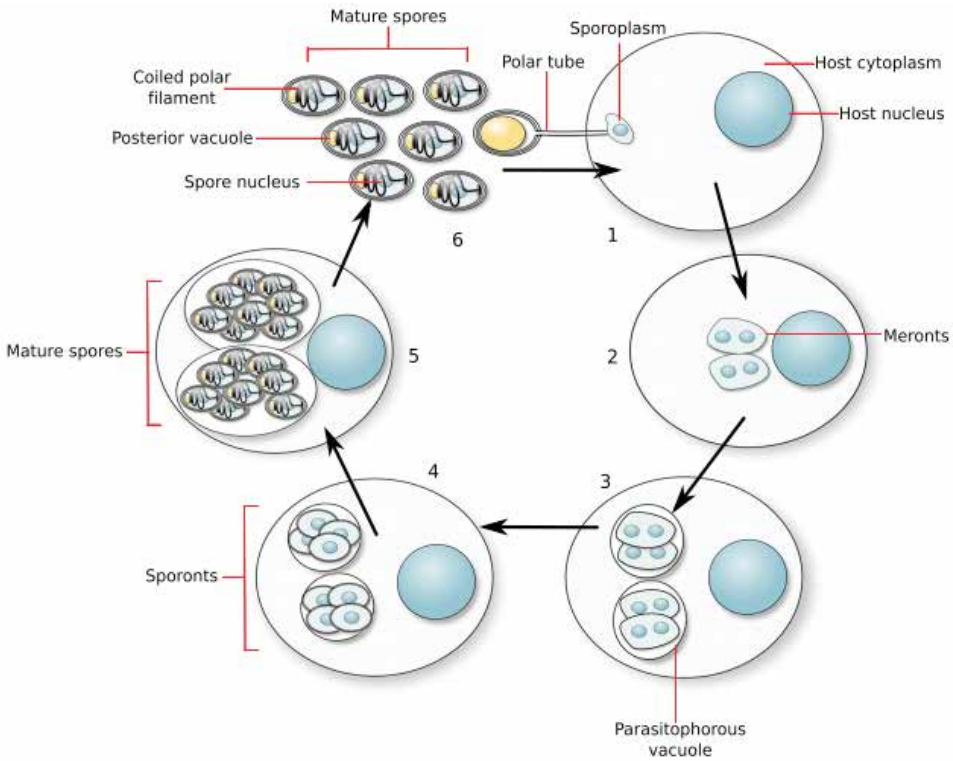


Fig : Model of spore germination. (A) Dormant spore, showing polar filament (black), nucleus (grey), polaroplast and posterior vacuole. (B) Polaroplast and posterior vacuole swelling, anchoring disk ruptures, and polar filament begins to emerge, everting as it does so. (C) Polar filament continues to evert. (D) Once the polar tube is fully everted, the sporoplasm is forced into and (E) through the polar tube. (F) Sporoplasm emerges from the polar tube bound by new membrane. (Keeling *et al*., 2002)

Life cycle

The life cycles of microsporidia have 3 phases: infective, proliferative, and sporogonic. Once inside the host cell, the parasite is referred to as a meront and it begins a stage of growth and division characterized by a high degree of interspecies variation. Often the host cell reorganizes around the parasite, so that the

parasite can be found surrounded by host organelles such as endoplasmic reticulum (ER), nuclei, or mitochondria. Host cells may also transform in shape and size, often enlarging induced by microsporidian parasite called as xenoma. Thus host cell is induced to enlarge and undergo many rounds of nuclear division resulting in an enormous plasmodium filled with parasites: mature spores at the center with the earlier stages radiating toward the periphery of the xenoma. The onset of sporogony is marked in some species by the separation of diplokaryotic nuclei and in other specific cases by meiosis. In most species, this stage of the life cycle is also accompanied by some degree of division, although the number of sporoblasts (presporal cells) produced varies among species from two (bisporous) to many (polysporous). Following division, the extrusion apparatus (including the polar filament, polaroplast, and posterior vacuole) begins to develop. As the extrusion apparatus nears complete formation and the sporoblasts approach maturity, the cells decrease in size and the chitinous endospore layer develops. *E. hepatopenaei* spores are ovoid in shape measuring (1.1- 0.7 µm in fresh preparation) and containing 5–6 visible coils of the polar filament. Polar filament precursors and other spore organelles formed within the sporogonal plasmodium and packaged into pre-sporoblast units prior to budding of sporoblasts to the host-cell cytoplasm. Once complete, the mature spores are released. In shrimp, *E. hepatopenaei* spores are released into the pond water via white feces where they can infect other animals.



Life cycle of a Microsporidian parasite (Watson *et al*.,2015)

Transmission

Virulence of the microsporidian parasite mainly depends on the mode of transmission. Horizontal transmission relies on sufficient number of available parasites (particularly when spores are released into water) in-turn leads to select for high parasite replication and virulence. Whereas, vertical transmission requires host reproduction to occur and thus will select for

reduced virulence. Some microsporidian parasites (such as *Edhazardia aedis* in mosquito hosts) can switch between low-virulence vertical transmission in well-resourced (reproducing) hosts and high-virulence horizontal transmission in poor-resourced hosts. Vertical transmission usually only occurs via female hosts (sperm size precludes infection), a situation which selects for reproductive manipulation to increase the relative frequency of infected hosts. Microsporidia are the only eukaryotic parasites known to induce sex ratio distortion via male killing or feminisation (eg *Amblyospora californica* causes benign infection in female mosquito larvae). In, *Gammarus duebeni, Nosema granulosis* perturbs development of the androgenic gland in juvenile, leading to feminisation and subsequent transmission. *E.hepatopenaei* can readily be transmitted among individuals through cohabitation (spores released into the water through shrimp faeces) and as well as through healthy shrimp cannibalizing those that were moribund or dead infection. The possible ways of *E.hepatopenaei* vertical transmission route (trans-ovum) is poorly understood. So far, no secondary hosts are known to be involved in transmission of *E.hepatopenaei*.

Conclusion

In shrimp aquaculture, a microsporidian parasite *E. hepatopenaei*, the causative agent of hepatopancreatic microsporidiasis had resulted in significant economic losses, in many shrimp farming nations. There are no specifically distinctive gross signs for this microsporidian infection. Shrimp hepatopancreas is the target organ for EHP infection. EHP infection can be transmitted horizontally through cohabitation and oral route and possibly vertical transmission. The studies on the biology, life cycle and transmission of emerging microsporidian *E. hepatopenaei* are limited. Microsporidian–host relationships provide numerous examples of parasite–host coevolution. Parasite-host coevolution, vertical transmission, role of intermediate host and any possible role of EHP in zoonotic infection (as closely related with human parasite *E.bineusi*) and host sex distortion need to be studied.

