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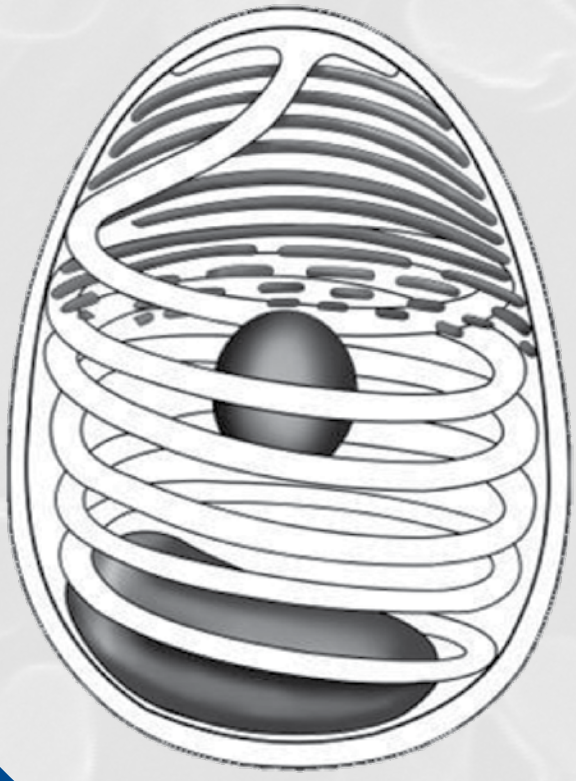
RECENT ADVANCES ON

DIAGNOSIS AND MANAGEMENT OF *Enterocytozoon hepatopenaei* (EHP) IN BRACKISHWATER SHRIMP AQUACULTURE



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Recent Advances on Diagnosis and Management of *Enterocytozoon hepatopenaei* (EHP) in Brackishwater Shrimp Aquaculture

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Overview of Hepatopancreatic microsporidiosis (HPM) in shrimp

K. P. Jithendran

Shrimp farming has emerged as one of the most important sectors in commercial aqua farming and diseases are the major threats faced by the industry. Hepatopancreatic microsporidiasis (HPM) in cultivated shrimp is an important emerging disease caused by microsporidian *Enterocytozoon hepatopenaei* (EHP). Since the target organ of EHP is the hepatopancreas, the disease was termed as hepatopancreatic microsporidiasis. *E. hepatopenaei* was first reported as an unnamed microsporidian in growth retarded black tiger shrimp, *P. monodon* from Thailand in 2004. Later this parasite was characterized in detail and taxonomy was elucidated in 2009. The spore ultra structure of this parasite also resembled another uncharacterized microsporidium previously reported in *P. monodon* from Malaysia in 1989 and from the post larvae (PL) of diseased *P. japonicus* examined in Australia in 2001. These early studies indicated potential for infection with EHP-like microsporidians in several penaeid shrimp species, leading to the proposal that EHP-like microsporidians were endemic within the Australia, Asia Pacific region prior to the expansion of shrimp aquaculture. Subsequently, its spread was recorded in many shrimp farming nations and now EHP outbreaks in shrimp farms have become common in Asian countries including south-east Asian countries and Latin America.

HPM outbreaks were reported widely in China, Indonesia, Malaysia, Vietnam, Thailand, India, Venezuela, Mexico, Brunei, Philippines, and Republic of Korea causing 20-30% production loss. So far, EHP infection has been reported in three known cultured species of penaeid shrimp i.e. *P. monodon*, *P. vannamei, P. stylirostris* and one suspected species (*P. japonicus*). Though the susceptibility of different life cycle stages are not clear, post larvae (PL-7 onwards), juveniles, sub adults and brood stock are found to be affected by the parasite.

HPM is one of the major threats for the shrimp farming industry due to associated growth retardation and significant losses in several shrimp farming countries. In India, the disease emergence has been recorded in *P. vannamei* since 2014 by CIBA and RGCA as a part of National Surveillance Programme on Aquatic Animal Diseases (NSPAAD), mainly in Andhra Pradesh and Tamil Nadu. Since then, EHP epizootics and spread was severe in the east coast of India. Recently, the disease has been recorded in the west coast of India and inland saline areas in Haryana Punjab and Rajasthan. It is also unlikely that EHP has been a

pre-existing disease in Indian shrimp aquaculture, as national level data never indicated its presence in other dominant species *P. monodon*. Initially EHP has not been taken seriously due to low prevalence, and no substantial loss due to mortality as compared to WSSV. However recently, the epizootics of EHP in India have been reported to be very high. The EHP infection initially detected in the hatchery reported to be increased to 96.6% after transferring shrimp to the pond. It appears that the disease entered India in the recent past through infected brood stock and the geographical spread was attributed mainly through transport of infected seeds to other parts of the country.

The emergence and spread of EHP in India will have a significant impact on the shrimp production. In India, the probability of occurrence of this disease during the period 2018-19 in 7259 ha of shrimp farming area in 23 coastal districts was reported as 17%. The production loss due to the disease during the period 2018-19 was also calculated as 0.77 million tonnes with a revenue loss of US$ 567.62 million.

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Biology of *Enterocytozoon hepatopenaei* (EHP)

K. P. Jithendran, T. Sathish Kumar and M. Poornima

Microsporidians are obligate, unicellular, spore-forming eukaryotic organisms belonging to the phylum Microsporidia. These organisms are ubiquitous, widely distributed in nature, with more than 1200 identified species. Several of them are known to infect most of the vertebrates, invertebrates, and some genera of protists. Almost half of the identified microsporidian parasites are reported from aquatic hosts and some described as pathogens of penaeid shrimp and finfish. A unique feature of the microsporidian is the spore's polar tubule which pierces the host cell and through which the infective sporoplasm is transmitted. The microsporidian infection in aquaculture species causes reduced growth rate, increased morbidity and decreased productivity in culture farms.

Taxonomy of Microsporidia

Initially, these groups of organisms were considered to be Microspora and Microsporidia. At present, they are classified under the phylum Microsporidia. Earlier the phylum microsporidia were regarded as protozoans and ranked under the kingdom Protista based on the conventional identification of the spore's morphological features, life cycle, and interaction with the host. With advanced genetic studies (ribosomal RNA sequencing) carried out recently have revealed that the phylum Microsporidia is related to the Kingdom Fungi. In the phylum microsporidia, a total of 1200 species were reported under 187 genera. Many microsporidians, except few, are host-specific and thus indicating their host specificity. Fifty genera are pathogenic to crustacean hosts such as Malacostraca, Maxillipoda, Ostracoda, and Branchiopoda. In Malacostraca, 20 genera are known to infect crab, shrimp, lobster, amphipod, and isopod hosts. It is evident that Clade VI of the Terresporidia has hosts in different trophic strata, especially in crustaceans and vertebrates. The family Enterocytozoonidae was positioned under the Clade IV of Terresporidia.

Pathogens belonging to the family Enterocytozoonidae infect hosts inhabiting marine and freshwater aquatic environments at different trophic levels including hyperparasitic copepods, decapods, fish, and mammals. The marine shrimp microsporidian is named *Enterocytozoon hepatopenaei* based on its distinctive ultrastructural characters and it was placed under the family Enterocytozoonidae. *E. hepatopenaei* and *E. beiniusi* develop inside the host cell cytoplasm, whereas the development of other species of the genera *Enterospora* and *Nucleospora* is intranuclear. *E. hepatopenaei* shares a number of identical traits with *E.*

bieneusi such as localization of sporangial plasmodium, budding of sporoblasts, and morphological features such as posterior vacuole, and two rows of polar filaments in 5-7 coils. Considering the spore size, marine habitat, and 16% difference in the 18 SSU (small subunit) rRNA gene sequence, a new name *E. hepatopenaei* within the family Enterocytozoonidae was accepted for this shrimp microsporidian

Phylogenetic classification of *Enterocytozoon hepatopenaei*

Phylum: Microspora (Sprague, 1977)

Class: Microsporea (Delphy, 1963)

Order: Microsporida (Balbiani, 1882)

Family: Enterocytozoonidae (Cali and Owen, 1990)

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

Species: *hepatopenaei* (Tourtip et al. 2009)

Biology and Life Cycle of Microsporidians

The spore is the only infective stage of a microsporidian which lives outside the host. The spore size ranges from 1-20 µm, but mostly in the range of 1-5 µm. The spores of EHP are monokaryotic, oval-shaped, and measure about 1.1 ± 0.2 µm × 0.6 ± 0.2 µm. Mature EHP spores contain a single nucleus, polar filament with 5-6 coils, a posterior vacuole, and an anchoring disk at the anterior end of the polar filament. Spores are protected with a thick cell wall composed of plasmalemma with an endospore (10 nm) and exospore (2 nm). The endospore layer of the microsporidian spore is usually composed of complex proteins and α-chitin layers and can withstand pressure during germination. In comparison, the exospore layer comprises of proteins and mediates communication with the environmental stimuli. The spore wall is also composed of spore wall proteins (SWP), which are reported to be involved in host-parasite interaction. In EHP, the spore wall protein EhSWP1 is characterized by heparin-binding motifs (HBM) at its N-terminus and a Bin-amphiphysin-Rvs-2 (BAR2) domain at its c-terminus. EhSWP1 is localized and distributed in both the endospore and exospore layers. It is also believed that EhSWP1 is a significant protein involved in the host-parasite interaction by attaching spores to the host cell surface.

Microsporidians have a relatively simple life cycle and contain two major forms - meronts and spores in three developmental phases such as infective, proliferative and sporogonic phases. The two life cycle forms are unique to microsporidians with significant morphological variations among different species. The infectious form of a microsporidian is

the resilient spore which can endure the extracellular environment for a long time. Typically, the life cycle of a microsporidian starts once the spore enters the host cell. The spore fires a polar tubule which pierces the host cell and through this tubule, the infective sporoplasm heads straight into the host cytoplasm via spore germination. Often, the host cell endoplasmic reticulum (ER), nuclei, and mitochondria are found surrounding the pathogen. In the proliferation phase, the injected sporoplasm develops into meronts through merogony (binary or multiple-fission) resulting in branched multinucleate plasmodial forms. In sporogony, the cell membrane of meronts thickens and forms sporonts.

In the genus *Enterocytozoon*, during sporogony, precursors such as the polar filament and anchoring disk develop before the cleavage of the sporangial plasmodium in the cytoplasm. The sporonts develop into sporoblasts and form mature spores. Before the maturation of sporoblasts, the extrusion apparatus including the polar tube, polaroblast, posterior vacuole, and other spore organelles develop within the sporangial plasmodium and are packed into the pre-sporoblast. After maturation, the shrimp hepatopancreas (HP) cell swells, ruptures, releases mature spores into the aquatic pond surroundings through the faeces, and transmits it to other shrimps. A mature spore is protected with a thick, chitinous endospore. This protective layer makes the spore dormant and resistant to environmental stress and maintains spore viability for a long duration in the aquatic habitat.

Metabolism

Hallmarks of microsporidians are their extreme streamlining of genomes and organelles. Recent comparative genome analysis of the 3.26- Mbp genome of EHP and those of their relatives in the family Enterocytozoonidae revealed that, like other microsporidia, EHP cannot generate ATP by either glycolysis or oxidative phosphorylation. Of the 10 enzymes in the glycolytic pathway, EHP and *E.bieneusi* (the only other member in the genus Enterocytozoon) possess only hexokinase and glyceraldehyde 3- phosphate dehydrogenase genes but lack phosphofructokinase and pyruvate kinase, the two glycolytic enzymes capable of generating ATP by substrate-level phosphorylation. Enzymes in alternative ATP-generating pathways were also surveyed and found missing from the genomes of all species in the family Enterocytozoonidae. Considering the coverage of every Enterocytozoonidae genome under comparison, the absence of these enzymes reflects genuine loss during evolution, rather than the failure to retrieve these genes during sequencing. On the other hand, some ATP-consuming enzymes have been retained. These include a potentially

functional hexokinase as well as ATP-costly pyrimidine and purine biosynthesis pathways. It has been postulated that the simultaneous loss of ATP-generating capacity and conservation of ATP consuming features may serve to set up an ATP sink in the plasmodium that draws on host ATP through ATP/ADP transporters abundant in the EHP genome, similar to a scenario reported in Chlamydiae.

Comparative genomic investigation also revealed that glucose-6- phosphate dehydrogenase (G6PD) is conserved in the family Enterocytozoonidae. It converts glucose-6-phosphate produced by hexokinase into 6-phosphoglyconolate and in the process releases the reducing equivalent, NADPH, that can scavenge harmful reactive oxygen species (ROS). It has been conjectured that the conservation of G6PD might have been due, in part, to the protection it might provide endo-parasites against oxidative damage from ROS that hosts deploy as an immune strategy. In contrast to EHP that possesses only one copy of hexokinase, other members of the family Enterocytozoonidae possess multiple hexokinase genes for redundant isozymes that are purportedly secreted into the host cytosol to disturb host metabolism in favor of parasite development. The prime reason why EHP is such a threat to the shrimp industry is its potential for shrimp growth retardation. In part, this probably occurs by generation of an ATP sink, but proteomic and metabolomic reports suggest that at least two other mechanisms might also contribute to stunted growth (Ning et al., 2019). One is an increase, after infection, in the level of a juvenile hormone called methyl farnesoate (MF) due to the upregulation of farnesoic acid O-methyltransferase (FAMet), a primary enzyme in the juvenile hormone biosynthesis pathway. This is accompanied by downregulation of Juvenile hormone esterase-like carboxylesterase 1 (JHEC1), the enzyme that degrades MF. The other possible mechanism is upregulation of ecdysteroid regulated-like protein (ERP) which results in a decline in ecdysteroid that is necessary for stimulating ecdysis.

Spore germination and infectivity

Spore germination is induced by different environmental triggers specific to species and habitat. *In vitro* germination of spores is achieved by many physical and chemical inducing factors such as changes in pH, dehydration followed by rehydration, hyperosmotic settings, presence of anions or cations, and exposure to ultraviolet light or peroxides. Inside the host cell, the spore initiates an exciting series of subcellular events called spore germination. Spore germination is an osmotic event triggered by the calcium/calmodulin-

binding on the spore surface. It is believed that the next step is the inflow of water into the spore, possibly with ionophore molecules/aquaporins of the spore membrane.

Spore germination starts with swelling of the polaroplast, and expansion of the posterior vacuole resulting in swelling of the spore. Swelling of the spore ultimately increases the osmotic pressure. Increasing pressure inside the spore ruptures the thinner apex of the spore case and expels the polar tube/injection tube by eversion. Expansion of the posterior vacuole forces the cytoplasm and nucleus through the tube. The ejected polar tube length may range from 50–500 µm in length. The whole event of spore germination can be completed within a few seconds. The ejected polar tube/injection tube can penetrate any obstacle, including other microsporidian spores.

Spore germination is a rapid strategy carried out by spores to infect the host cell. A unique feature of the microsporidia spore is the possession of an infiltrating organelle polar tube/ polar tube filament for invading host cells. The development of microsporidia in host cells is generally intra-cytoplasmic, although some genera are reported to develop inside the host nucleoplasm. A microsporidian spore is a single cell compact pressure vessel like structure which fires the sporoplasm into the host cell. The piercing apparatus of spores includes three parts - polar tubule/injection tubule, polaroblast, and a posterior vacuole. The polaroblast acts like an accordion-like membrane for storage, whereas the posterior vacuole acts as a pressure-building organelle to initiate the firing.