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Update of investigations on *Enterocytozoon hepatopenaei* (EHP) in India by ICAR-CIBA, Chennai

Hepatopancreatic microsporidiosis caused by *Enterocytozoon hepatopenaei* (EHP) have been reported to occur widely in China, Indonesia, Malaysia, Vietnam and Thailand. A special warning was also issued to the aquaculture sector in Mexico. EHP is a microsporidian parasite reported in black tiger shrimp farming in Thailand in the year 2009 in slow growing shrimp. EHP affects the shrimp hepatopancreas (HP) and thereby possibly leads to slow growth of shrimp. Studies suggest that EHP is not an exotic pathogen but it is endemic to Australasia. EHP also infects *Penaeus vannamei* and it is transmitted in shrimp directly by the oral route. Although EHP does not cause mortality, it seems to be associated with growth retardation in *P. vannamei*.

ICAR CIBA initiated investigations on EHP in India since 2014. Since then, the investigations carried out by CIBA have indicated that EHP has become an endemic problem in Indian shrimp farming and requires urgent attention by the stakeholders. The following is the gist of investigations carried out on this important pathogen over the last two and a half years.

A first report on the "Emergence of *Enterocytozoon hepatopenaei* (EHP) in farmed *Penaeus (Litopenaeus) vannamei* in India was published in the journal 'Aquaculture', with a statement of relevance: EHP, a microsporidian parasite is an emerging pathogen having serious economic consequences to cultured shrimp in Asia (Rajendran et al, Aquaculture, 2016, 454: 272-280). This report forms the first record of the emergence of the parasite in farmed *P. vannamei* from India, and presents a comprehensive account of the pathogen and its impact on the host.

National consultation was held on "Managing Enterocytozoon hepatopenaei (EHP) infections in Brackishwater aquaculture in India: A one day national consultation was held on "Managing *Enterocytozoon hepatopenaei* (EHP) infections in Brackishwater aquaculture in India" on 19th January 2016, at CIBA Chennai. Stakeholders from the brackishwater aquaculture sector, representing scientific research institutions, universities, shrimp hatchery operators, shrimp farmers, feed manufacturers, members of Society of Aquaculture Professionals (SAP), Seafood Exporters Association of India (SEAI), officials from the Ministry of Agriculture, Department of Animal Husbandry Dairying and Fisheries, Coastal Aquaculture Authority, Marine Products Export Development Authority (MPEDA), Rajiv Gandhi Centre for Aquaculture (RGCA) and state Govt. officials participated the consultation meeting. The consultation concluded that the pathogen was transmitted in shrimp directly by the oral route and caused stunted growth condition in shrimp leading to significant economic losses to the farmers. Presently, there is no drug or therapeutic options available for the control of EHP infection in shrimp. Hence, adoption of better management practices and proper biosecurity measures along with stocking of EHP free shrimp seeds is the only way to keep the pathogen away from shrimp farming system. An advisory on managing EHP in shrimp farms was also prepared and published online and distributed to the stakeholders.

Prevalence of EHP in Indian shrimp aquaculture: Active disease surveillance was carried out in 141 shrimp farms covering coastal states such as, Andhra Pradesh, Tamil Nadu, Gujarat and West Bengal during 2015-16 and in 155 shrimp farms during 2016-17. The EHP emerged as a new challenge to Indian shrimp farming, which was detected in 26.7% and 23.6% of the shrimp farms respectively for the years 2015-16 and 2016-17 in the maritime states, particularly Andhra Pradesh (AP) and Tamil Nadu (TN).

However, during recent months, EHP is being increasingly detected in larval shrimp in hatcheries, and has become a matter of great concern. Although increasing efforts have been made by CIBA, CAA, MPEDA, RGCA in promoting use of PCR tested seed by the shrimp farmers, the move is yet to be taken seriously by the stakeholders. Considering this, it is likely that EHP is likely to become a bigger problem in the coming years.

Transmission of EHP: Investigations on the transmission of EHP indicated that it could be transmitted to healthy SPF vannamei shrimp on two weeks of cohabitation with infected shrimp and after seven days of feeding with hepatopancreas from the EHP affected shrimp. Another interesting observation was that EHP could be transmitted through soil as confirmed by PCR upon 15 days of exposure of healthy shrimp to pond-soil. Screening of other fauna such as polychaete worms, green mussel and clams suggested that these biota may not have a role in the transmission of EHP, as revealed by PCR tests.

***Improved microscopic method for detection of EHP in shrimp*:** Light microscopic examination of the stained clinical smear is an inexpensive method of diagnosing microsporidian infections even though it does not allow species level identification. EHP spores could be relatively easily detected by microscopic examination of modified Ryan-blue trichrome staining method. This method enables differential diagnosis of microsporidian spores by a characteristic staining pattern of pinkish-red which could be easily distinguished from similarly staining particles.

***EHP diagnostics*:**

Specific detection of EHP can be done using DNA based molecular diagnostic tools such as PCR, real time PCR, *in situ* hybridization (ISH) and loop mediated isothermal amplification (LAMP) methods (Tourtip et al., 2009; Tangprasittipap et al., 2013; Suebsing et al., 2013; Liu et al., 2014; Tang et al., 2015). The AAHED lab of CIBA has been testing shrimp samples using PCR protocols of Tourtip et al., 2009; Tangprasittipap et al., 2013 and Tang et al., 2015). Recently, a nested PCR assay to avoid false positive detection of EHP in the environmental samples in shrimp farms has been published (Jaroenlak et al, 2016). According to this report, since many PCR protocols target the EHP small subunit ribosomal RNA (SSU rRNA) gene (SSU-PCR), which can give false positive test results due to cross reactivity of the SSU-PCR primers with DNA from closely related microsporidia, to overcome this problem, a sensitive and specific nested PCR method was developed for detection of the spore wall protein (SWP) gene of EHP (SWP-PCR). More recently, Tang et al (2017), compared the nucleotide sequence similarities in 18S rRNA gene (1095-bp), in β-tubulin (583-bp) and spore wall protein (431-bp) genes between the Venezuela EHP and SE Asia isolates; and the results showed 99%, 93% and 91% identities, respectively.

CIBA's EHP diagnostic kit: CIBA has developed an indigenous and cost effective diagnostic kit for detection of EHP by nested PCR based on the sequence of 18s small subunit rRNA (SSU rRNA) gene of the EHP isolates in India. This kit is designed for the specific and sensitive detection of as low as 10 copies of EHP DNA in the sample. This kit is cost effective, user friendly and can be performed consistently. The kit contains all the essential materials and reagents for DNA isolation and PCR, and packaged as single or double tube format to suite the diagnostic laboratories. An inbuilt reaction control using a house keeping gene (β-actin gene) will ensure perfectness of nucleic acid extraction and the PCR reaction.

ICAR-CIBA is in the process of developing an EHP spore wall gene based PCR kit and would be commercialised soon for the benefit of the aquaculture community. Additionally, a real time PCR kit for more accurate and specific diagnosis of EHP would also be made available to the stakeholders.

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Methods for detection of *Enterocytozoon hepatopenaei* (EHP) in shrimp

Introduction

Hepatopancreatic microsporidiosis (HPM) is an important disease of cultivated shrimp caused by *Enterocytozoon hepatopenaei* (EHP). Microsporidia belonging to the Phylum: Microspora; Class: Microsporea; Order: Microsporida; Family: Enterocytozoonidae and Genus: Enterocytozoon, are obligate intracellular parasites known to infect a wide range of eukaryotic hosts. Development of this parasite generally occurs within the cytoplasm of the host-cell via nuclear proliferation, and spore formation (sporogony). EHP was first reported from the slow growing black tiger shrimp *Penaeus monodon* in Thailand during 2004 and later it was described in detail during 2009. EHP could also infect *P. vannamei* by the oral route and it is confined to the shrimp hepatopancreas (HP). It is associated with severe growth retardation in *P. vannamei* without causing mortality. HPM outbreaks were reported widely in China, Indonesia, Malaysia, Vietnam and India by many researchers. Thus, it has been considered as an economically important disease. Hence, it is compulsory to monitor *P. vannamei* and *P. monodon* for EHP in broodstock and post larvae (PL). To serve this purpose, fast and accurate diagnosis is paramount important to ensure the biosecurity in shrimp hatcheries and grow-out farms. Different diagnostics for this emerging disease is discussed in this chapter as there is an urgent need for control and prevention.

Clinical signs

There are no specifically distinctive gross signs of infection by EHP. The infection may be suspected with the occurrence of unusually retarded growth with reduced feed conversion ratio (FCR) in the absence of other diseases. EHP must be confirmed by microscopic and/or molecular methods.

Direct microscopy

Wet mounts of fresh HP tissue squash and/or faecal strands (non-lethal method) are examined with Giemsa or modified trichrome staining under light microscopy. Diagnosis is made by the demonstration of characteristic spores under oil immersion (100 x objective) in light microscope. The spores are very small (1.1±0.2 by 0.6-0.7±0.1 µm) and show the presence of a polar filament of 4-5 coils. This method may not be useful in detecting the spores which are present in scant numbers. But there is need to find out the correlation between the parasitic load and growth retardation.

Histopathology

Among all other diagnostic methods, histopathology plays very significant role in demonstrating the actual infection i.e., presence of pathogen with sequential pathological changes in the cells and tissues. The target organ for HPM in shrimp is identified as HP. Hence, HP is collected in 10 times the volume of Davidson's fixative (95% ethanol 330mL + 37% formalin 220mL + glacial acetic acid 115mL + distilled water 335mL per litre). Dead shrimp should never be collected to avoid the confusion in interpretation due to autolytic and decomposed changes. Only live, moribund, or compromised shrimp should be selected for fixation and histological examination. Selected shrimp are collected on ice and the smaller shrimps (<1 g) are fixed directly by immersing in samples to Davidson's fixative ratio of 1:10 for 24 h and larger shrimps (>1 g) are fixed for 48 h in a wide mouth plastic bottle. For larger shrimp, the fixative is injected into iced live animals @ 10% of shrimp body weight, first into HP, then into the ventral sinus after removing haemolymph so that the fixative is equally distributed along the course of the circulatory system. The cuticle is slit open on both sides of the shrimp from the 6th abdominal segment to the rostrum using scissors before placing it in the fixative. After 24-48 h fixation in Davidson's fixative, the samples are transferred to 70% ethanol for long storage with appropriate sealing and labelling. The HP tissue is processed by standard histopathological technique such as dehydration with ascending grades of alcohol, clearing with xylene, infiltration/impregnation in paraffin, embedding/

blocking, sectioning @ 4–6 µm thickness and staining with routine histological haematoxylin and eosin (H&E) stains. HP tubule epithelial cells show the presence of cytoplasmic, basophilic inclusions under oil immersion lens (100x) containing clusters of elliptical to somewhat ovoid spores. Spore formation is observed exclusively in cytoplasm of B cells. Extensive infection of the medial and proximal tubule epithelial cells of the HP is observed in the absence of spores. Sometimes free spores released from lysed cells may be seen in the tubule lumens. Infected tissues reveal several developmental stages, including the early sporogonal multinucleated plasmodia and mature spores (1.1±0.2 x 0.6-0.7±0.1 µm). Plasmodia of the microsporidian (ePm, early plasmodium; lPm, late plasmodium) may also be seen in tissue sections but cannot be used in as a confirmatory diagnosis in case of the absence of spores. In some situations, spore production may be low or not yet initiated, making confirmatory diagnosis difficult or impossible. In such cases, PCR detection is recommended.

In situ hybridization (ISH)

In situ hybridization (ISH) is an another important tool to determine the location of pathogen nucleic acid in tissue sections of PCR positive animals in order to know whether they are active (infected) or passive (uninfected) carriers. It can be done using digoxigenin-labeled (DIG-labeled) probes for the small subunit ribosomal RNA (SSU rRNA) and spore wall protein (SWP) gene. Final detection is performed with anti-digoxigenin antibody conjugated to alkaline phosphatase that is visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. However, specificity of the *in situ* hybridization reaction can be relatively low.

Transmission electron microscopy (TEM)

Small pieces (2 mm^3) of HP tissue are fixed in 2.5% glutaraldehyde and 4% paraformaldehyde, or 6% glutaraldehyde for transmission electron microscopy (TEM). The tissues are processed as per the TEM protocol and visualised with the following details. Different stages of the parasite are described, from early sporogonal plasmodia to mature spores in the cytoplasm of host-cells. The multinucleate sporogonal plasmodia exist in direct contact with the host-cell cytoplasm and contain numerous small blebs at the surface. Binary fission of the plasmodial nuclei occurs during early plasmodial development and numerous pre-sporoblasts are formed within the plasmodium. Electron-dense disks and precursors of the polar tubule develop in the cytoplasm of the plasmodium prior to budding of early sporoblasts from the plasmodial surface. Mature spores are oval, measuring 0.7-1.1 µm and contain a single nucleus, 5–6 coils of the polar filament, a posterior vacuole, an anchoring disk attached to the polar filament, and a thick electron-dense wall. The wall is composed of a plasmalemma, an electronlucent endospore (10 nm) and an electron-dense exospore (2 nm).

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a technique very often used in molecular biology by selectively amplifying a particular targeted segment of DNA using specific primers. PCR derives its name from one of its key components, a DNA polymerase used to amplify a piece of DNA by *in vitro* enzymatic replication. As PCR progresses, the DNA thus generated is itself used as template for replication which is further exponentially amplified. Recent past years, PCR has become indispensable technique in medical and biological research. The representative shrimp samples are collected in 95% ethanol for EHP detection by PCR. EHP is diagnosed by PCR targeting the SSU rRNA gene (SSU-PCR) but it can give false positive results due to cross reactivity of the SSU-PCR primers with DNA from closely related microsporidia. This is challenging for investigating and monitoring EHP infection pathways. To overcome this problem, a sensitive and specific nested PCR method is available targeting the spore wall protein (SWP) gene of EHP (SWP-PCR). The new SWP-PCR does not produce any false positive results from closely related microsporidia. In addition, SWP-PCR method is 100 time more sensitive than that of the existing SSU-PCR method. Due to its greater specificity and sensitivity, the SWP-PCR method can be used to screen for EHP in HP, faeces, feed and environmental samples for potential EHP carriers. Recently, ICAR-CIBA has developed a nested PCR

targeting the SSU rRNA which can detect the EHP as low as 10 copies of parasite DNA in the shrimp sample.