Conclusion

Microspordia are obligate intracellular pathogens and spores are the infective material of microsporidians for transmitting disease. In shrimp aquaculture, recently the emergence of *E. hepatopenaei* (EHP) has caused major havoc in shrimp production. Studies on the biology, life cycle, and transmission of the emerging microsporidian, *Enterocytozoon hepatopenaei,* are limited. The pathogen-hostrelationship in microsporidians has illustrated several instances of parasite-host coevolution.

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Enterocytozoon hepatopenaei (EHP): Clinical manifestation, pathology, epidemiology and transmission

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

Diseases usually are indicated by gross signs. A clinical sign is defined as an objective manifestation of disease and observable phenomenon that can be identified. Each disease entity has a constellation of signs more or less uniquely its own. Externally visible clinical signs are often used for presumptive diagnosis of suspected infections in shrimp. However in the case of EHP, there are no known distinctive visual clinical signs in shrimps but known to cause severe unusual growth retardation over time which leads to an increased variability in size. Size variation may not be apparent during early days of culture (DOC). EHP is confined to the shrimp hepatopancreas, infects the hepatopancreatic tubules limiting the assimilation of nutrients and results in growth retardation. In a more advanced stage, EHP-infected shrimp typically display soft shells, lethargy, loss of appetite, reduced feed intake, empty midguts and whitish hindgut. EHP infection in pond conditions may happen continuously leading to progressive damage to the hepatopancreas with different degree of infection. Hence the signs of EHP appear as size variation, overall slow growth and loss during harvest. In hatcheries, EHP can be suspected if post larvae grow unusually slower and show size variation. In addition, the coefficient for correlation between severity of EHP infection and size is low. The coefficient of variation in shrimp size or weight is the best indicator for its impact on production is in a culture pond. In a normal pond this would be in the range of 10% or less but in a severely infected EHP pond it would be in the range of 30% or more such that the harvest would be unprofitable. In shrimp farms, EHP does not cause mass mortality. Severe infection by EHP in shrimp farms can increase the susceptibility to other bacterial infections (*Vibrio* spp.) and result in low level daily mortality. The feed conversion efficiency was drastically affected resulting in severe economic loss to the farmers.

EHP is often associated with White Feces Syndrome (WFS). Farmers report EHP infected shrimp exhibiting white faeces syndrome as early as 23 DOC, often with a phase of recovery. A 100% prevalence of EHP in WFS affected shrimp farms has been recorded in the states of Tamil Nadu and Andhra Pradesh in India. White faecal strands floating on surface of an affected pond is one of the clinical signs of EHP infection. However, white faeces syndrome is not a consistent feature as compared to slow growth and size variation and both

conditions are not always concurrent. For instance, a pond can still test positive with EHP even after shrimp has stopped producing white faecal strings, suggesting that the association between EHP and WFS is indirect. Conversely, there are EHP-free ponds that exhibit white, floating mats of faecal strings containing vermiform structures called aggregated transformed microvilli (ATM). Furthermore, it is known that shrimp may produce white faeces in response to other causes such as heavy gregarine infections, vibriosis and hemocytic enteritis. Since the relationship between EHP and WFS appears to be conditional and suggested that WFS could be an indicator of EHP in shrimps.

Pathology

The impact of Microsporidian infection on hosts has been reported in several cases in wild, culture and laboratory experiments of aquatic organisms. However, most microsporidian diseases may result in chronic infection with low mortalities. There are four contact methods that have been described in the way microsporidians avoid the lethal response of the host. The methods are Type I: Direct contact e.g., *Nosema* and *Enterocytozoon*, Type II: Indirect contactby parasite-produced isolation e.g., *Anncalia, Pleistophora, Brachiola,* and *Tubulinosema*, Type Indirect contact by host-produced isolation e.g., *Endoreticulatu*s, and Type IV: Indirect contact by host- and parasite-produced isolation e.g., *Trachipleistophora, Encephalitozoonintestinalis*. The shrimp microsporidian EHP infect the host cell by direct contact method. With regard to EHP, the host interactions are largely unknown and studies and research on host-parasite interactions are in the early stages.

As EHP is an intracellular spore-forming parasite, it replicates within the cytoplasmic area of the tubule epithelial cells in the hepatopancreas. When EHP enters the ponds it is highly difficult to eradicate. Spores of EHP in fecal pellets or dried cadavers have shown to be viable up to six months and retain infectivity for over a year under aqueous conditions. The spores released in the environment are activated when external factors are suitable, and infect host cells. Once the spores enter the gut of the shrimp they start infecting the hepatic cells via extending a highly specialized polar tube. As the infection progresses there is an increase in the number of spores since they replicate within the host cells. The new spores thus formed then infect other healthy cells. EHP infection in pond conditions may happen continuously leading to progressive damage to the hepatopancreas with different degree of infection. Infections with the massive, production and release of spores by cell lysis, together

with sloughing of whole cells containing spores results in a loss of integrity of the HP tubule epithelium and may be accompanied by some shrimp mortality. The spores, sloughed cells and debris from lysed cells accumulate in the midgut making it the white fecal strings. In addition, the cells that do lyse to release spores normally in a dispersed manner over time allows for cell renewal and leaves the HP structure more-or-less intact. This allows for long term infections that result in no external signs of disease but may cause retarded growth.

Epidemiology

An EHP infection can occur at a wide range of salinity (2 - 30 ppt). The prevalence and the severity of the EHP infections were reported to be higher at 30 ppt than at 2 ppt and 15 ppt in Venezuela. It has been reported that calcium is an important second messenger that activates many cell events and calcium influx might be, in part, responsible for the activation of microsporidian spore discharge at higher salinities. In India there are some shrimp farming areas in high and low salinity, and the prevalence of EHP seems to be lower at lower salinities (below 5 ppt), as was observed during a shrimp disease survey in Andhra Pradesh in 2019. The difference in the severity of the EHP infection at the three different salinities was probably due to the differential effect of salinity on spore germination. One of the critical phases in the spore germination is the increase of intra-spore osmotic pressure. The difference in salinities led to a hypotonic environment at 2 ppt and 15 ppt compared to hypertonic environment at 30 ppt. It is possible that the hypertonic solution enhances the germination of the spore by increasing the spore activation process.

Ammonia and nitrite concentration could influence the prevalence of EHP infection to the shrimps in the super-intensive farms. A significant correlation between ammonia and nitrite with the prevalence of EHP infections was reported. There was a high prevalence of EHP infection with the increase in nitrite concentration and ammonia in shrimp ponds. The concentration of a range 1 mg/l to 1.2 mg/l of ammonia and nitrite could influence EHP infection prevalence in the shrimp farms. The accumulation of ammonia and nitrite might impact shrimp production by reducing survival, growth performance, and damage of hepatopancreas of *P. vannamei,* which provides the chance for the opportunistic pathogen like EHP to infect the shrimp.

Transmission

Microsporidians are reported to be transmitted by both horizontal and vertical modes of transmission. In general, the mode of transmission determines the virulence of a

microsporidian pathogen. Horizontal transmission is highly pathogenic as a large number of spores are released into the water. Shrimp microsporidian EHP is transmitted horizontally by ingestion of spores released from feces of infected shrimp, cannibalism and cohabitation. In cohabitation, healthy shrimps ingest (fecal-oral route) spores released into the water by infected shrimps. Transmission has been demonstrated in laboratory co-habitation experiments using infected shrimp separated by mesh cages from naïve shrimp. In cannibalism, healthy shrimps are infected by feeding (oral route) the infected moribund or dead shrimps. Rapid and intense EHP infection established experimentally by HP injection and reverse gauge of EHP inoculum in naïve shrimps and in shrimp post larvae (PL) by adding the tissue homogenate infected with EHP. The oral challenges proved that EHP spores alone are infectious via ingestion. Since reverse gavage is accepted as an unhindered route that allows direct exposure of the shrimp HP to bacteria and toxins, it proved that spores produced in the HP (similar to bacteria in size) could spread EHP internally to uninfected HP tubule epithelial cells (autoinfection).

The possible ways of *E. hepatopenaei* vertical transmission route (trans-ovum) is poorly understood. A study reported positive PCR test results for EHP in nauplii, zoeae 1 and zoea 2 stages from EHP-infected female broodstock. But the study lacked clear histological examination. Further *in situ* hybridization (ISH) test results with EHP-infected juvenile shrimp and broodstock have shown positive signals for EHP in the HP tissue but none in the ovaries or testes of the same specimens, making trans-gonadal transmission unlikely. At this stage it is not possible to rule out potential for EHP transmission via vertical routes as comprehensive in depth research work with larger sample numbers and all life stages are to be studied. But there is possibility of vertical transmission of EHP from the infected female brooders can still pass infections horizontally to their offspring in hatcheries via release of feces containing spores into spawning tanks. Once some of the larvae become infected, they will rapidly transmit the infection horizontally via spores in their feces or via cannibalism. EHP can be transmitted orally by ingestion of infected live feeds such as brine shrimp *Artemia salina*, polychaetes, and molluscs.

Recently, severe EHP infections in shrimp farms have become common, and these infections are resulting in substantial economic losses to producers. The white feces are composed, almost completely, of massive quantities of EHP spores, gut mucus, remnants of sloughed tissues from hepatopancreas tubules infected with EHP. Massive numbers of EHP

spores in the ‘‘white feces” of WFS-affected ponds, suggest that EHP is associated with WFS. It is possible, that EHP rapidly multiplies in hepatopancreatic tubule epithelium cells, then the degenerated epithelial cells and the large numbers of EHP spores release into the feces. The white feces containing large quantities of EHP can break down and sink to the pond bottom. The associated EHP can be ingested by shrimp, results in re-infection, and ultimately will increase the severity of the infection.

Conclusion

The microsporidian parasite *E. hepatopenaei*, (EHP) causes hepatopancreatic microsporidiosis (HPM) disease in shrimp. The disease does not cause mass mortality but affects shrimp production due to growth retardation, and causes severe economic loss in shrimp farming. Shrimp hepatopancreas is the target organ for EHP infection. There are no specifically distinctive reported gross signs for this microsporidian infection in shrimp. However, in a pond, shrimp with growth retardation, white gut, and pond with floating white fecal threads can be an indicator for EHP infection. EHP infection can be transmitted horizontally through the cohabitation and oral routes, and possibly by vertical transmission.

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Economic burden of *Enterocytozoon hepatopenaei* (EHP) to Indian shrimp farming

P. K. Patil

Global farmed shrimp trade worth US$ 17.7 B is dominated by India (24.9%) which is the leading supplier of shrimp to the US (US$ 2.3 B) and the second-largest supplier to the EU ($0.9 B) during 2018. Shrimp farming contributes to economic growth and provides significant employment opportunities in developing countries. Shrimp farming is an important economic activity earning a foreign exchange US$ 4.89 B in 2019-20 generating 14 million jobs in the country. Being the second-largest aquaculture producing country, India produces 17.5% of the global farmed shrimp. Following the introduction of specific pathogen-free (SPF) Pacific white shrimp, *Penaeus vannamei* in 2009, the establishment of Aquaculture Quarantine Facility (AQF) practices and screening of imported broodstock for OIE listed diseases have led to substantial improvement in shrimp health and production. Scientific interventions for economic sustainability of the sector through the adoption of biosecurity measures and better management practices were the corner stones of the sector.

The microsporidian, *Enterocytozoon hepatopenaei* (EHP) has become a significant threat to shrimp aquaculture in recent years and has been widely reported from major shrimp producing countries such as China, Thailand, Indonesia, Malaysia, Vietnam, India, Bangladesh and Venezuela affecting the economic sustainability, production and supply of shrimp in the global market. Since 2016, the disease is being reported from different shrimp farming regions of India. The affected farms suffer significant growth retardation and low-level mortality during the culture period. In the absence of apparent disease symptoms farmers continue feed and apply other inputs, incurring substantial expenditure.

Following the introduction of *P. vannamei* to the Indian farming and the subsequent practice of screening broodstock at the national quarantine and post larvae before stocking have led to the substantial improvement in shrimp health and production. Additionally, scientific interventions through the development of molecular diagnostic tools, adoption of biosecurity measures, and better management practices helped in the sector's economic sustainability. However, these financial gains were short-lived due to mounting losses due to infectious diseases and stress due to deteriorating environmental conditions following farm intensification. Economic effects of diseases are more evident to farmers, and their management has a cost that has to be weighed against profits. A thorough understanding of

disease impact on shrimp productivity and comprehensive economic loss is of utmost importance for formulating various health management efforts. The economic cost of disease could be directly due to the loss of stock and the expenditures to control/manage the infection. Indirect cost may include loss of employment in hatcheries, feed mills and processing exports, etc. and the consequent loss to the exchequer.

To allocate the limited resources to control these infections, it is essential to understand the risk factors involved and the economic loss quantification. The national drop in shrimp production in Thailand (1996-97, 2013), China (1993) and in Brazil (2006) has been attributed to the widespread occurrence of infectious diseases. Several studies have reported the economic loss worth billions of US dollars and employment losses due to EHP in different countries at the other period.

Estimating the national level economic cost of *Enterocytozoon hepatopenaei* (EHP)

Microsporidian infection, *Enterocytozoon hepatopenaei* (EHP) in addition to other endemic diseases continue to cause significant economic losses in shrimp farming worldwide including India. Since its first report in 2009, microsporidian pathogen *Enterocytozoon hepatopenaei* (EHP) has emerged as a significant threat in all the shrimp farming countries worldwide. Being an intracellular microsporidian parasite, EHP multiplies in the cytoplasm of hepatopancreas and midgut leading to size variation, growth retardation loss of production. Disease incidences due to microsporidian parasites have been reported worldwide, but there are few studies on economic loss experienced by the world shrimp industry. As the resources are scares and challenges are many, there is an urgent need to prioritize the research in developing and implementing strategies and specific policies to reducing the losses due to EHP and infectious diseases in general to Indian shrimp farming. A study in Thailand has speculated economic loss due to EHP as US$ 76.4 M.

We have recently reported an economic loss due to infectious diseases in Indian shrimp farming. Questioner based survey on the disease's occurrence and the associated loss in terms of mortality and employment was conducted. Economic loss to India's aquaculture sector due to infectious disease was estimated with particular reference to EHP based on the difference between expected and actual production. Factors like mortality, FCR, stocking density, culture period, average body weight and the survival rate were found to influence the economic loss due to EHP. Our study estimated the economic loss due to infectious diseases

in shrimp with special reference to EHP and WSSV. The questionnaire-based survey covering 23 shrimp farming coastal districts of India estimated the probability of EHP occurrence as 17% with production loss of 1.80±0.24 ton per ha per crop. The total production loss calculated per crop due to EHP was found to be 77,370 tons worth US$ 380 M. The overall annual economic loss due to infectious diseases to Indian shrimp farming was estimated to be US$ 1,028.55 M which includes US$ 571.03 M due to EHP.