Real-time PCR (qPCR)

Real-time PCR, also known as quantitative PCR (qPCR), is a molecular technique based on the PCR. It monitors the amplification of a targeted DNA molecule in real-time during the PCR, not at the end as in conventional PCR. PCR products are quantified in qPCR by two common methods either by a non-specific fluorescent dyes that intercalate with any double-stranded DNA or by using the sequence-specific DNA probes labelled with a fluorescent reporter and quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of real time PCR platforms. Based on these principles, qPCR for EHP is developed with sequence-specific DNA probes and commercialised by few companies.

Loop-mediated isothermal amplification (LAMP)

Traditional PCR or qPCR detection methods are restrictive because they require high investment in equipments and expertise in molecular methods. As an alternative, loop-mediated isothermal amplification (LAMP) method has been developed capable of detecting only a few copies of target nucleic acid under isothermal conditions utilizing self-recurring strand-displacement DNA synthesis initiated by specially designed primer sets. This technique can be achieved simply by using a water bath or an inexpensive heating block. LAMP amplicons had been initially visualized by agarose gel electrophoresis stained with ethidium bromide. But recent development shows that the LAMP products may also be assessed indirectly either by the amount of white magnesium pyrophosphate precipitate formed as a by product of the LAMP reaction, by measuring fluorescence from incorporation of DNA intercalating agents such as SYBR Green I, by using a lateral flow dipstick format, or by using SSU rRNA based gold nanoparticle (AuNP) probe (LAMP-AuNP assay). This technique is useful in the field condition for diagnosis of EHP without sacrificing sensitivity or specificity. It significantly reduces the time and cost for molecular detection of EHP in shrimp. Nevertheless, LAMP has a disadvantage of susceptibility to false positives due to carry-over or cross contamination.

Serologic assays

Serological assays such as immunofluorescence, immunoperoxidase, enzyme-linked immunosorbent assay (ELISA) and Western blot are developed in diagnosis of human microsporidiosis. But, the sensitivity and specificity of these methods for detecting antimicrosporidial antibody are not known because no comparative evaluations have been published. In shrimp, these methodologies are not directly applicable since they do not have antibodies producing defence mechanism.

Conclusion

Disease diagnosis plays a vital role in disease prevention and control. The disease prevention and control strategy is the best practice for successful hatchery and grow out culture practices in shrimp industry. In last two decades many lethal viral pathogens like WSSV, Taura syndrome virus etc. are discovered in shrimp culture and their pathogenesis and prophylaxis have been elucidated in detail. However, similar interest in parasitic diseases for brackishwater shrimp and finfish culture is lacking probably due to less incidence. Recent outbreaks of HPM in shrimp warrant the researchers to pay attention towards the parasitic infestation. Moreover, in view of the change in climatic condition, it is necessary to concentrate on parasitic infections in wild and cultured shrimp and fish.

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Non-lethal method for detection of *Enterocytozoon hepatopeanei* in shrimp

Introduction

Infectious diseases pose one of the most significant threats to the Indian aquaculture industry. Till date, a series of disease issues have hit the aquaculture production with the recent emergence of a microsporidian parasite *Enterocytozoon hepatopenaei* (EHP) in farmed shrimp. The disease entity hepatopancreatic microsporidiosis (HPM) caused by *E. hepatopenaei* was first detected black tiger shrimp *Penaeus monodon* in Thailand in 2004, but taxonomically described in 2009. *Enterocytozoon hepatopenaei* is an intracellular spore-forming parasite; it replicates within the cytoplasmic area of the tubular epithelial cells in the hepatopancreas causing destruction of the organ, poor assimilation of nutrients resulting growth retardation often referred as slow growth syndrome (SGS). In 2010, EHP was reported as being associated with 'white faeces syndrome' (WFS) in Vietnam, but further laboratory experiments failed to show the association with WFS, although transmission has been demonstrated. Severe infections by EHP can increase the susceptibility for other bacterial infections like *Vibrio* spp. in shrimp farms and could manifest mild mortality. Many workers demonstrated heavy infections of EHP spores in shrimp manifesting white faeces syndrome. EHP spores are extremely hardy and EHP can be transmitted horizontally between shrimp, particularly in earthen rearing ponds. As a result, EHP infection can spread progressively and is believed to intensify with successive shrimp crops. The problem is likely to be exacerbated in biofloc based shrimp culture.

Diagnosis of *Enterocytozoon hepatopeanei* in shrimp

As there are no specific clinical signs for EHP infection, but reported to be associated with stunted growth and/or white faeces syndrome and no mortality, confirmatory diagnosis is tricky. Diagnosis of EHP infection can be carried out in specimens such as hepatopancreas, faeces or pond sediments by traditional staining techniques, histology or *in situ* hybridization techniques. However, these techniques are time consuming, expensive and need specialized laboratory facilities. Species identification usually requires detection by PCR techniques and more advanced genomic characterisation. Histology of infected hepatopancreas reveals several developmental stages, including the early sporogonal plasmodia and mature spores. The plasmodia are multinucleate and mature spores are oval shaped, measuring 1.1 × 0.6-0.7 µm and have a polar filament with 5-6 coils. These spores are excreted through faeces, which is the infective form of the parasite and hence ideal sample for non-lethal diagnostic techniques. However, these tiny spores are indistinguishable from large volume of tissue/faecal debris in diagnostic samples and poor differentiation of spores from other debris materials by conventional stains. **Pleopods, uropods or haemolymph etc. are not suitable for diagnosis of EHP** by any of the above mentioned techniques. In this chapter we will examine the non-lethal method of EHP diagnosis by various techniques.

Detection of EHP in shrimp faeces

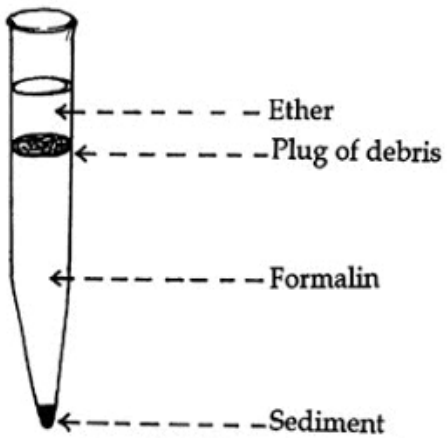
Microscopic detection by wet smear technique

Microscopically, a typical microsporidian spores contain a single nucleus, coiled polar filament, a posterior vacuole, an anchoring disk attached to the polar filament, and a thick electron-dense wall. Unique feature of this parasite is its very small spore size as compared to other microsporidians and hence these features may not be discernible. Difficulty in confirmatory diagnosis of microsporidians by conventional parasitological techniques arise largely due very small size of the spores and non-specific staining of sample debris originated from faeces or biopsy tissues by conventional stains such as Giemsa, Haematoxylin & Eosin (H&E) etc. A highly diluted wet smear of suspected faecal sample may be observed directly under a phase contrast microscope for the presence of spores. The smear preparations were allowed to dry at room temperature, fixed in methanol for 15 min and stained with Giemsa. The same type of dried smear preparations can be fixed in 5% neutral buffered formalin for 15 min and stained with haematoxylin and eosin. The stained smears were dehydrated, mounted in

DPX following standard procedure and observed under a microscope.

Purification of microsporidian spores from faecal samples

The efficiency of microscopic observation can be increased many-fold by concentration of samples by density based floatation techniques. The purification and concentration of microsporidian spores from faecal samples by sedimentation by water-ether method is given here. Briefly, Collect the faecal strings and homogenise with distilled water and filtered through fine mesh to remove the larger debris. The filtrate was then transferred to a 15 ml centrifuge tube. Add 3 ml of diethyl ether to the centrifuge tube.



Formal ether sedimentation technique

Thoroughly vortex the homogenate for 1 min and centrifuge at 5,000 rpm for 15 min. Carefully remove the fat layer and supernatant and collect the pellet (Fig. 1). Resuspend the pellet in 5 ml distilled water and centrifuge at 5,000 rpm for 5 min. Repeat the washing procedure twice and finally collect the pellet and resuspend in 1 ml distilled water. Check for the presence of spore using oil immersion objective of a light microscopy (Fig 2 A). The spore may be stored at room temperature or at 4^{o}C for future study.

Modified Trichrome Staining

A thin smear of purified spores can be stained by conventional Giemsa (Fig. 2 B) and modified trichrome stain (Ryan-Blue method) for microsporidians. To enhance the differentiation of spores, a microsporidia-specific staining using modified trichrome stain may be useful.

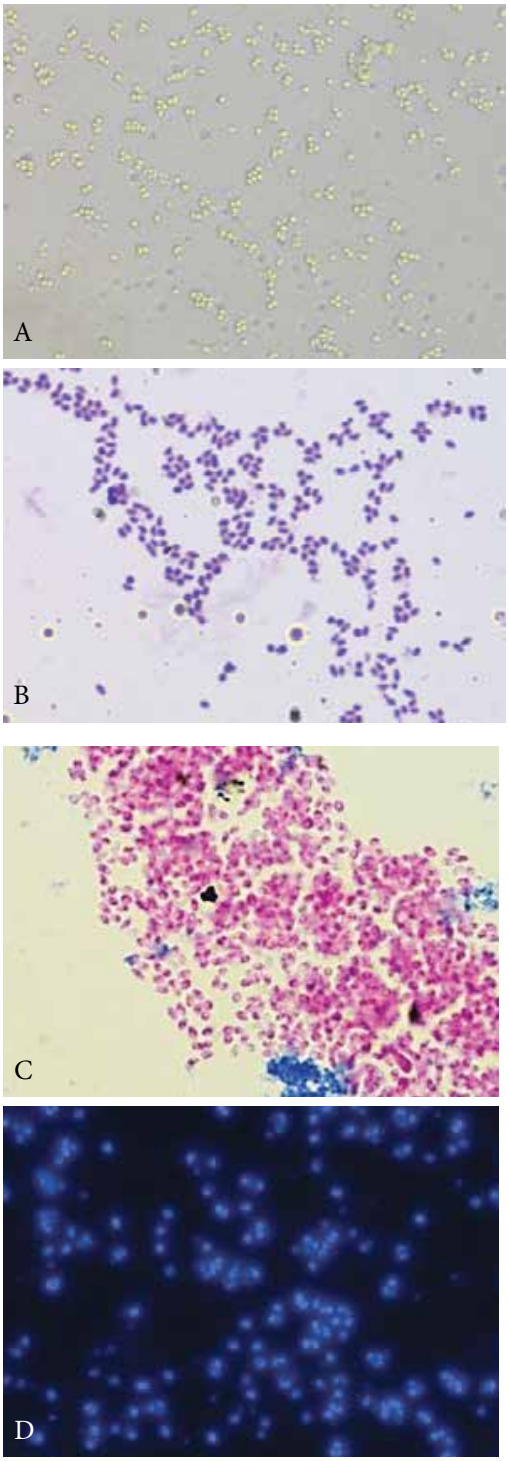


Fig. 2. Microscopic detection of purified spores of *Enterocytozoon hepatopenaei* from *Penaeus vannamei* A- Fresh spores; B- Giemsa stain; C- Modified trichrome stain (Ryan-Blue method); D- Calcofluor White stain. 100x (all digitally enlarged view)

Briefly, trichrome stain solution was prepared by mixing 6 g of chromotrope 2R (Sigma), 0.5 g of aniline blue (Sigma), and 0.25 g of phosphotungstic acid (SRL, laboratories) with 2 ml glacial acetic acid. After the mixture was allowed to stand for 30 min, 100 ml of distilled water was added and the pH was adjusted to 2.5 with 1 M Hydrochloric acid. Following methanol fixation for 10 min, slides were stained in trichrome-blue for 90 min, rinsed for 10 s in acid alcohol (0.45% acetic acid in 90% ethanol) and then for 10 s in 95% ethanol. Slides were then transferred through two changes of 95% ethanol for 5 min each, transferred into 100% ethanol for 10 min and then transferred into xylene for 10 min, followed by mounting in DPX. The stained slides were observed under a microscope for detailed studies of microsporidian spores. Spores appear as ellipsoidal staining pink-red with a transverse band of polar filament or central non-staining zone (Fig. 2 C).

Rapid staining and detection of microsporidian using chemoflourescent stains

Microsporidia stain poorly with hematoxylin-eosin and hence we explored the use of chemoflourescent agents such as Calcofluor white for quick identification of *E. hepatopenaei* spores in hepatopancreatic tissue or faecal smears from shrimp. Fluorochrome stains, have a high affinity for chitin and has been used to detect microsporidia in urine, stool, mucus, and tissue sections in human beings. Calcofluor White is a non-specific fluorochrome that binds with cellulose and chitin contained in the cell walls of yeasts, fungi and parasitic organisms. Evans blue present in the stain act as a counterstain and diminishes background fluorescence of tissues and cells. Staining specimens with Calcofluor White Stain is a rapid procedure and has been described as a rapid method for the detection of detection of yeasts, pathogenic fungi, as well as microsporidian parasites. Calcofluor White Stain can be mixed with a potassium hydroxide mixture to clear up the specimen to facilitate visualization.

Formulation per Litre

Calcofluor White M2R	1.0 g
Evans Blue	0.5 g

Recommended Procedure

- Put the specimen (faecal sample/ hepatopancreas/purified spores) to be examined onto a clean glass slide.
- Add one drop of Calcofluor White Stain (Sigma) and one drop of 10% Potassium Hydroxide.
- Place a coverslip over the specimen. Let the specimen stand and stain for 1 min.
- Cover the slide with a paper towel and gently press to remove any excess fluid.
- Examine the slide under UV light at x100 to x400 magnifications.

Interpretation of Results

The stained slides may be observed through a fluorescent microscope Invitrogen EVOS FL Auto Cell Imaging System, and viewed at wave length between 345 Abs peak and 455 Em peak of DPAI illumination light cube. The microsporidian spore appears as bluish-white or turquoise oval halos (Fig. 2 D). The Calcofluor staining protocol is sensitive method and fast, require ~ 15 min to perform.

Nonlethal techniques using PCR

As mentioned earlier non-lethal screening of precious broodstock can be also be done using Polymerase Chain Reaction (PCR) described in this manual. Arguably one of the most powerful laboratory techniques ever discovered, PCR combines the unique attributes of being very sensitive and specific with a great degree of flexibility. As described in earlier chapters, this technique is being used as a powerful tool for rapid diagnosis of EHP as well. PCR protocol using primers designed based on small sub unit ribosomal RNA (SSU rRNA) gene and spore wall protein (SWP) gene are currently available. Recently, many commercial kits based on conventional nested PCR and real time PCR are available.