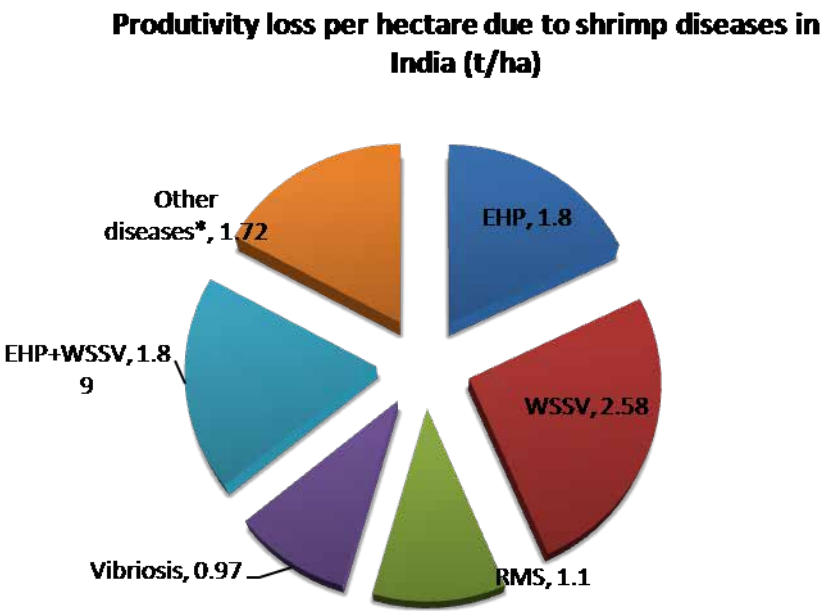

Fig. 1. Productivity loss due to shrimp diseases in India

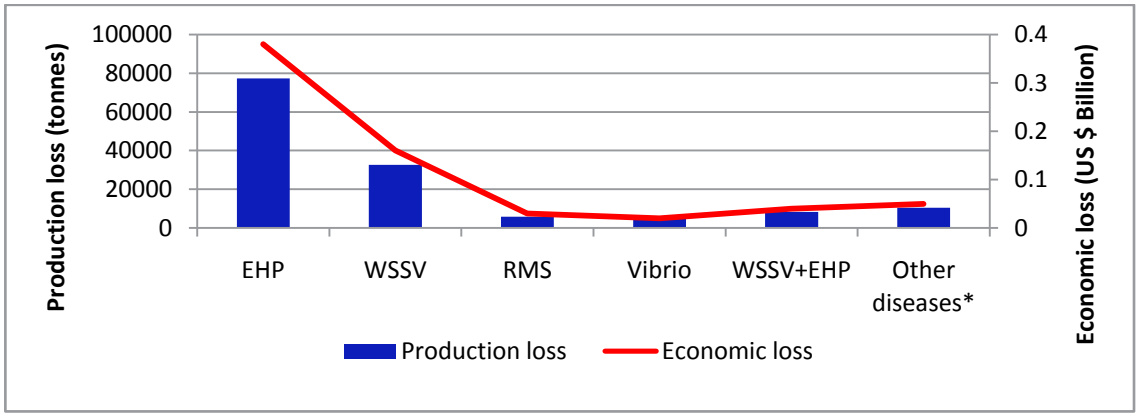

Fig. 2. Production and economic loss due to shrimp diseases in India

Generally the studies conducted to estimate the economic loss in shrimp aquaculture consider culture period of 120 days (average number of days usually needed for producing market size shrimp) and the productivity obtained at the end of the culture period is used as

the benchmark for economic loss estimation. Methodology to estimate PDO, production loss, economic loss and employment loss are given below.

Estimation of the probability of disease occurrence (PDO)

The probability of disease occurrence (PDO) index is computed using the proportion of disease occurrence at the farm level. The state-wise PDO index for each of the diseases is worked out by using the total culture area in the state and the farm area affected by the particular disease. The production loss due to particular disease at the national level is calculated using the weighted average of PDO for the state based on the culture area and the production data for each of the shrimp farming states.

Estimation of economic loss

Economic loss for each of the farms is calculated using the average farm gate price (Rs.per kg) for different size of shrimp harvest for particular region. Loss of production in monitory terms for each area is calculated using the PDO factor for each disease, loss of production (t per ha per crop) and the total shrimp culture area (ha). The economic loss is quantified by the production difference between healthy crop and diseased crop. The economic burden of the disease is estimated in terms of loss in production which may be attributed to poor quality, stunted growth and loss in employment in terms of man-days.

Estimation of employment loss

The loss of man-days in the farms is calculated based on the reduction in duration of culture (no. days) and number of workers employed. The number of man-days was calculated assuming one labour is employed per ha of farming area for the entire period of culture. The number of man-days lost is valuated based on the average daily wages in the concerned region.

Outbreaks of diseases in the surrounding area were the primary factor determining the intensity of the disease while the bulk of the financial burden was attributed to expenditure incurred on account of steps taken to contain the infectious agents from spreading. Further, cessation of farming activity due to disease outbreaks leading to emergency harvest causes loss of employment. Models for estimation of economic loss due to aquatic animal diseases should include an estimate of loss undergone due to premature harvest and sale of small size animals and the cost incurred to mitigate the disease which adds to the cost of production.

These economic losses could be reduced through effective implementation of better management practices (BMP) which is mandatory for *P. vannamei* farms in the country.

The data suggested that the regular occurrence of EHP and WSSV led to the severe reduction in production followed by reduced stocking densities in future crops. Steps taken to reduce the severity and spread of diseases through the application of disinfectants and other antimicrobial agents could further add to the cost of production. Distress harvest during the disease outbreaks leading the discharge of large quantities of pond water containing the pathogen loads further enhances the chances of spread of pathogen. While EHP spores are highly resistant to harsh environmental exposures; introduction of the pathogen into the farm would result in the continuous build-up in the system.

The study revealed EHP with a 17% probability of occurrence accounted for a production loss of 0.77 M tons, with a corresponding revenue loss of Rs. 3977 crores (US$ 567.62 M). The total employment loss due to major shrimp diseases was estimated to be 1.65 M man-days worth US$ 7.07 M. The overall probability of infectious disease occurrence in the country was at 49% leading to an annual loss of 0.14 M ton shrimp worth US$ 1.02 B. Economic loss due to shrimp diseases in Indian shrimp farming warrants prioritized implementation of better management practices (BMP) and biosecurity protocols along with policy interventions to reduce the direct and indirect losses.

Effective implementation of scientific farming could help reduce the impact of diseases as estimated economic loss comprised mainly the direct loss due to mortality and expenditures to control and manage EHP and other infectious diseases. The study of economic impact assessment of diseases helps decide the proportionate investment in national aquatic animal health management programs. The study revealed EHP is one of the major threats for the Indian shrimp farming, leading to substantial economic losses, including the consequent loss of jobs. In addition to disease surveillance, implementing better management practices would reduce the loss and minimize financial losses. Further, region-specific modifications in stocking density, culture period, and targeting size at harvest could mitigate the losses. Prioritizing the research areas, including disease forecasting and policy interventions would help the sector's economic sustainability.

Estimating the farm-level economic cost of *Enterocytozoon hepatopenaei*

Estimation of economic consequences of diseases in shrimp farming gives insight into the risk factors addressed at local levels. The biological losses expressed in monitory terms at

farm level would help in the effective allocation of resources to develop suitable control and prevention strategies to achieve the economic sustainability of the farming systems. The direct cost of EHP or any disease affected farms is expenditure incurred on basic costs of farming, expenditures towards prevention and treatment, the extraordinary cost to manage the disease, production loss and the reduced unit price for small size harvest. In addition, indirect costs of the disease include loss of employment, reduction in national production and foreign exchange earnings. Analysis of farm-level economic losses will help to develop the cost-effective strategies for managing the disease, assist the farmers to take an informed decision on reducing the risks and ultimately determine the economic viability of the aquaculture activity. Hence there is an urgent need to develop a stochastic model to estimate the economic loss due to EHP and identify the critical risk factors associated at the farm level.

Recently we have developed a stochastic model to estimate the economic loss due to EHP to Indian *P. vannamei* shrimp farms and identify the associated key risk factors at the farm level. Using Monte Carlo simulation model in excel @ Risk was used to estimate the cost of production and revenue of healthy and affected shrimp farms. The occurrence of the EHP was found to be positively correlated with the stocking density and the higher FCR. We estimated the farm level loss due to EHP at ₹ 61,778 (US$ 813) per ton shrimp production. In the EHP affected farms, the significant factors negatively influencing the net returns were expenditure on feed (0.51), seed (0.19) and labour (0.18). There was a significant regional variation in the economic impact of EHP at the farm-level.

Cost of production

The production cost includes expenditure on pond preparation, seed, feed, labour, consultation, health management, power and cost of disease and expressed as per ton production of shrimp. Net returns are calculated as gross returns (biomass harvested*farm gate price) - the cost of production. Lost profit (LP) for the EHP affected farms is estimated as the difference between net returns of affected and healthy farms.

Direct cost of EHP

The direct cost in the stochastic model describing the cost of the disease in commercial shrimp operations, include Biological Loss (BL), Treatment Cost (TC), Extraordinary Cost (EC) and Prevention Cost (PC). The cost of biological loss is calculated based on the difference in the biomass harvested in EHP affected and healthy shrimp farms. The TC includes expenditure on chemicals, nutritional supplements and other healthcare

products to manage the disease while the EC includes expenditure to hire labour for treatment and manage the disease and PC includes pond preparation and consultation expenditure.

We have used stochastic model to quantify the economic loss due to EHP at the farm-level and variations in the determinant factors among different farming regions of the country. The farm-level economic loss estimations are necessary to understand the associated risk factors influencing the economic outcome of the disease, which can be addressed in a targeted approach. Generally, it is observed that there is an association between the farms with higher stocking density and loss due to EHP, which might be due to the challenges in management of stress. The EHP affected farms show significant reduction in the harvest size with higher FCR.

Our analysis using the model demonstrated that the EHP infected farms incur considerable loss due to reduced average harvest weight, increased cost of feed in addition to expenditure on treatment and prevention. The reduction in production is the strongest stochastic variable in the EHP specific biological loss. In the absence of any insurance plan in Indian shrimp farming presently, these losses have to be borne by the farmers. Recent initiatives for covering the Indian shrimp farming under insurance could help partly recover these losses in future.

The economic loss due to EHP was shown to be correlated with higher FCR. Reduction in production due to EHP infection was shown to be partially compensated by harvesting at a particular size and appropriate timing for reducing the cost of feed and for realizing farm-gate price commensurate with the size of shrimp. In the absence of effective therapeutic measures, treatment included expenditure on account of feed supplements to reduce the stress and improve the growth. The increase in extraordinary cost might be due to additional labour required for pond management during the disease. The EHP spores accumulate in the pond soil and require the higher application of disinfectants. Hence the expenditure on pond preparation and professional consultation was included in the cost of prevention.

The influence of farm gate price on the economic impact suggests the importance of shrimp size, which fetches a higher unit price. Reduction in the animal's size is the primary determinant in reducing the profitability in EHP. Loss of production and expenditure on feed were the significant risk factors responsible for influencing the economic loss, as indicated by the sensitivity analysis confirming the effect of EHP on growth retardation and continuous feeding by farmers in the absence of any observed mortality. As expected, loss of production

was the major component influencing the direct loss positively while higher farm gate price was influencing negatively. Lower production leads to reduced supply of the commodity in the market and price escalation in the endemic regions.

In addition to the cost of disease, farmers lose their net income due to lower sale prices during disease incidence. In endemic areas, farmers are forced to harvest smaller shrimp, further reducing the wider market accessibility. A significant difference in the state-wise average farm gate prices of shrimp was not observed during the study period. The reduced losses in farms of Gujarat might be due to premium harvest size, as the farm gate prices per unit of shrimp are directly proportional to the size at harvest. Using the hard data we have estimated the biological effect of EHP on reduced production, harvest size and their correlation with the cost of prevention. The estimated loss of profit in EHP affected farms was ₹1, 15, 200 per ton (US $ 1,576) of shrimp production.

Cost-benefit analysis of intervention

Using cost-benefit models, the information generated on biological losses could be expressed in monetary figures. Measures to reduce the economic impact by identifying components contributing to the loss would benefit the individual farmers and the sector in general. Combining biological and economic models would help identify the most effective cost-benefit analysis to address the issue at the national level. The losses due to EHP could be overcome by practicing lower stocking density, scientific pond preparation and testing of seeds before stocking. The short crop cycle is one of the critical factors helping in the sustainability of the sector despite the threat of infectious diseases. Identification and generating the appropriate information on the specific cost of the disease would help in the efficient use of resources for economic control of the disease. The effect of measures like reducing the expenditure on feed by harvesting the appropriate size of shrimp would help reduce the losses.

Conclusion

Among the infectious diseases EHP has become the major disease in Indian shrimp farming, causing substantial economic losses and the consequent loss of employment. Revenue loss by EHP alone is more than two times than that of WSSV contributing more than half of the national economic loss due to all other diseases combine. In addition to disease surveillance implementation of better management practices would help in reducing the loss of production and minimize economic losses. It is important to assess the economic

impact of important diseases especially EHP for appropriate investment in disease control programs. Reduction in economic loss could be achieved through region-specific adjustments in stocking density and culture period and targeting for appropriate average body weight at harvest. Prioritizing the research, especially the application of artificial intelligence in disease forecasting and implementation of required policy interventions are warranted for the economic viability of the sector.

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Co-infection of *Enterocytozoon hepatopenaei* (EHP) with other pathogens

S. K. Otta

Co-infection is observed as a natural phenomenon in many of the organisms where the host gets simultaneously infected with more than one pathogen. It is generally considered that this event is not inconsequential to the host. Different pathogens can interact with the host immune system differentially and thus changing the overall pathogenesis. Similarly, in favoring their own survival and multiplication, certain pathogens can decide the virulence of co-infecting pathogen. Therefore, this affects the overall treatment strategy and sometimes it becomes difficult to provide appropriate treatment to the host.

The co-infection event has also been observed in shrimps and reported for many pathogens. Particularly, simultaneous infections by a multiple number of viral pathogens are considered to be very common in shrimp.

Enterocytozoon hepatopenaei (EHP), a hepatopancreatic microsporidian, has been an emerging pathogen affecting cultured shrimps in many of the countries including India. As this pathogen at present is considered as a major problem for the shrimp farmers, researchers are putting considerable effort to know more about it. Some of these efforts have resulted to provide knowledge regarding the co-infection of EHP with many other pathogens. In fact, much before this pathogen got its present name, it has been affecting the cultured tiger shrimps (*Penaeus monodon*) since as early as 2003 and was reported to cause monodon slow growth syndrome where it was also found to be co-infected with many of the opportunistic pathogens such as monodon baculovirus (MBV), hepatopancreatic parvo virus (HPV) and *Vibrio* sp.

During the recent research developments, EHP has also been reported to be simultaneously present with a number of other pathogens in cultured *Penaeus vannamei*. The present chapter attempts to bring an update on such co-infection status.

Co-infection of EHP with shrimp viruses

Co-infection of EHP with Taura syndrome virus (TSV)

Co-infection of EHP with TSV was reported in one of the vannamei farms in Venezuela. Presence of EHP in the ponds was confirmed by histopathology, *in situ* hybridization and PCR. Sequence similarity of this EHP strain was assessed with south East Asian strains through the amplification of 18s rRNA, β-tubulin and spore wall protein genes.

Interestingly, TSV was also detected from the same shrimps by RT-PCR and histopathology. Considering the virulence of both the pathogens, continuous monitoring of ponds were suggested.

Co-infection of EHP with white spot syndrome virus (WSSV)

During the routine surveillance of *vannamei* farms from Tamil Nadu and Andhra Pradesh of India, simultaneous infection of shrimps with EHP and WSSV was recorded. In a kind of first report, only about 2% of shrimps screened were found to be positive for both the pathogens. During this study, a multiplex PCR assay was developed for simultaneous detection of both the pathogens. The authors suggested using this multiplex PCR detection method for quick and specific detection of both these pathogens in shrimp larvae before stocking in ponds to avoid the spread of these virulent pathogens.

Similarly, a small percentage of shrimps (7.6%) from east coast of India were also reported to have co-infection with both EHP and WSSV. A study conducted on screening of pathogens from both *vannamei* and *monodon* firms along the east coast of India and reported EHP to be dominant pathogen in *P. vannamei* compared to *P. monodon,* whereas MBV was the dominant in *P. monodon*. Though EHP was also recorded in *P. monodon*, co-infection of this pathogen with WSSV or MBV was not recorded.

Co-infection of EHP with infectious myonecrosis virus (IMNV)

Co-infection of EHP with IMNV in *P. vannamei* was recently reported from shrimp farms of India. During a survey along the east coast of India, a major number of ponds (7 out of 12) were reported to have co-infection of both IMNV and EHP. Presence of both the pathogens in the same shrimp was detected both by histopathology and PCR. Interestingly, shrimps with single infection of EHP didn't show any clinical symptoms where as co-infected shrimps showed clinical symptoms for both the pathogens. Both the pathogens were also found to have sequence similarity with the respective pathogens from India and other parts of the country.

Co-infection of EHP with hepatopancreatic parvovirus (HPV)

This report involving simultaneous occurrence of both EHP and HPV in *P. vannamei* samples was also from the east coast of India during the recent time. In this study, about 5.2% of the farms were reported to have co-infection of both these pathogens. While doing electron microscopy study, typical structures of microsporidian parasite and icosahedral virus like

particles were observed. Latter during PCR analysis, HPV was found to be present along with EHP.

Co-infection of EHP with bacteria

Since EHP infections were often accompanied by opportunistic pathogens, particularly Vibrios, the risk factor associated with EHP was verified through an experimental challenge with the acute hepatopancreatic necrosis disease (AHPND) causing *Vibrio parahaemolyticus* (AHPND-VP) and through a case study involving bacteria associated septic hepatopancreatic necrosis (SHPN). In the experimental challenge when EHP shrimp was infected with AHPND-VP, it caused significantly higher mortality than AHPND-VP single infection. Similarly, 57% of shrimps with both EHP and AHPND-VP infection showed severe hepatopancreas necrosis and sloughing whereas only 11% of AHPND-VP single infection animals showed such severity. When the same authors compared individual shrimp displaying histological signs of SHPN with the shrimp from the same ponds without these signs, a strong association was found between SHPN and EHP, indicating that shrimp with EHP have an increased susceptibility to SHPN. Thus the authors came to a conclusion that EHP is a risk factor both for AHPND and SHPN. At a latter point of time, it was reported detection of co-infection of both EHP and AHPND in naturally infected samples and the authors developed a duplex recombinase polymerase amplification for simultaneous visual diagnosis of both the pathogens.

In an investigation, it was observed that growth variation to cause large, medium and small size of shrimps was directly related to the number of EHP spores present in the shrimp. The authors also found that intestinal bacteria of small size shrimps were more similar to medium size shrimps and different from large size shrimps indicating intestine microbiota to be severely affected by EHP infection.

White feces syndrome (WFS) of shrimp is often associated with EHP. Through an investigation, it was found that the mid-gut and hepatopancreas of WFS shrimps had more EHP spores than the non-WFS shrimps. Further, HP microbiome study indicated dominance of *Vibrio* spp, *Propionigenium* spp. and EHP in EHP-WFS shrimp and *Propionigenium* spp. were uniquely high in EHP-WFS shrimp. This clearly indicated the association of specific bacteria and EHP in bringing specific pathological features.

As has been mentioned earlier where EHP was found to be present along with WSSV in the same farm, the authors also reported simultaneous presence of *V. parahaemolyticus*

and EHP in some of the shrimp farms. In this investigation, about 6.1% of samples were found to have both EHP and VP. Application of probiotics was found to have inhibitory effect against those isolated VP.

Conclusion

Like other pathogens, shrimp EHP can also occur as co-infection with other infectious agents such as shrimp viruses and bacteria. Since this pathogen is comparatively new, many more specific investigations and targeted surveillance are still necessary to find out the association of this pathogen with other shrimp pathogens. However, with the existing information, it appears that association of EHP with many of the other shrimp pathogens can increase the severity of disease conditions and increase mortality. Therefore, specific management protocols need to be developed to avoid many of the secondary infections and keep the EHP specific infection under control.

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Role of carriers, intermediate hosts and vectors in transmitting hepatic microsporidiosis

P. Ezhil Praveena

Microsporidians are pervasive in nature and found to be affecting many hosts from insects to humans. Microsporidians considered as protozoan parasites belonging to the phylum Microspordia are eukaryotic obligate intracellular protozoans infecting insects, fish and mammals. They are considered as opportunistic pathogen. The microsporidian spore is highly resistant in nature and can remain viable for long. The infected shrimp shed the spore and contaminates the water and soil. The aquatic animals like freshwater fish, marine lobsters, crabs, copepods and shrimp have been found to be infected with various genera of microsporidia but *Enterocytozoon hepatopenaei* infects Penaeid shrimps especially *Penaeus vannamei* and in the recent years it has severely damaged *P.vannamei* culture because it causes severe damage to the hepatopancreas and thereby affecting the growth of the shrimp. In India incidence of this infectious agent has increased in the recent years and the farmers are experiencing the brunt of this disease. In ponds, EHP can readily be transmitted among individuals through cohabitation and as well as through healthy shrimp cannibalizing those that were moribund or dead, ingesting spores from the water, from sediment, from eating EHP-infected live feeds. Besides this some play the role as disease carriers and they aids in disease progression in shrimp. They are;

Polychaetes

The EHP has the potential to persist in wild polychaetes as long as the worms inhabit contaminated soil sediments and polychaetes were apparently capable of carrying infectious spores for some period after ingestion of EHP-contaminated feed or soil sediments. The results support the popular view that use of wild-caught polychaetes as live-feed to shrimp broodstock can increase the risk of passive or mechanical transmission of EHP leading to contamination risk in hatcheries. The EHP-positive gravid worms did not transmit the microsporidian parasite to their progeny.

Mussel

Mytilopsisleucophaeata, false mussel was found to be capable of carrying infectious spores for some period and can serve as a mechanical or passive carrier. It was reported that *M. leucophaeata* can accumulate EHP spores released from EHP-infected shrimp. Further it

was found that spore amplification and infection are not seen in them. However, they can serve as passive carriers and can transmit infective spores to naïve shrimp if removed from EHP-infected pond and moved directly to an uninfected shrimp pond.

Crab

Crab were collected from shrimp farms in Nellore district of Andhra Pradesh and has been screened for EHP by PCR and it was found to be positive for EHP indicating that they could act as carriers of EHP

Acetes sp.

PCR screening of non-penaeids (*Acetes* sp.) collected from vannamei farms gave positive amplification for EHP by PCR indicating their potential role in transmitting the disease

Artemia

It was reported that sufficient copy numbers of EHP DNA could be detected from the commercial Artemia and hence they can be suspected to be carriers for EHP. Another study also reported that Artemia could be a vector transmission for EHP in shrimp. Histological examination of infected adult Artemia revealed spores in them, which was confirmed by PCR.

Other Potential carriers

Molluscs, squids, Frozen Artemia mass and aquatic insects

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Role of gut microbiome in white feces syndrome (WFS)

Sujeet Kumar

Introduction

Health management has become a serious issue in the rapidly growing shrimp culture. There are growing evidences, suggesting that shrimp health is intimately linked to the gut microbiome. These microbes colonise the shrimp gut and contributes in the digestion and improvement of shrimp immunity. However, many a times change in gut microbes lead to microbial imbalance causing manifestation of disease. Our knowledge in this field has started unravelling with the arrival of high throughput sequencing (HTS) which enhances our understanding in the beneficial and harmful role of microbes.

White feces syndrome

White feces syndrome (WFS), has emerged as serious problems in Indian shrimp farming. The study conducted at ICAR-CIBA suggests that WFS alone is responsible for up to 20-30% production loss of farmed shrimp. The WFS appears in shrimps from approximately 2 months of culture onwards. This is characterised by formation of vermiform bodies consisting of Aggregated Transformed Microvilli (ATM). The ATM has originated by sloughing from epithelial cells of the shrimp hepatopancreatic tubules. When the occurrence of ATM is severe, it leads to the formation of white faecal strings in shrimp and lead to floating fecal strings.

Gut microbiome in healthy shrimp

Most studies carried out in shrimp suggest the wide presence of Proteobacteria in aquatic environment and gut. The phylum Proteobacteria is highly diverse in terms of physiology, morphology, and genetics. They are Gram-negative, and most are facultative or obligate anaerobes. Among the proteobacteria, the Gammaproteobacteria, is the largest class and have often been described as the most common bacteria in the gut of giant tiger shrimp (*Penaeus monodon*) and Pacific white shrimp (*Litopenaeus vannamei*). This class, mainly comprises *Vibrio* and *Photobacterium* spp. Many *Vibrio* spp. produce chitinolytic enzymes explaining their dominance in a chitin-rich environment of crustacean gut. Several *Vibrio* spp. are known to cause losses to the aquaculture industry. Despite that, *Vibrio* spp. are often described as the dominant genus within the shrimp gut microbiota. Several other taxa such as

Firmicutes, Bacteroidetes, Fusobacteria and Actinobacteria are also known to be present in shrimp gut.

Microbial involvement in White faeces syndrome (WFS)

WFS is thought to be linked to the presence of the microsporidian parasite *Enterocytozoon hepatopenaei* (EHP). It is true that many of the white feces affected animals often contain densely packed EHP spores. However, white feces could also be a common characteristic of several health conditions. Therefore, intensive work taking the advantage of molecular high throughput sequencing is being carried out to find the involvement of microbes in possible pathogenesis of WFS. When comparing bacterial gut profiles of WFS infected shrimp and asymptomatic individuals, there was an increase in *Candidatus* Bacilloplasma (Tenericutes) and *Phascolarctobacterium* (Firmicutes) along with a decrease in *Paracoccus* (Proteobacteria) and Lactococcus spp. (Firmicutes), which correlated with a significant reduction in overall diversity of the bacterial community. An increase in *Candidatus Bacilloplasma* and a reduction in overall richness and diversity in WFS-infected guts has also been confirmed in another study. Further, they also found the over representation of other bacterial genera such as *Vibrio, Photobacterium* and *Aeromonas* in WFS affected farms whereas Shwanella, Chitinibacter, and *Rhodobacter* population were higher in healthy farms. The latter study demonstrated that 36.7% of healthy shrimp that received intestinal microbiota transplants (IMTs) from WFS-infected donors eventually became infected with the disease. Conversely, WFS-infected shrimp receiving IMTs from healthy donors recovered from the disease. In another study, It was reported that *Vibrio* isolates such as *V. sheinaloensis* and *V. parahaemolyticus* could cause experimental shrimp to develop a similar histopathology to the naturally WFS-affected. Further, *Acinetobacter* and *Candidatus Bacilloplasma* dominated the bacterial communities of hepatopancreas and gut from grossly healthy shrimp, respectively. Therefore, the emerging new research is pointing towards the multifactorial cause of white fecal syndrome with possible involvement of microbial species.

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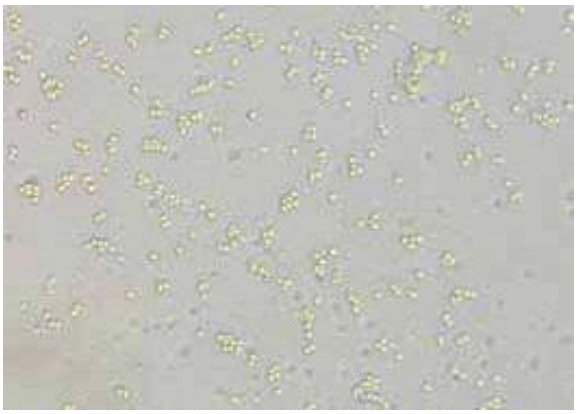
Microscopy including wet smear, special staining, SEM and TEM

P. Ezhil Praveena, T. Sathish Kumar and K. P. Jithendran

Microscopy

Wet mount/ Squash method

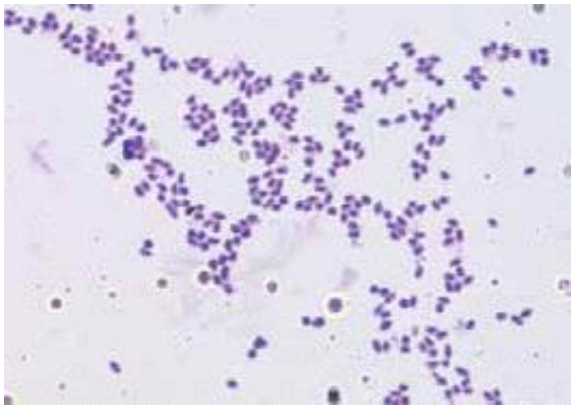
Microscopically, a typical microsporidian spores contain a single nucleus, coiled polar filament, a posterior vacuole, an anchoring disk attached to polar filament, and a thick electron-dense wall. Unique feature of this is very small spore size as compared to other microsporidians and hence these features may not be discernible. Wet mounts of fresh hepatopancreas (HP) tissue squash and/or faecal strands are examined by this method. Diagnosis is made by the demonstration of characteristic spores under light microscope. The spores are very small (1.1±0.2 by 0.6- 0.7±0.1 µm). This method may not be useful in detecting the spores which are present in scant numbers. Trained or experienced personnel are required for identification of the spores.



Microscopic detection of wet mount of spores of *Enterocytozoon hepatopenaei* from *Penaeus vannamei* feces

Wet smear technique

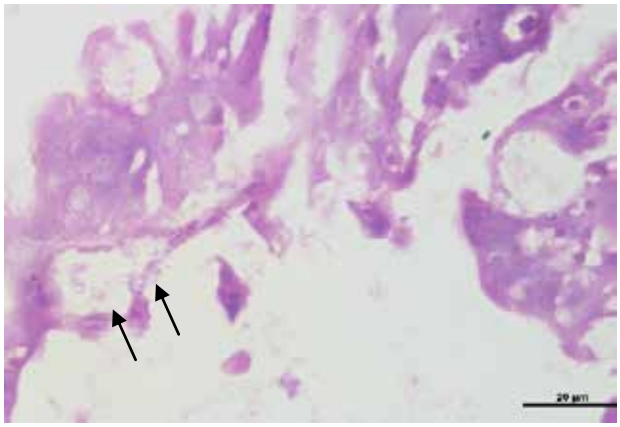
By using wet mount or squash method it will be difficult to identify the microsporidians spore due very small size. Hence staining is advisable to differentiate the spore by conventional stains such as Giemsa, Haematoxylin & Eosin (H&E) etc. The smear preparations from the feacal sample / infected hepatopancreas were made in a glass slide and allowed to dry at room temperature, fixed in methanol for 15 min and stained with Giemsa/ H&E. The stained smears can be observed under microscope using oil immersion.