Polymerase Chain Reaction (CIBA Kit)

Step 1- ***Reaction set up***

Component	Vol. ml / Reaction (25 ml)	Vol. ml / (6 reactions)
Water	9.5 ml	57 ml
2x Master Mix	12.5 ml	75 ml
Forward primer (10 µM	1.0 ml	6 ml
Reverse primer (10 µM)	1.0 ml	6 ml
DNA template	1.0 ml	6 ml
	25 ml	150 ml

PCR cycling conditions

Cycle	Duration of cycle	Temperature °C)
1	5 min	95
35	30 sec	95
	30 sec	55
	30 sec	72
1	5 min	72

Use 1 ml of the PCR product from the step 1 PCR reactions as template to proceed to step 2 (nested) PCR.

Step 2 (Nested) - ***Reaction set up***

Component	Vol.ml / Reaction (25 ml)	Vol. ml / (6 reactions)
Water	9.5 ml	57 ml
2x Master Mix	12.5 ml	75 ml
Forward primer (10 µM	1.0 ml	6 ml
Reverse primer (10 µM)	1.0 ml	6 ml
First PCR Product (template)	1.0 ml	6 ml
	25 ml	150 ml

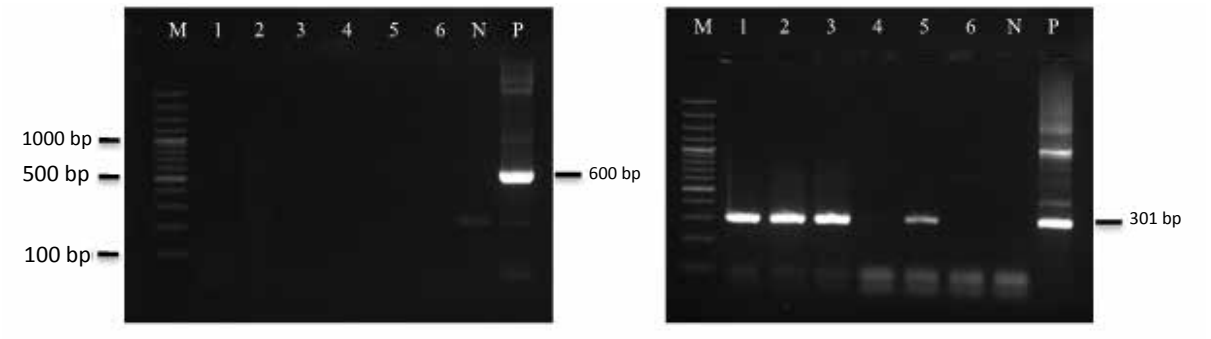
PCR cycling conditions for step 2

Cycle	Duration of cycle	Temperature (°C)
1	5 min	95
35	30 sec	95
	30 sec	55
	30 sec	72
1	5 min	72

After completion of the PCR, run 10 ml of PCR products in 1.6% agarose gel electrophoresis using 5 ml of the DNA ladder in the kit visualise under UV-trans-illuminator.

Interpretation of results

Positive samples in first step will show two bands at 600 and 240 bp corresponding to the amplicon from EHP and house keeping (β-actin), respectively. Negative sample will show single band at 240 bp only. Nested reaction will show one band at 301 bp of EHP and 240 bp amplicon corresponding to housekeeping gene (if included in the second step).



Lane M : 100 bp DNA ladder

Lane 1 : Sample 1 , Lane 2 : Sample 2 , Lane 3 : Sample 3

Lane 4 : Sample 4 , Lane 5 : Sample 5 , Lane 6 : Sample 6

Lane N : (-) Control, Lane P : (+) Control

Fig. 3. Non-lethal screening of broodstock using faecal sample using CIBA primer set

Conclusions

Molecular detection assays for aquatic pathogens are being developed at an increasingly rapid rate. Unfortunately, the assays often have not been validated against traditional techniques, and most of these assays have not been thoroughly tested for inclusivity or specificity. In addition, it is important to realize that the polymerase chain reaction (PCR) detects DNA and not necessarily a viable pathogen. To confirm the presence of a viable pathogen, PCR should be used in conjunction with other methods that allow visualization of the pathogen in tissue, such as histology or *in situ* hybridization with DNA probes. Nonetheless, the development of sensitive and specific molecular detection assays has greatly increased our ability to rapidly and specifically diagnose important pathogens in aquatic organisms.

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Management of Enterocytozoon hepatopenaei (EHP) in shrimp aquaculture

Introduction

The Indian shrimp aquaculture showed massive enhancement in production since the introduction of the exotic species *Penaeus vannamei* (Pacific white leg shrimp) in the year 2009. Presently, Indian shrimp aquaculture sector is dominated by this species over the indigenous black tiger shrimp (*Penaeus monodon*) since the availability of specific pathogen free (SPF) and specific pathogen resistant (SPR) shrimp seeds. Last few years Indian shrimp industry has been gripped by a small spore forming microsporidian protozoan called *Enterocytozoon hepatopenaei* which are ubiquitous in the environment and can cause stunted growth in shrimps thereby affecting the profit earning of the shrimp farmers. *Enterocytozoon hepatopenaei* are transmitted horizontally and reported to cause severe growth retardation in *P. vannamei* and control of them in pond is a herculean task for the shrimp growers. Hence farmers have to monitor this pathogen at the broodstock, PL and rearing ponds in order to get uniform growth of shrimp.

Management of this infection can be achieved by carrying out strict biosecurity at various levels like maturation facilities, hatcheries, farms and ensuring out certain best practices.

Broodstock management

The imported stocks of Specific Pathogen Free (SPF) labelled *P. vannamei* may also be positive for EHP, since it is not on the OIE list of reportable diseases. Therefore, SPF suppliers or quarantine agencies are responsible for endorsing SPF status to these imported shrimps. Moreover, these broodstock shrimp are usually fed with live feeds (*e.g*., polychaetes and clams) obtained from local sources or from importers. The agency should ensure, what they are feeding to the animals are free from the EHP spores and thereby can ensure a good seed supply to the farmers. There are reports that some live polychaetes from local and imported sources can have EHP. This can be prevented by use of frozen feeds which would kill EHP. Other option would be pasteurization (heating at 70°C for 10 min) of the feeds and also gamma irradiation would also kill major shrimp viruses. Alternatively, polychaetes could be selected and tested for shrimp pathogens and then reared as broodstock feed in biosecure settings (*i.e*. SPF polychaetes). Further, non-lethal testing of broodstock faeces for the presence of EHP can be carried out to make sure the animal is free from the infection. This problem could be resolved by adding EHP to the SPF list of both suppliers and quarantine agencies. Another best approach for maturation and hatchery facility is avoid use of wild, captured, live animals (e.g., live polychaetes, clams, and oysters) as feeds for broodstock. Additional important perilous factor is use of pond reared broodstock for seed production where in the broodstock shrimp may be a carrier for EHP. Awareness has to be created to curtail this practice.