Microscopic detection of wet mount of spores of *Enterocytozoon hepatopenaei* from *Penaeus vannamei* feces by Giemsa stain – 100X

Histopathology

Among all other diagnostic methods, histopathology plays very significant role in making confirmatory diagnosis i.e., presence of pathogen with sequential pathological changes in the cells and tissues. The target organ for EHP in shrimp is HP. Dead shrimp should never be collected to avoid the confusion in interpretation due to autolytic changes. Only live, moribund, or compromised shrimp should be selected for fixation and histological examination. The collected tissue should be processed following standard protocols and stained by Haematoxylin & Eosin and examined under microscope. Spore can be seen in the tubular epithelial cells as well as inside of the lumen of the tubules as ovoid bodies and wherever the epithelial cellular damage is more spores can be seen in clusters. Sometimes free spores released from lysed cells may be seen in the tubule lumens. Infected tissues may reveal several developmental stages, including the early sporogonal multinucleated plasmodia.

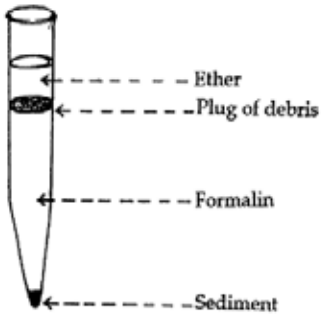


Spore seen inside the tubular epithelial cells of HP – H&E 100x

Detection of EHP in shrimp feces

Purification of microsporidian spores from fecal samples

The efficiency of microscopic observation can be increased many-fold by concentration of samples. 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 homogenize 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. 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 a light microscopy.

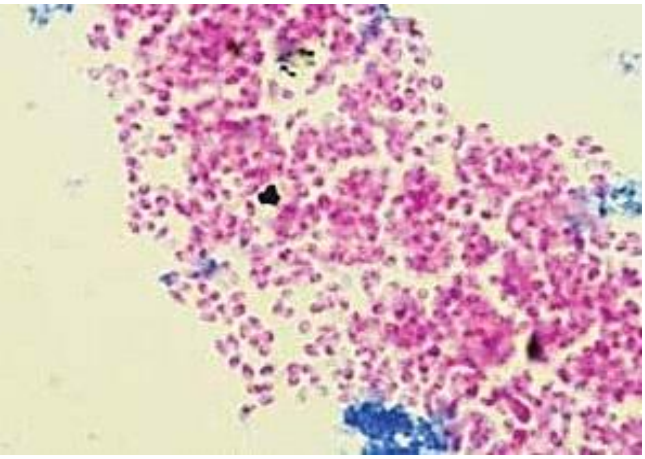
Special Stains – EHP spore detection

Besides the H& E stain EHP spore can be demonstrated from the tissue section by using toluidine blue Ignatius et al., 1997; phloxine (Aldama-Cano et al., 2018), Giemsa (Rajendran et al., 2016), or hematoxylin and eosin. A modified trichrome staining method was developed, which improved the identification of EHP spores (Praveena et al., 2018).

Modified Trichrome Staining

A thin smear of purified spores can be stained by 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. 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 phosphortungstic 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. Spores appear as ellipsoidal staining pink-red with a transverseband of polar filament or central non-staining zone.



Microscopic detection of spores of *Enterocytozoon hepatopenaei* from *Penaeus vannamei by* Modified trichrome staining- 100X

Detection of microsporidian using fluorescent stains

Calcofluor white (CFW) is a disodium salt of 4, 4'-bis (4 anilino-bis-diethylamino-5-triazine-2-ylamino) 2, 2'-stilbene-disulfonic acid which specifically binds to β-1,3 and β-1,4 polysaccharides in cellulose and chitin and emits fluorescence at a wavelength of 395~ 415 nm, which is not specific to EHP spores. This fluorescent stain can be used for rapid detection method of yeasts and pathogenic fungi, including Microsporidia, Acanthamoeba and Pneumocystis, and is all types of samples, like fresh, fixed, frozen, and paraffin-embedded tissues can be stained. CFW stain is a mixture of calcofluor white (1g/L) and Evans blue (0.5g/L), act as a counterstain and diminishes background fluorescence of tissues and cells. However, CFW may be irritating to the eyes, and Evans blue may be carcinogenic.

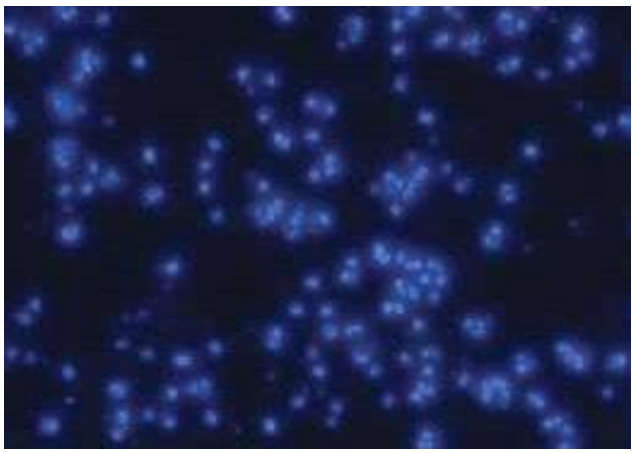
Utmost care is recommended. Calcofluor white stain can be mixed with a potassium hydroxide mixture to clear up the specimen to facilitate visualization.

Procedure

Faecal sample/ hepatopancreas/purified spores to be examined placed 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. The slide was placed at room temperature (25°C) for 5~10 min. Cover the slide with a paper towel and gently press to remove any excess fluid. Examine the slide under a fluorescence microscope at the excitation wavelength of 340~380 nm and emission wavelength of 400 nm with a 100× oil objective.

Interpretation of results

The microsporidian spore appears as bluish – white or turquoise oval halo. The staining method is sensitive and fast, requires ~15 min to perform.



Microscopic detection of spores of *Enterocytozoon hepatopenaei* from *Penaeus vannamei by* Calcoflour white Stain

In situ hybridization (ISH)

In situ hybridization (ISH) is another important tool to determine the location of pathogen nucleic acid in tissue sections of PCR positive animals. It can be done using digoxigenin-labeled (DIG- labeled) probes for the small subunit ribosomal RNA (SSU rRNA) or for spore wall protein (SWP) gene. The gene probe was labelled with digoxigenin-11-deoxyuridine triphosphate (dUTP) in a PCR reaction according to the manufacturer's instructions. Following PCR, the digoxigenin-labelled DNA probe was prepared stored at 20°C. The Davidson's fixed shrimp tissue were processed, embedded in paraffin, and sectioned (4 µm thick), deparaffinization, hydrated, proteinase K digestion, and post-fixation,

sections were overlaid with hybridization solution containing the EHP probe. Slides were placed on a heated surface at 90^0C for 10 min and hybridized overnight in a humid chamber at 42^0C. Final detection was performed with anti-digoxigenin antibody conjugated to alkaline phosphatase. The slides were counterstained with bismark brown and examined using a light microscope.

Transmission electron microscopy (TEM)

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

Scanning electron microscopy (SEM)

Hepatopancreas of EHP infected were rapidly fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer at 4°C, dried using Hexamethyldisilazane (HMDS) as per the method described by Nikara et al., (2020) and Berger et al. (2016). Processed samples can be examined and imaged in Scanning Electron Microscope. The tubular surface changes as well the spore attachment in the tubule can be visualized.

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Polymerase Chain Reaction (PCR) for early diagnosis of *Enterocytozoon hepatopenaei* (EHP)

R. Ananda Raja, T. Bhuvaneswari and J.J.S. Rajan

Enterocytozoon hepatopenaei (EHP) causing hepatopancreatic microsporidiosis (HPM) is a newly emerging disease of cultivated shrimp in Asia. It is associated with severe growth retardation in chronic infections and may even cause low continuous mortality in the case of very severe infections. Current production loss to HPM in India is estimated to be in hundreds of millions of US dollars per year as mentioned in previous chapter elsewhere. Transmission of this disease may come to shrimp grow out system via post larvae (PLs) used to stock ponds or via environmental sources such as live carriers or spores in water and sediments. Once transmitted to single host/shrimp in the pond, infected host can spread the infection horizontally to other uninfected animals. The PLs are infected in hatcheries through broodstock in non-biosecure facilities. EHP SPF broodstock may be contaminated by live feeds such as polychaetes and molluscs, which ultimately leads to severe economic loss in the shrimp industry. Hence, early diagnosis of EHP in PLs, and screening of broodstock, live feeds and other environmental hosts are of paramount important for success of shrimp aquaculture industry. A molecular technique called Polymerase Chain Reaction (PCR) is routinely used worldwide in the field of disease diagnosis targeting the presence of the specific DNA molecule as highly precise, accurate and sensitive method. Hence, PCR is also employed worldwide in diagnosis of EHP as well. There are many ready to use PCR kits available in the market, but it is essential to understand the basics of the PCR. This chapter is aimed to explain about the same and methodology adopted to diagnose the EHP.

Polymerase Chain Reaction (PCR)

PCR works with the basic principles of selectively amplifying a particular segment of DNA by *in vitro* enzymatic replication using specific primers. It was first explained by H.G.Khorana in 1971 but the further advancement was incomplete by primer synthesis and polymerase purification issues. In 1983, Kary Mullis successfully amplified the DNA for which he was awarded Nobel Prize in Chemistry in 1993. Recent past years, PCR has become indispensable technique in medical and biological research. PCR is routinely used worldwide in the shrimp industry for the diagnosis of various diseases including *Enterocytozoon hepatopenaei* (EHP).

Components and reagents

As a thumb rule, all the chemicals and reagents used for PCR should be of molecular biology grade. Clean and sterilized condition should be well maintained in all operations as a prerequisite.

Buffer solution

PCR is carried out in a buffer that provides suitable chemical environment for optimum activity with stability of the DNA polymerase. The buffer pH is stabilized between 8.0 and 9.5 by Tris-HCl. Potassium ion (K^+) is a common component in buffer which promotes primer annealing.

Magnesium ion (Mg^{2+}) concentration

TheMg^{2+} plays major role in the yield of PCR product by forming a complex with dNTPs, primers and DNA template. Hence, the optimum concentration of $MgCl_2$ should be augmented within the range of 1.5 to 4.5 mM in the PCR mixtures for each PCR reaction. The concentration of $MgCl_2$ should be selected empirically, starting from 1 mM and increasing in 0.1 mM steps, until a sufficient yield of PCR product is obtained. The $MgCl_2$ concentration is independent of the GC/AT ratio of the primers and the numbers of DNA band(s) amplified. Too few Mg^{2+} ions result in a low yield of PCR product, and too many increase the yield of non-specific products with multiple bands. However, lower Mg^{2+} concentrations are desirable when fidelity of DNA synthesis is critical. If the DNA samples contain EDTA or other chelators, the $MgCl_2$ concentration in the reaction mixture should be raised proportionally.

Deoxyribose nucleotide triphosphates (dNTPs)

dNTPs are employed in PCR to expand the growing DNA strand binding with the complementary DNA strand by hydrogen bonds with the help of Taq DNA polymerase. The concentration of each dNTP in the reaction mixture is usually 200 µM. Inaccuracy in the concentration of even a single dNTP dramatically increases the misincorporation level. So it is very important to have equal concentrations of each dNTP i.e., dATP [adenine], dCTP [cytosine], dGTP [guanine], dTTP [Thymine]. When maximum fidelity of the PCR process is crucial, the final dNTP concentration should be 10-50 µM.

DNA polymerase

Similar to DNA replication in a live organism, PCR requires a DNA polymerase enzyme that makes new strands of DNA using existing strands as template. In 1959, Arthur

Kornberg was awarded the Nobel Prize for identifying the first DNA polymerase in 1957. In 1969, Thomas Brock isolated and identified a new species of thermophilic bacterium, *Thermusaquaticus* from which the enzyme *Taq* DNA polymerase was produced in 1976. *Taq* DNA polymerase is world widely used in PCR reaction. It should be stored at -20 ^{o}C in a non-frost free freezer, typically in 50% glycerol. It should never be allowed to reach room temperature and gloves should be worn while handling to avoid contamination. Before opening a new tube of enzyme, it is spun briefly as there is often enzyme in the cap. When pipetting enzyme from a stock tube, the end of the tip is just plunged far enough into the enzyme to get what is needed to avoid excessive adherence of enzyme to the peripheral tips. Enzyme should never be added to unbuffered water to avoid its denaturation. Usually 0.5-1.0 U of *Taq* DNA polymerase is used in 50 µL of reaction mix. Higher *Taq* DNA polymerase concentrations may cause synthesis of nonspecific products. Normally, ready to use 2x Master mix is available in the market which contains buffer with $MgCl_2$, dNTPs and*Taq* DNA polymerase. It can be readily used with the standardised protocols.

Primers / oligonucleotides

Primers are short, single-stranded DNA sequences used in the PCR techniques which are also referred to as oligonucleotides. They are usually 15-30 nucleotides in length. Longer primers provide higher specificity. A pair of primers (forward and reverse) is used to amplify the region of interest from the sample DNA. The two ring structure purines in DNA are adenine (A) and guanine (G) while the single ring pyrimidines are cytosine (C) and thymine (T). The CG content in primers should be 40 – 60% and distributed uniformly throughout of the primer. To avoid nonspecific priming, more than three G or C nucleotides at the 3'-end of the primer should be avoided. The primers should have neither intra-complementarity nor inter-complementarity in order to avoid primer-dimer and hairpin formation. The melting temperature of flanking primers should not differ by more than 5°C, so the GC content and length must be chosen accordingly. The annealing temperature should be approximately 5°C lower than the melting temperature of primers.

The melting temperature (Tm) of the primers is calculated using the following formula:

$$Tm = [(\ Percentage\ of\ GC\ content\ in\ primers \text{ X } 0.41\ ) + 62.3\ ] - [\ \left[{}^{500}\!/_{\text{Pr}imer\ \text{length}} \right] - 5\ ]$$

The details of the published primerare mentioned in the following table.

Table: The details of the published primers for detection of EHP

S. No.	Primer name	5' Primer sequence 3'	Amplicon size (bp)	Target gene	Reference
1.	MF1	CCGGAGAGGGAGCCTGAGA	951	SSU rRNA	Tourtip et al., 2009
	MR1	GACGGGCGGTGTGTACAAA			
2.	ENF779	CAGCAGGCGCGAAAATTGTCCA	779	SSU rRNA	Tangprasittipap et al., 2013
	ENR779	AAGAGATATTGTATTGCGCTTGCTG			
3.	ENF176	CAACGCGGGAAAACTTACCA	176	SSU rRNA	
	ENR176	ACCTGTTATTGCCTTCTCCCTCC			
4.	EHP-510F	GCCTGAGAGATGGCTCCCACGT	510	SSU rRNA	Tang et al., 2015
	EHP-510R	GCGTACTATCCCCAGAGCCCGA			
5.	SWP_1F	TTGCAGAGTGTTGTTAAGGGTTT	514	SWP	Jaroenlak et al., 2016
	SWP_1R	CACGATGTGTCTTTGCAATTTTC			
6.	SWP_2F	TTGGCGGCACAATTCTCAAACA	148	SWP	
	SWP_2R	GCTGTTTGTCTCCAACTGTATTTGA			

Autoclaved molecular grade ultra-purified water

Autoclaved molecular grade ultra-purified water free of detectable inhibitors, contaminants or enzymatic activity is used to make up the desired volume for the PCR reaction.

DNA template

It is the sample DNA (50-100µg) such as genomic double-stranded DNA (gDNA), complementary DNA (cDNA), and plasmid DNA that contains the target sequence to be amplified.

Thermal cycling reaction

This is carried out on an automated programmed thermal cycler, which can heat and cool the reaction mix in tubes in a very short time.

Initial denaturation

The PCR is begun with initial denaturation to separate the double-stranded template DNA into single strands so that the primers can bind to the target region and initiate extension. The initial denaturation should be performed at 94-98°C for a period of 1-5 minutes depending on the nature of the template DNA. This interval may be extended up to 10 minutes for GC-rich templates. If the initial denaturation is no longer than three minutes at 95°C, *Taq* DNA polymerase can be added into the initial reaction mixture. If longer initial denaturation or a higher temperature is necessary, *Taq* DNA polymerase should be added only after the initial denaturation, as the stability of the enzyme dramatically decreases at

temperatures over 95 °C. For EHP diagnosis, the initial denaturation is standardised at 95°C for 5 minutes for both first and second step PCRs.

Denaturation

During the denaturation, the double-stranded template DNA melts open and separate to single strands which are important in PCR reaction. Incomplete denaturation of DNA results in the inefficient utilization of template in the first amplification cycle leads to a poor yield of PCR product. Usually denaturation for 0.5-2 minutes at 94-95°C is sufficient. If the amplified DNA has a very high GC content, denaturation time may be increased up to 3-4 minutes. Alternatively, additives such as glycerol (up to 10-15 vol.%), DMSO (up to 10%) or formamide (up to 5%) may be used to facilitate DNA denaturation.

Annealing

Usually the optimal annealing temperature is 5°C lower than the melting temperature of primer-template DNA duplex. Incubation for 0.5-2 minutes is usually sufficient. The annealing temperature should be optimized by increasing it stepwise by 0.5-2°C to avoid nonspecific PCR products. The primers are jiggling around, caused by the Brownian motion and ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer) the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer which does not break anymore. In case of addition of additives such as glycerol, DMSO or formamide to facilitate DNA denaturation, the annealing temperature should be adjusted experimentally, since the melting temperature of the primer-template DNA duplex decreases significantly when these additives are used. The amount of enzyme in the reaction mix should be increased since DMSO and formamide inhibit *Taq* DNA polymerase by approximately 50%. Alternatively, a common way to decrease the melting temperature of the PCR product is to substitute dGTP with 7-deaza-dGTP in the reaction mix.

Extension

It is the final stage in PCR cycle which is performed to extend the primer sequence from the 3' region to the end of the amplicon with the help of DNA polymerase. Usually, the extending step is performed at 70-75 °C. The rate of DNA synthesis by *Taq* DNA polymerase is highest at this temperature. A 1-minute extension is appropriately sufficient to synthesis the

PCR fragments up to 2 kb and may be further increased by 1 minute for each 1 kp. Primers that are on positions with no exact match get loose again (because of the higher temperature) and don't give an extension of the fragment. The polymerase adds complementary dNTP's to the template from 5' to 3', reading the template from 3' to 5'. Because both strands are copied during PCR, there is an exponential increase of the number of copies of the gene. But, polymerization is not strictly doubling the DNA at each cycle in the early phase. The number of PCR cycles depends on the amount of template DNA in the reaction mix and on the expected yield of the PCR product. Usually, 30-40 cycles of these three major steps such as denaturation, annealing and extension are sufficient depending on the amplified product size.

Final extension

After the last cycle, the samples are usually incubated at 72 °C for 5-15 minutes to fill-in the protruding ends of newly synthesized PCR products. Also, during this step, the terminal transferase activity of *Taq* DNA polymerase adds extra A nucleotides to the 3'-ends of PCR products.The final extension is standardised at 68°C for 5 minutes for both first and second step PCRs in diagnosis of EHP.

Gel documentation

After amplification of the targeted DNA fragments, the PCR product should be visualized and verified in UV transillumination to know the amplification of the product of interest. Agarose gel at 0.8-2% in 1x Tris Boric acid EDTA (0.09 M Tris borate; 0.002 M EDTA; pH 8) electrophoresis buffer is prepared with ethidium bromide (0.2 µg per mL of agarose). The PCR product is mixed with the desired gel loading dye (bromophenol blue and xylene cyanol) and glycerol mixture and slowly loaded into the wells of the submerged agarose gel using a micropipette. Electrophoresis is carried out by applying the voltage @ 1-5V/cm distance between two electrodes and continued until the dye migrates to the appropriate distance in the gel. Finally, the gel is examined under the UV light in a transilluminator for band visualization at the targeted region.

Record maintenance

It is essential to maintain the record to know the shortfalls if any. The results of each sample analysed should be well recorded and maintained for future reference and to assess the performance of the laboratory in diagnosis over a period of time.

Conclusion

PCR is a useful tool for rapid identification of pathogens and has become inevitable for any molecular biologist, since it has gained its importance in modern molecular biology over a period of last two decades. The same technique has become vital in diagnosis of various diseases in aquaculture, developing ds RNA therapy and vaccines to shrimp and fish diseases, developing transgenic fish, and so on. PCR has also been widely applied to the detection of shrimp pathogens either from the host or carriers so that the risk of disease can be controlled. EHP is one among the pathogens which needs to be detected at the early stage in broodstock, larvae and post larvae, which will help to discard the batch before taking them into culture practice.

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Quantitative detection of *Enterocytozoon hepatopenaei* using Real Time Polymerase Chain Reaction (qPCR)

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Quantitative polymerase chain reaction (qPCR) also known as real time PCR (RT-qPCR) is one of the most rapid molecular technique in amplification of target DNA of interest. It determines targeted gene copy numbers with reference to standard curve of defined concentration, without a need for visualization in agarose gel electrophoresis. Real time detection of amplified DNA attached to fluorescent dye markers were measured in relation to reaction progress with the use of PCR instruments developed for detecting changes in fluorescence in a single tube. This technique is sensitive, specific, quantifiable and more convenient for assaying large no of samples. *Enterocytozoon hepatopenaei* infections of shrimp in earthen ponds are reported with clinical signs increasing with respect to degree of infection. Hence the quantitation of infection in terms of amount of EHP in infected shrimp is essential to adopt the management practices in culture system.

Sample preparation and nucleic acid extraction

For detection and quantification of EHP in shrimp a good quality sample is most crucial for qPCR and to get accurate results the sample should not be contaminated or degraded. When isolating DNA for qPCR, it is necessary that the tissues are stored properly, free from contaminants that may inhibit the reaction. DNA can be extracted from shrimp gut tissues, hepatopancreas and feces, live feeds such as polychaetes, artemia, planktons, crab, water infected with EHP, soil infected with EHP. Purification of DNA from cellular proteins, cell membranes and extra cellular matrices is achieved in chemical cell lysis, the lysis steps can be tailored to specific tissue and sample types, as well as steps in chemical removal of protein constituents for optimization of DNA extraction. Kit based methods with columns or magnetic beads may also require optimization for specific nucleic acid types to provide pure, concentrated nucleic acid. The concentrations of nucleic acids were measured using the UV absorbance at 260nm and 280nm in spectrophotometer. An A_{260} reading of 1.0 is equivalent to ~50 µg/mL of pure doublestranded DNA.

Primer and amplicon design

- Both primer design and careful choice of target sequence are essential to ensure specific and efficient amplification of the products.

- Target sequences should be unique, 75–150 bp long with a GC content between 50% and 60%, and should not contain secondary structures.
- It is recommended that primers should have a GC content of 50–60% and a melting temperature of 55–65 C.
- Long G or C stretches in the primer should be avoided, but it is recommended to have G or C at the end of the primers.

A number of programs are available to design primer pairs and pick target sequences. For instance, Primer-Blast (www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi?_LINK_LOC=BlastHomeAd),is a program developed by NCBI that uses the algorithm Primer3 available in public domain. Primer sequences are to be compared (blasted)with the user-selected databases to ensure they are unique and specific for the gene of interest. The program MFOLD (http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi) can be used to analyze the amplicons for potential secondary structures that may prevent efficient amplification. Ideally, two sets of oligonucleotides should then be ordered and tested for their performance in a qPCR.

qPCR validation

A validated qPCR assay is one that has been assessed for the optimal range of primer annealing temperatures, reaction efficiency, and specificity using a standard set of samples. This will assure that the reaction conditions, buffers, and primers have been optimized and that the cDNA samples are not contaminated with inhibitors of Taq polymerase. Practical web source tools are available for qPCR design and validation.

Establishment of a standard curve to evaluate PCR efficiency

The efficiency of a PCR is a measure of the rate at which the polymerase converts the reagents (dNTPs, oligonucleotides, and template cDNA) to amplicon. The maximum increase of amplicon per cycle is 2-fold representing a reaction that is 100% efficient. It is important to measure reaction efficiency as it is indicative of problems with the qPCR that can cause artifactual results. Low efficiency reactions are generally the result of primer–dimers or nonspecific amplicons. The most common causes of both high and low reaction efficiencies are poorly calibrated pipettes or poor pipetting technique. A standard curve is generally used to determine the reaction efficiency for any qPCR. The template for this typically is a sample of cDNA or spiked plasmid cDNA in a sample extract. We recommend initially producing a

10-fold dilution series over eight points starting from the most concentrated cDNA sample, to ensure the standard curve covers all potential template concentrations that may be encountered during the study (i.e., broad dynamic range). For each dilution, a standard qPCR protocol should be performed in triplicate for all the primer pairs to be used in the experiment and Cq values determined. The standard curve is constructed by plotting the log of the starting quantity of the template against the Cq values obtained. The equation of the linear regression line, along with Pearson's correlation coefficient (r) or the coefficient of determination (r 2), can then be used to evaluate whether the qPCR assay is optimized.

Real Time PCR studies for detection of EHP

The taxonomical classification of EHP has been supported by small subunit rRNA (SSU rRNA)-based phylogenetic studies. The small subunit ribosomal RNA (SSU rRNA) gene, a housekeeping gene, is a universal diagnostic target in EHP molecular detection methods. Several EHP diagnostic methods have been developed based on the SSU rRNA, including PCR, qPCR, in situ hybridization, and loop mediated isothermal amplification assays. But it is well known that the SSU rRNA gene is highly conserved among microsporidia, which may give false-positive test results. For example, the EHP's SSU rRNA sequence shared a 90% similarity to that of *Enterosporacanceri* which infects marine crabs. Therefore, a more specific PCR method is needed for EHP diagnostics.

A SYBR Green I based qPCR assay was established to detect EHP. With relatively higher sensitivity and by a quantitative method, this method provides a useful technique to support the detection and exploration of studies for controlling EHP. Among the quantitative detection methods, TaqMan probe based qPCR assay uses a specific probe which can hybridize with the target sequence with a high specificity. Therefore, the TaqMan probe based qPCR assay could effectively prevent influences caused by nonspecific products in the PCR and further improve the accuracy of quantitative assay and has been extensively applied in the detection of aquatic animal pathogens. In detecting EHP in farm samples, the positive detection rate of TaqMan-based EHP qPCR (93.5%) was noticeably higher than nested PCR (77.4%), while it was a slightly higher than SYBR Green I qPCR-EHP (88.7%). Compared with nested PCR and SYBR Green I qPCR-EHP, the diagnostic specificity of TaqMan-based EHP qPCR was reported 100%, while that of nested PCR and SYBR Green I qPCREHP were 82.8% and 96.6%, respectively, indicating that TaqMan based EHP qPCR had a higher diagnostic sensitivity. Further the correlation of the quantitative results of TaqMan-based

EHP qPCR and SYBR Green I qPCR-EHP was high and the value comparability was good. Although SYBR Green I fluorochrome method is applied to the quantitative analysis of DNA, the lack of specific fluorescent probe could cause interference of primer dimers and nonspecific amplification products, thereby, affecting the accuracy of results and even might produce false positives.

The β-tubulin gene sequence has sufficient variability for molecular diagnosis at the species level. Recently a qPCR assay based on the β-tubulin gene sequence for EHP-specific quantification was developed. This assay was applied to examine shrimp and feces samples to quantify the level of EHP present. The method was also reported to be applicable for screening live shrimp feeds and water samples. Using this qPCR, it was found shrimp feces contained high numbers of EHP copies, indicating that this may act as an important source of infection in ponds. Substantial amounts of EHP (as DNA copies) were detected in the Artemia samples by this technique. Live shrimp feeds, including polychaetes, squids, and Artemia biomass, have been suspected to be carriers for EHP Thus the quantification method for EHP based on the β-tubulin gene, applicable in EHP management in shrimp farming., so EHP quantification by qPCR in live shrimp feeds can be used as a tool to aid in disease prevention in broodstock populations.

The polar tube, a highly specialized invasion organ, is one of the important taxonomic indexes of microsporidia. Up to now, there are five polar tube proteins (PTP1–PTP5) located on the polar tube identified. PTP2 gene encoding a 35-kDa protein was identified from many microsporidian genomes. The PTP2 gene was also reported to be a single copy in the EHP genome. Due to these unique properties, the PTP2 gene was selected as the EHP detection target in molecular diagnostic tools. A SYBR Green I fluorescence quantitative PCR method based on the PTP2 gene has been established. The integrated method of qPCR and microscopy to quantify EHP spores reported for the detection of EHP in shrimp farming. It was recommended for early detection for EHP-infection, suitable for real-time monitoring of EHP in the field during the whole period of shrimp farming and provides a reference for the epidemiological study of EHP. The details of primers for qPCR are enlisted in following table for quick reference.

Table: Primers details for qPCR

Sl.No	Primer ID	Sequence	Reference
1	ENF185	GTAGCGGAACGGATAGGG	Liu et al., 2016
		CCAGCATTGTCGGCATAG	
2	F157	AGTAAACTATGCCGACAA	Liu et al., 2018
	R157	AATTAAGCAGCACAATCC	
	Taqman Probe	FAM-TCCTGGTAGTGTCCTTCCGT-TAMRA	
3	EHP-PTP2-F	GCAGCACTCAAGGAATGGC	Wang et al., 2020
	EHP-PTP2-R	TTCGTTAGGCTTACCCTGTGA	
4	EHP-bTub-F2	GATTTGAGAAAATTGG CGGTTAA	Piamsomboon et al., 2019
	EHP-bTub-R2	TTCTGAACACAGAGGCGCATA	
	TaqMan probe	FAM-TGATTCCATTTCCACGACTGCAC TTTTTC-TAMRA	

Note: FAM = fluorophore; TAMRA = quencher

Conclusion

RT-qPCR is the method of choice pathogen detection. In order to assure accurate and reproducible, quantitative data, strict standard operating procedures should be followed. The key steps for RT-qPCR method include experimental design with appropriate number of biological replicates and proper control samples, adherence to strict experimental protocols for acquisition, processing, and storage to assure reproducibility and minimize standard deviations between replicates, qPCR experiments with proper primer design, choice of target sequence and reference genes, and technical replicates. EHP infections are highly prevalent and require careful monitoring of EHP levels in shrimps and their live feed using a highly specific diagnostic method. Quantification of EHP in live feeds, pond environments, or feces becomes more important for EHP management. The qPCR based quantification methods for EHP are applicable in EHP management in shrimp farming.