Hatchery management

Disinfection of all hatchery facilities like all the surfaces, tanks, pipelines and other accessories should be soaked with 2.5% sodium hydroxide solution with a minimum of three hours of contact time and then drying for about a week followed by rinsing with acidified chlorine (200 ppm and pH less than 4.5) before the start of production cycle will prevent EHP. It is always advisable to wash eggs and nauplii with iodine, formalin etc., that will kill the surface microsporidial spores, if any. There are fears that some hatcheries mix these local broodstock with imported SPF stocks to reduce the cost of production which will increase the biosecurity risks of shrimp farmers who are often unaware of the production history of the PL they purchase. Hence, it is always advisable to screen the PLs procured by the farmers from the hatcheries by using nested PCR to ensure EHP free seeds before stocking.

Farm Management

At the farm level biosecurity plays an important role in preventing this residual protozoan disease. Farm management has many aspects and they are:

(i) **Larval management:**

PL stocking density plays an important role,

whereas increased stocking may produce stressful environment for shrimp, increasing their chances of disease expression. Farmers should always buy seeds from registered shrimp hatcheries and should stock quality healthy seeds free from EHP, in addition to WSSV and IHHNV.

(ii) **Pond management:**

To maintain farm productivity, farmers should always follow better management practices like providing adequate time (at least 3-4 weeks) for the ponds to dry after every harvest. The ponds having history of disease should be disinfected using appropriate protocols. High organic loads typically relate to spore loads. Spores can be inactivated by physical and chemical methods. Heat treatment significantly inactivated spores. There is likely some intermediate vector, and until we are sure what it is, use strategies to properly treat sediments before stocking. As spores typically are resistant to a wide variety of environmental conditions, treat pond bottom with a very caustic material to bring the pH to 12 and kill many of the spores. Killing all of them may not be possible. It has been recommended that earthen ponds be disinfected with very heavy use of calcium oxide, or quick lime, applied at a level of 6,000 kg/ha or greater. Pond bottoms must be completely dry. Plough the quick lime into the dried sediments to adepth of 10 to 12 cm; then moisten the sediments to activate the lime. If the application is done properly, the pH of the soils will rise to 12 or more within days and then gradually return to normal as the quick lime becomes calcium carbonate.

(iii) **Feed management:**

Feed management is an important factor in successful shrimp culture. The feed should be given in right amount to shrimp for proper growth. Insufficient feeding may lead to cannibalism, while excess feed will deposit at the bottom of the pond, augmenting bacterial growth and compromising water quality maintenance and thereby giving stress to the animal.

(iv) **Water management:**

Water management is another crucial element of disease control in shrimp aquaculture. Water is often pumped into the farm from water sources, such as the sea, estuary or canal, which may introduce pathogens that can cause diseases. The use of water reservoirs with proper duration of water ageing appears to be a regular practice on shrimp farms that can reduce disease occurrence. Water ageing prior to stocking decreased the odds of EHP cases in shrimp ponds. Treatment of water bodies before stocking PL with Calcium hypochlorite at the concentration of 18 mg/l can eradicate wild crustaceans and significantly reduced the prevalence of microsporidian infection.

Conclusion

The disease emergence and its spread is due to the various linked events involving the interactions between the host, the environment and the pathogens. It would be always cautious to monitor and encourage producers to consider standard management strategies such as the lowering of stocking densities, maintaining a good water quality, and the use of EHP-free shrimp PLs for stocking, improved better management practices, routine farm biosecurity measures and responsible trade practices to prevent epizootics in aquaculture. Shrimp health is a shared responsibility, of every stakeholder's contribution to minimize losses and sustain productivity.

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PCR Protocols

PCR protocol for detection of *Enterocytozoon hepatopenaei* infection in shrimp

Sample collection

For PCR detection, fresh or ethanol-preserved samples of PL from hatchery or whole hepatopancreas in case of grow-out may be used. As the microsporidian spores are expelled out through faeces, fresh faecal sample from precious or specific pathogen-free (SPF) broodstock may also be used as non-lethal sample for analysis.

DNA Extraction

Take 20-30 mg of sample in 150 µl lysis buffer (50 mM Tris pH 8.0- 606 mg, 1mM EDTA- 37.2 mg, 500 mM NaCl- 2.922 gm, 1% SDS – 1 gm make up to 100 ml with Proteinase K @ 10 µg/ml) and homogenize the sample with the micropestle.

Add 350 µl of lysis buffer to the homogenate and mix well by vortexing.

Incubate the sample at 95°C in dry bath for 1 min.

Cool the lysate to room temperature and centrifuge the sample at 12,000 rpm for 10 min.

Collect 200 µl of supernatant into fresh tube with a micropipette and add 500 µl of 70% ethanol and mix by invert mixing.

Centrifuge the tube at 12,000 rpm for 10 min and decant the supernatant without disturbing the DNA pellet.

Invert the tube on a clean dry tissue, wipe and air dry the pellet.

Reconstitute the DNA pellet in 200 µl of water or TE buffer (1 ml 1M Tris (pH 8.3) and 200 µl 0.5 M EDTA (pH 8.0) and adjust the volume to 100 ml of de-ionized water).

Use 1 µl of this sample as DNA template for the PCR.

Quantification of DNA

The concentration of DNA should be determined by measuring the absorbance at 260 nm in a spectrophotometer. For quantification of DNA, take 10 µl of DNA and dilute in 990 µl of TE buffer. Take the O.D at 260 nm and the concentration of DNA to be calculated as follows. The concentration of DNA should be adjusted to 50 µg/µl with TE buffer for PCR analysis. The extracted DNA can be kept for long period at 4°C.

1 OD of dsDNA at 260nm = 50 µg/ml

Therefore, DNA concentration (µg/ml) = $\frac{\text{O.D x Dilution factor x 50}}{1000}$

PCR Primers for the detection of *Enterocytozoon hepatopenaei*

Primers	Sequence	Amplicon Size	Reaction condition	Reference
SSU rRNA				
ENF779	CAGCAGGCGCGAAAATTGTCCA	First step 779 bp	Initial denaturation at 94°C, 3 min 35 cycles of - Denaturation at 94°C, 20 sec - Annealing at 58°C, 20 sec - Extension at 72°C, 45 sec Final extension at 72°C, 5 min	Tangprasittipap *et al*. (2013)
ENR779	AAGAGATATTGTATTGCGCTTGCTG			
ENF176	CAACGCGGGAAAACTTACCA	Nested 176 bp	Initial denaturation at 94°C, 3 min 35 cycles of - Denaturation at 94°C, 20 sec - Annealing at 64°C, 20 sec - Extension at 72°C, 20sec Final extension at 72°C, 5 min	
ENR176	ACCTGTTATTGCCTTCTCCCTCC			
Spore wall protein				
SWP 1F	TTGCAGAGTGTTGTTAAGGGTTT	First step 514 bp	Initial denaturation 95°C, 5 min 30 cycles of - Denaturation at 95°C, 30 sec - Annealing 58°C, 30 sec - Extension 68°C, 45 sec Final 68°C, 5 min	Jaroenlak *et al*. (2016)
SWP 1R	CACGATGTGTCTTTGCAATTTTC			
SWP 2F	TTGGCGGCACAATTCTCAAACA	Nested 148 bp	Initial denaturation 95°C, 5 min 20 cycles of - Denaturation 95°C, 30 sec - Annealing 64°C, 30 sec - Extension 68°C, 20 sec Final 68°C, 5 min	
SWP 2R	GCTGTTTGTCTCCAACTGTATTTGA			