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Loop-mediated isothermal amplification (LAMP) detection of *Enterocytozoon hepatopenaei* (EHP)

T. Sathish Kumar

Introduction

Diagnostics plays a crucial part in aquatic biosecurity, and timely use of this tool helps in successful aquatic animal health management and a successful crop. Most advanced molecular diagnostics such as PCR and qPCR are commonly used in aquaculture for routine screening compared to human/veterinary disease diagnostics. These advanced diagnostic methods need expensive thermal cycling equipment for amplification, gel electrophoresis/real-time monitoring (fluorimetry) for detection, and trained/ skilled personnel for analysis.

LAMP is a simple, efficient, and highly selective diagnostic technique that can detect the pathogen under isothermal settings in a short period of time. LAMP results can be quickly inspected for color change with the naked eye by in-tube colorimetric indicators without any toxic staining materials. The closed-tube LAMP assays were demonstrated to avoid cross-contamination and opening of tubes after amplification. Visual detection of closed-tube LAMP amplicons by naked eye was achieved by the accumulation of magnesium pyrophosphate metal indicator dyes such as Hydroxynaphthol blue, malachite green, etc., intercalating dyes such as SYBR green I, Eva green, etc and pH-indicator dyes such as phenol red, neutral red, cresol red, m-cresol purple, etc..

Principle

The LAMP assay is mainly based on the auto cycling and high strand displacement activity mediated by the enzyme Bst polymerase at isothermal conditions. The LAMP assay includes an initial step and a combination of amplification step with an elongation/recycling step. The LAMP amplification is carried out at isothermal temperature (60–65 °C), which is optimum for Bst polymerase activity. LAMP primers include two inner primers (forward inner primer (FIP) and backward inner primer (BIP) and two outer primers (forward outer primer (F3) and backward outer primer (B3). Inner primers consisted of two different sequences that recognized a sense and an antisense sequence of the target DNA, while the outer primers recognized only one external sequence of the target DNA. In order to accelerate the reaction process two additional loop primers are also used in the LAMP amplification

LAMP assay for the field level detection of EHP

Many LAMP protocols have been demonstrated for the rapid diagnosis of EHP. Real-time LAMP and visual LAMP assays have been established for the diagnosis of EHP. Suebsing et al., 2013 established a loop-mediated isothermal amplification (LAMP) assay combined with colorimetric nanogold (AuNP) for the rapid detection of EHP. Few Real-time LAMP assays had been demonstrated for the sensitive detection of EHP. All these LAMP protocols were developed based on SSU-rRNA gene sequence. In our earlier study also, we demonstrated a closed tube visual LAMP detection of EHP based on SSU rRNA, where the aerosol cross-contamination after amplification was avoided by adding the SYBRTM green I dye by using needles and syringes into the reaction vials without opening the tubes. However, SSU rRNA primers were reported to produce false-positive results. Recently an EHP LAMP protocol based on spore wall protein (SWP) sequence colorimetric nanogold (AuNP) for the specific and sensitive detection has been reported. Later, in 2021 ICAR-CIBA developed a closed tube LAMP protocol based on spore wall protein (SWP) sequence using simple indicator dyes such as phenol red and hydroxynaphthol blue.

Fig. Closed tube EHP LAMP using phenol red and HNB dyes (Sathish Kumar et al., 2021)

This LAMP assay showed 100% sensitivity, 100% specificity in clinical diagnostic evaluation when compared with SWP-PCR. Also the LAMP assay showed the analytical sensitivity as ten copies per reaction vial, equivalent to SWP-PCR. The LAMP assay is rapid, can diagnose the EHP microsporidian within 40 minutes in a simple dry bath. Also, the end

results can be visualized with naked eye. LAMP assays do not need any post-amplification harmful chemicals, expensive equipment, and skilled personnel. Thus, this LAMP protocol is a comparatively simple, highly sensitive, and highly specific EHP diagnostic assay.

Conclusion

LAMP diagnostics, in particular, has wide applications in various fields and certainly suited for testing at points of care. The EHP LAMP can be performed with minimum skills by using a simple dry bath, and the results can be easily visualized with the naked eye. This LAMP assay does not need any high resource setting/skilled personnel and has a great potential to be a rapid, on-farm diagnostic tool. Additionally, this field-friendly, low-cost, robust assay can be an effective tool for EHP transmission studies, epidemiology, and routine surveillance.

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Management of *Enterocytozoon hepatopenaei* (EHP) in shrimp hatcheries and farms

Vidya Rajendran, T. Sathish Kumar and K.P. Jithendran

Enterocytozoon hepatopenaei (EHP) is an obligate intracellular microsporidian parasite which infects and proliferates in hepatopancreatic tubules and thereby impairing its digestive and absorptive functions. EHP affected shrimps display slow and retarded growth with high FCR (2.9 to 3.0), often found to be associated to White feces syndrome, however its association is often refutable. A microsporidian infection was first detected from a growth retarded *Penaeus monodon* in Thailand (2004) and later identified as EHP (2009). The first case of EHP in India was reported from farmed *vannamei* shrimps from South east coast in 2016. Since then more cases were reported and in a short span of time EHP has emerged as a serious threat to Indian shrimp farming industry with a prevalence rate of 27% in 2019 with a production loss of 1.80 ± 0.24 ton/ ha/crop (2021).

Significance of EHP

EHP is a spore producing microsporidian parasite currently classified as a kind of fungi and can be detected only through microscopy and specific PCR tests. It spreads horizontally through oral route by consuming spores via cannibalism and cohabitation with infected shrimps. EHP often associated with slow growth. However its conclusive association with white feces syndrome is still debatable. There are no specific signs and symptoms associated to EHP infections in shrimp. Only regular monitoring of intake of feed, slow growth and monitoring hepatopancreas can assist EHP detection in shrimps.

Microscopic and PCR diagnosis are available to detect the infection as the earliest, however many of times the low level infection remain undetected and find its entry to shrimp hatcheries and grow out systems. The recommended methods to contain the infection are only through enforcing tight biosecurity which additionally involves routine disinfection using recommended chemicals before stocking the brooders and post larvae. Many of the disinfection methods involve the inactivation of spores targeting the polar tube or polar flagella extrusion which is essential for infecting the target animal. A high pH (9) usually activate spore to extrude the flagella and release spore contents which make them inactive for successive infection. Spores are hardy and thick walled hence they can survive from two

weeks to even 1 year depending on environmental condition. Use of chemicals and drugs are regulated for aquaculture activities; however certain permitted chemicals can be effectively used in controlling EHP in hatcheries and farms.

EHP prevention and management in hatcheries

Disease prevention and management in hatcheries are based on a biosecurity plan. It involves preventing the entry and establishment of infectious agents in the hatchery facility using prophylactic measures, e.g. sterilisation of input water, quarantine of brood stock, use of vaccines, probiotics, and bacteriophages. Once infection establishes in the hatcheries biosecurity plans enforce to shut down the facility, culling of infected stocks and initiate disinfection process. Disinfection involves the killing of established infectious agent using physical and chemical methods which are recommend by regulatory bodies, e.g. chlorination in hatcheries and therapeutic treatment of infected animals.

Biosecurity

Biosecurity means a set of management and physical measures designed to reduce the risk of introduction, establishment and spread of pathogenic agents (EHP) to, from and within an aquatic animal population. Biosecurity plans apply important principles from HACCP (Hazard Analysis and Critical Control Points) and quality assurance systems.

Identification of critical control points in EHP prevention in hatcheries

Critical control points are the physical locations or production steps at which shrimp may be exposed to disease pathogens/EHP. Each of these represents a critical control point. Through following critical control points pathogen/EHP can enter the hatchery.

- Infected brooder shrimp (wild or imported) and spores adhered to eggs
- Untreated water sources
- Contaminated diets: Algae, live feeds, polychaetes, frozen foods, commercial feeds
- Vectors: Personnel, contaminated equipment and vehicles

Disinfection process

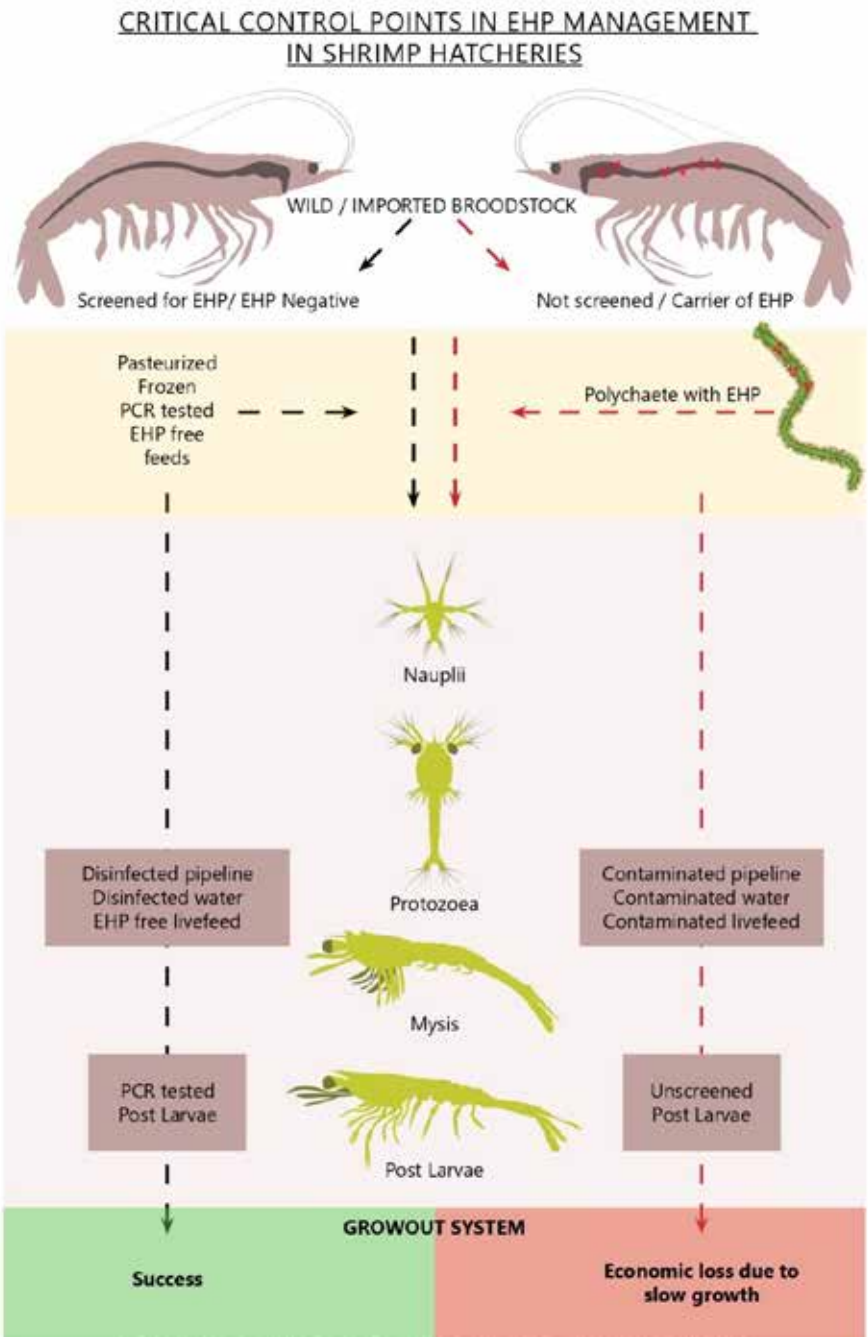
Disinfection of tanks and pipelines

Treat tanks, pipelines, tools and accessories in hatcheries with 2.5% sodium hydroxide for 3h and wash them with disinfected water followed by dry out them for 7days.

Sodium hydroxide will increase the pH to 9 which aids ejection of polar flagella and make them ineffective for initiating infection (http://ciba.res.in/stuff/ehp_adv.pdf).

Screening of brood stock

The major resource of hatcheries is wild or imported brooder shrimps. Practice screening of brooders for EHP including all the other potential disease threats (OIE listed) may eliminate the disease risk in larval production and enhancing biosecurity in shrimp hatcheries. It is recommended to use high quality diets to promote shrimp health.



Screening of brooder feed

Hatcheries use live polychaetes for feeding brood stock to enhance spawning efficiency which often carries EHP. Try feeding frozen or pasteurised polychaetes or SPF polychaetes which can eliminate the entry of this important pathogen.

Contaminated Artemia cysts

Artemia cysts used for hatchery operation may sometimes get contaminated with EHP spores and other disease agents. So, screening or use of specific pathogen free Artemia cysts may reduce the infection chances in larval production.

Screening PLs

Prior to release the seed stock from hatcheries check the health status of post larvae by testing for EHP and other listed disease agents. However, the testing can only indicate the status of the animal whereas the farms if already contaminated with disease agents become potential threats to successful culture.

Regular monitoring

Examine the faecal threads of brood stock and PLs for EHP and closely monitor the intake of feed and growth rate. These observations can guide the farmer to detect the infection early and take adequate measures to reduce the economic loss.

EHP management in shrimp farms

Farm Management

EHP is contagious and spread through spores from infected shrimp to others. Spores are method of reproduction in microsporidians and infected shrimp can produce large number of spore and shed to the environment where they live. These spores may enter either laterally through oral route or deposited in the pond bottom and available to re-infect the post larvae in the successive cultures. Spores cannot be removed due to its microscopic nature and therefore can only be deactivated using certain chemicals and disinfectants. Hence preparation of shrimp ponds is critically important prior to stocking. At the farm level biosecurity plays an important role in preventing this residual protozoan disease. Farm management has many aspects and they are:

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 other important pathogens.

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 are typically related to spore loads. Once spores are in ponds, it is very difficult to eradicate the disease. 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 (https://enaca.org/?id=1064). Pond bottoms must be completely dry. Plough the quick lime into the dried sediments to a depth of 10 to 12 cm; then moisten the sediments to activate the lime. If the application is done properly, the pH of the soils will rise to 12 or more within days and then gradually return to normal as the quick lime becomes calcium carbonate.NACA also suggested >15 ppm KMnO4 or >40 ppm chlorine to inactivate spores in the soil (https://enaca.org/?id=1064).

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.

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 with Calcium hypochlorite at the concentration of

18 mg/l before stocking PL can eradicate wild crustaceans and significantly reduced the prevalence of microsporidian infection.

Continuous monitoring

Health and growth of shrimps must be monitored regularly and ensure healthy feed and effective aeration and water movement in the pond. Once slow growth and low feed intake is observed, check for EHP by PCR. Once EHP is diagnosed in an on-going culture avoid over feeding and try to provide high protein diet. Post-culture disinfect the water and dry the pond bottom for two weeks. Prior to next culture, remove mud or black soil from pond bottom.

Conclusion

The disease emergence and its spread are 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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Disinfectants and therapeutics in the treatment and control of *Enterocytozoon hepatopenaei* (EHP)

T. Sathish Kumar

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.

Recently, *Enterocytozoon hepatopenaei* (EHP) (measure about 1.7 ×1 μm), has 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 characterized from *P. monodon* from Thailand. 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, the power house of the animal, and infection in hepatopancreas may cause impairment in metabolism and ultimately result in stunted growth. EHP infection can be transmitted horizontally through an oral route (cannibalism, predation) and possibly by vertical transmission (trans-ovum). So far, no intermediate hosts have been known to be involved. Thus, EHP is being emerged as a serious threat to the shrimp farming industry. The studies on prophylactic and therapeutics strategies for the treatment and control of EHP are very limited. This chapter discuss about the potential disinfectants and drugs in the control and treatment of EHP.