Setting up the PCR Reaction

Once we choose the appropriate substrate DNA and PCR primer sequences, the PCR reaction can be set up as follows. Take care not to cross-contaminate the reagents, especially the templates and primers. Pipetting order in general; we may add water first and the enzyme last. For a large number of reactions, it is good practice to first set-up a master mix of the common reagents and then aliquot them, rather than to pipette the reagents separately for each individual tube. An example for a typical reaction of 25 µl set up is shown below:

Step 1 (First step PCR)

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

Step 2 (Nested PCR)

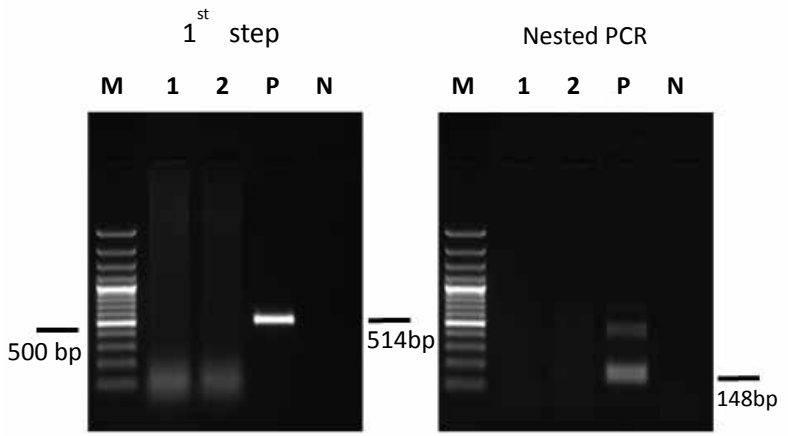
Reagents	**Vol (µl)**	**Vol (µl) (X) no. of reactions**
Water	9.5	
2x Master Mix (contains Buffer with $MgCl_{2,}$ dNTPs, Taq DNA polymerase)	12.5	
Forward primer (10 µM)	1	
Reverse primer (10 µM)	1	
First PCR product (template)	1	
* Original Rxn. Vol. 25 µl	25 µl	

The reaction conditions for each step are provided above along with references

Observations and Documentation

After completion of the PCR, 1.6% agarose gel prepared either in 1x Tris-Acetate-EDTA buffer (1 litre 50x TAE–242 g Tris Base,55 ml Glacial Acetic acid and 37.2 g EDTA, pH 8) or 0.5 x Tris Boric acid EDTA buffer (1 litre 50x TBE – Tris base 540 g, Boric acid 275 g and EDTA 18.5 g, pH 8.0). Ethidium bromide is added to the molten agarose (0.5 µg/ml final concentration) when temperature reached 45-50°C and then poured into the casting tray. Once the gels are solidified, it is submerged in the tank with the same buffer. A total volume of 5-10 µl amplified product is directly loaded in sample wells and 4 µl of the 100 bp DNA ladder loaded to the marker well in gel to verify the size of the amplified product. After loading, the tank is connected to a power pack and electrophoresis is carried out at voltage of 80-120. Continue the electrophoresis until the dye migrates to the appropriate distance in the gel and visualise under UV transilluminator.

Representative PCR gel electrophoresis



M-DNA marker (100bp), Sample 1, Sample 2, P-Positive control, N-Negative control

Fig. 1.Agarose gel electrophoresis showing detection of EHP by PCR amplifying 514 and 148 bp products

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Detection of *Enterocytozoon hepatopenaei* (EHP) in shrimp pond soil

Shrimp pond is naturally inhabited by many microorganisms including several microsporidians hence monitoring of EHP spores and their elimination in natural environment is highly tedious. However, in the context of alarming rate of detection of EHP in farmed shrimps inspite of stocking post larvae from specific pathogen free brood stock raising alarms indicating the presence of infective spores in the rearing environment and causing secondary infections in the grow-out period. Several methods are available for recovering parasitic spores from environmental samples comprising floatation, sedimentation and density gradient separations. A simple method is devised to recover EHP spores from soil from shrimp ponds and briefed below.

Principle

A method originally recommended by Medema et al., (1998) is modified for concentrating EHP spores from pond soil and is found to be satisfactory in recovering EHP spores from pond soil under laboratory trials

Method

Concentration of EHP spores from pond soil

Collect 20-100 g of soil (sieved through 40 µ mesh) from shrimp ponds and add 100 ml of distilled water containing 1-2% Tween 80

↓

Mix the suspension for 15 min in a magnetic stirrer for homogeneity/vortex thoroughly

↓

Allow the suspension to settle for 15 min at room temperature

↓

Collect the supernatant and pelletize the suspension containing spores by centrifugation at 5000 rpm for 15 min

↓

This pellet can be used for extraction of DNA as recommended for EHP detection

DNA extraction from recovered EHP spores and PCR protocol: (Please refer to the article above)

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Trouble shootings in PCR

General trouble shootings

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

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

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

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Aquatic Animal Health and Environment Division
ICAR-Central Institute of Brackishwater Aquaculture (CIBA)

Indian Council of Agricultural Research (ICAR)

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A Profile

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

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

The AAHED of CIBA was the first to commercialise a white spot syndrome diagnostic kit to a premier Biotechnology company in the year 2002. The AAHED also produced kit for diagnosis of white tail disease in scampi in the year 2004. AAHED has the expertise and capacity to carry out all the proposed levels of Diagnostics of OIE listed brackishwater pathogens, and has been serving as a National Referral Laboratory.

The environment section of AAHED has the expertise to look into all aspects of abiotic parameters. Novel methods have been developed for the bioremediation and environmental monitoring of the brackishwater rearing systems, including hatcheries and grow-out farms. The section has developed climate smart solutions such as carrying capacity assessment tools for optimisation of brackishwater aquaculture development. The unit has expertise in climate related matters and capacity for environmental impact assessment.

AAHED, CIBA has published over 60 research publications in peer reviewed national and international journals, produced 15 Ph. Ds, who are currently employed in key positions in various Institutions in India and abroad.

Microbiology & Virology: Dr S.V. Alavandi, Dr M. Poornima, Dr. P. K. Patil, Dr. Sanjoy Das, Dr. Sujeet Kumar, Dr. T. Bhuvaneshwari, Dr. N. Lalitha

Biotechnology,Molecular Diagnostics & Aquaculture: Dr. K.K. Vijayan, Dr. S.K. Otta, Dr. Satheesha Avunje

Parasitology & Pathology: Dr. K. P. Jithendran, Dr. Ezhil Praveena, Dr. R. Ananda Raja, Mr. T. Sathish Kumar, Dr. Vidya Rajendran,

Soil & Water Chemistry: Dr. M. Muralidhar, Dr. R. Saraswathy, Mr. P. Kumararaja, Ms. C. Suvana

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