EHP spore inactivation

Artificial germination of microsporidian spores can be triggered in vitro by using a combination of nutrients, alterations in temperature, pH, hyperosmotic conditions, the presence of anions or cations, or exposure to ultra-violet light or peroxides. Triggering the artificial germination (extrusion of the polar tube) to render EHP mature spores non-infectious is extremely important, as the polar tube extrusion is the key to successful

transmission of microsporidian to host cell. Recently, evaluation of EHP spores viability by inducing extrusion of polar tubule was demonstrated with phloxin B. Microsporidians can be killed by boiling for at least 5 min and by application of several disinfectants including quaternary ammonium, 70% ethanol, formaldehyde, phenolic derivatives, hydrogen peroxide, chloramines, sodium hydroxide, or amphoteric surfactants. The viability of the purified spores has been studied by treating EHP spores with different chemical treatments such as calcium hypochlorite, formalin, potassium permanganate (KMnO4) and ethanol. It was found that 15ppm KMnO4 for 15min, 40ppm of 65% active chlorine for 15min or 10ppm of 65% active chlorine for 24h and 20% ethanol for 15min rendering complete inhibition of EHP spores activity. In our study in ICAR-CIBA the EHP spore artificial germination was achieved with 1%KOH at pH 11 for 15 min incubation. It was found that the overnight treatment of $KMnO_4$ at 10 ppm, Formalin at 150 ppm, Calcium hypochlorite at 40 ppm , Sodium hydroxide at 3%, Hydrogen peroxide at 2% cause 100% inhibition of EHP spore polar tubule extrusion and disintegration and denaturation of EHP spores

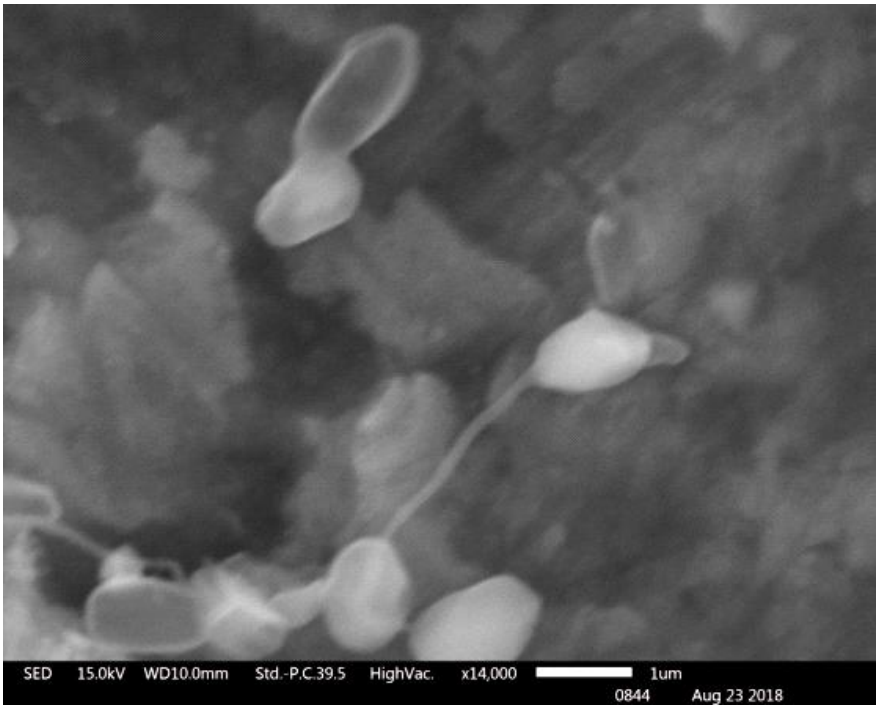

Fig: SEM image of a germinated EHP spore

Potential therapeutics for treating microsporidians

Microsporidia do not have true mitochondria and lack the tricarboxylic acid cycle for energy production. It entirely depends on other metabolic pathways such as the glycolytic

pathway, pentose phosphate pathway, and trehalose metabolism for energy production. The enzymes participating in the above pathways can be targeted as a potential target for evolving therapeutic strategies. Triosephosphateisomerase (TIM) an important enzyme in glycolysis, was inhibited by sulfhydryl reagents in *Trypanosomacruzi*, *T. brucei*, *Entamoebahistolytica*, and *Giardia duodenalis*. Microsporidian microtubules are formed by polymerization of tubulin protein; this polymerization can be inhibited by benzimidazole drugs such as albendazole, cambendazole, benomyl, carbendazim, fenbendazole, mebendazole, and triclabendazole. Methionine aminopeptidase 2 (MetAP2) is vital for tissue repair, protein degradation, and angiogenesis in microsporidians. Anti-angiogenic drugs - ovalicin and Fumagillin were reported to show anti-microsporidian activity. Chitin maintains the spore wall structure and is vital for infection and extracellular survival. Chitin synthesis can be inhibited by the activity of Nikkomycin and Polyoxin. Spore germination is calcium-dependent and can be inhibited by calcium antagonists (Verapamil, Lanthanum), calmodulin inhibitor (trifluoperazine, chlorpromazine), and calcium channel blocker (Nifedipine). Studies on treatment or therapeutic drugs available for controlling EHP are limited. There is no reported treatment, or therapeutic drug available for killing EHP spores. One study reported Fumagillin-B to be ineffective in controlling EHP spores.

Disinfectants in the control of EHP

Disinfectants may be useful in eliminating extracellular spores in the soil, water, and implements. It is recommended to use 2.5% sodium hydroxide solution (25gms NaOH/L fresh water) for control the epizootics of EHP in hatcheries. After 3 hrs contact time, NaOH should be washed off and dried for 7 days. Then it should be rinsed down with acidified chlorine (200 ppm chlorine solution at pH <4. 5). This treatment was recommended for all equipment, filters, reservoirs and pipes in hatcheries. For disinfecting shrimp earthen ponds CaO (quicklime, burnt lime, unslaked lime or hot lime) was recommended at 6 Ton/ha. CaO should be plowed into the dry pond sediment (10-12 cm) and then moisten the sediment to activate the lime and left untouched for 1 week before drying or filling. After application of CaO, the soil pH should rise to 12 or more for a couple of days and then fall back to the normal range as it absorbs carbon dioxide and becomes $CaCO_3$. Many disinfectants have demonstrated the complete inhibition of spore germination. It is reported that 15 ppm $KMnO_4$ for 15 min, 40 ppm of 65% active chlorine for 15 min or 10 ppm of 65% active chlorine for 24 h, and 20% ethanol for 15 min are effective in inhibiting spore germination. In the ICAR-

CIBA *in-vivo* challenge study, we found that potassium permanganate at 15 ppm, Calcium hypochlorite 20 ppm, sodium hydroxide 3%, and hydrogen peroxide 3% are effective in destroying EHP spores in the soil.

Conclusion

Microspordia are obligate intracellular pathogens that cause severe impact across all trophic levels from mammals to planktonic copepods. Spores are the infective material of microsporidians for transmitting disease. The epizootics of microsporidians in aquatic animals are known in all freshwater, brackish water and marine water environments. Shrimp hepatopancreas is the target organ for EHP infection. The studies on prophylactic and therapeutics strategies for the treatment and control of EHP are very limited. Better management practices and strict implementation of biosecurity protocols are the proper ways to prevent the disease.

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Collection and preservation of shrimp samples for diagnosis of *Enterocytozoon hepatopenaei* (EHP)

Sujeet Kumar and P. Ezhil Praveena

Sample collection

Acurate sampling and preservation of samples is one of the most crucial step for accurate disease diagnosis.

- Before sampling, note any gross changes (behavioural abnormalities, size variation, stunted growth, white fecal threads in the ponds, change in appearance, colour, lesions etc.)
- Moribund (approaching death) or freshly dead shrimp is the most appropriate sample, since autolysis takes place immediately.

Sending live shrimp samples for diseases diagnosis

A representative live sample of shrimp is the most ideal for diseases diagnostic lab as this enhance the usage of various techniques for diagnostic purpose. For this purpose, 5-6 shrimps showing the sign of stunted growth, size variation should be collected and pack in double polypack with oxygen. Pack these bags in a styroform or thermocol box to keep temperature cool. Place a couple of ice pack or gel pack while sending samples long distance. For smaller shrimp or post-larvae place adequate number ranging from 30 to 150 depending on size in the same manner. LABEL and dispatch through courier with completed history sheet (use separate photocopy for each batch of sample) within 24 hours.

Sending fixed shrimp samples for disease diagnosis

In case live sample delivery is not possible, the samples collected as above may be fixed appropriately as detailed below for PCR analysis or for histopathology.

Shrimp samples for PCR analysis

Smaller shrimp samples or dissected hepatopancreas from larger shrimps may be collected in 95% Ethyl alcohol in tightly capped container and submit to the disease diagnostic lab at the earliest. Remember, that hepatopancreas is the target organ for EHP, therefore, it must be included in the dissected sample.

Faecal sample for microscopy and PCR analysis

In addition to shrimp samples, faecal sample can be collected from the affected animals in normal saline and send to laboratory for microscopical examination as well as for PCR analysis for the presence of spore.

Shrimp samples for histopathology analysis

Fixation of sample: Shrimps are usually fixed in Davidson's OR Neutral Buffered Formalin (NBF) fixative (composition shown below) in a wide mouth plastic bottle.

Smaller shrimp may be fixed directly by immersing in appropriate fixative for 24 hours and for larger shrimps it should be in the fixative for 48 hours (sample size should not exceed $1/10^{th}$ of the volume of the fixative).

For larger shrimp (>1g), inject fixative into live animals, first in hepatopancreas, then the region anterior to the hepatopancreas, and lastly two injections into the abdomen, one anterior and the other posterior. Inject fixative @ 10% of shrimp body weight (e.g. 1 ml for a 10 g shrimp).

To ensure proper penetration of fixative, slit the cuticle open on both sides of the shrimp, from the 6th abdominal segment to the rostrum using scissors before placing in fixative. Seal the container, LABEL and dispatch with history sheet as soon as possible.

Collection of water samples

Water samples may also be collected for examination of various physicochemical parameters. Further, white, floating faecal strings from the affected disease ponds should be collected from the affected ponds and transported on ice.

Collection of soil samples

About 500gm of soil sample from the four corners and centre of the pond was taken and then packed in a zip lock pouch and label it properly and send it to the laboratory for diagnosis of any EHP spore. The collected soil should be representative of the pond as a whole.

Precautions for transportation of samples

- Glass containers are not recommended due to breakage.
- Wrap samples in a non-fibrous material.

- Samples over ice may be packed in zip-lock bags.
- Labels should be written with pencil.
- Complete proforma with contact details.

Preparation of fixatives

1. Davidson's Fixative 1000 ml

- Formalin (37% formaldehyde) 220 ml
- 95% ethanol 330 ml
- Distilled water (if not available, tap water) 335 ml
- Glacial acetic acid 115 ml

2. Neutral Buffered Formalin (NBF) 1000 ml

1. Formalin (37% formaldehyde) 100 ml
2. Distilled water (if not available, tap water) 900 ml
3. Sodium phosphate, monobasic monohydrate 4.0 gm
4. Sodium phosphate, dibasic anhydrous 6.5 gm

3. 10% Formalin 1000 ml

1. Formalin 100 ml
2. Distilled water 900 ml

4. 95% Ethyl alcohol 1000 ml

1. Absolute alcohol 950 ml
2. Distilled water 50 ml

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

T. Sathish Kumar

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 in-spite 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. ICAR-CIBA developed a simple purifying method to recover EHP spores from soil and also developed a simple DNA extraction procedure to extract DNA directly from EHP infected shrimp pond soil.

Purification of spores from soil

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 containing spores can be viewed under a microscope (or) DNA can be extracted from the pellet for EHP detection (Refer above article for DNA extraction)

DNA extraction from EHP infected shrimp pond soil

Soil sample collection

The soil sample (around 5 kg) should be collected from the four corners and centre of the infected pond and for the detection of EHP.

Before DNA extraction, the soil collected from each pond was pooled.

⬇

Then 250 mg of soil sample with 0.5 mm glass beads (Omni International, USA) was taken in a 2 ml tube, and 500 µl of DNA extraction buffer were added.

⬇

The mixture was vortexed at maximum speed for 15 min.

⬇

The sample was incubated at 65°C for 10 min and centrifuged at 12000 × g for 5 min at 4°C.

⬇

The supernatant was transferred to a fresh 2 ml centrifuge tube, and 100 µl of 3 M sodium acetate and 400 µl of 30% PEG (MW-8000) was added.

⬇

The mixture was allowed to precipitate at -20°C for 20 minutes and centrifuged at 12000 × g for 5 min at 4°C after thawing.

⬇

The resultant pellet was re-suspended in 500 µl of sterile TE buffer and 500 µl of chloroform: isoamyl alcohol mixture (24:1) and centrifuged at 12,000 × g for 5 min at 4°C.

⬇

The aqueous phase was transferred into a new tube, 500 µl of isopropanol (ice-cold) was added and centrifuged at 12,000 × g for 10 min at 4°C.

⬇

The precipitate was washed twice with 70% ethanol at 12,000× g for 2 min at 4°C. The DNA pellet was dissolved in nuclease-free water and preserved at -20°C.

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

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

T. Bhuvaneswari, R. Ananda Raja, J. J. S. Rajan and M. Poornima

Sample collection

For PCR detection, fresh or ethanol-preserved samples of post larvae (PL) from hatchery or whole hepatopancreas in case of grow-out monitoring may be used. As the microsporidian spores are expelled out through feces, fresh fecal 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 mMTris pH 8.0- 606 mg, 1mM EDTA- 37.2 mg, 500 mMNaCl- 2.922 gm, 1% SDS – 1 gm make up to 100 ml with Proteinase K @ 10 µg/ml) and homogenize the sample with the micropestle.
- Add 350 µl of lysis buffer to the homogenate and mix well by vortexing.
- Incubate the sample at 95°C in dry bath for 1 min.
- Cool the lysate to room temperature and centrifuge the sample at 12,000 rpm for 10 min.
- Collect 200 µl of supernatant into fresh tube with a micropipette and 500 µl of 70% ethanol and mix by invert mixing.
- Centrifuge the tube at 12,000 rpm for 10 min and decant the supernatant without disturbing the DNA pellet.
- Invert the tube on a clean dry tissue wipe and air dry the pellet.
- Reconstitute the DNA pellet in 200 µl of water or TE buffer (1 ml 1M Tris (pH 8.3) and 200 µl 0.5 M EDTA (pH 8.0) and adjust the volume to 100 ml of de-ionized water).
- Use 1 µl of this sample as DNA template for the PCR.

Quantification of DNA

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.

1 OD of dsDNA at 260nm = 50 µg/ml

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

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.

Method : The PCR procedure followed as described by Jaroenlak *et al.* (2016)

PCR Primers details for the detection of *E.hepatopenaei*

Primers	**Sequence**	**Amplicon Size**
Spore wall protein		
SWP 1F	TTGCAGAGTGTTGTTAAGGGTTT	First step 514 bp
SWP 1R	CACGATGTGTCTTTGCAATTTTC	
SWP 2F	TTGGCGGCACAATTCTCAAACA	Nested 148 bp
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 trans illuminator.

Representative PCR gel electrophoresis

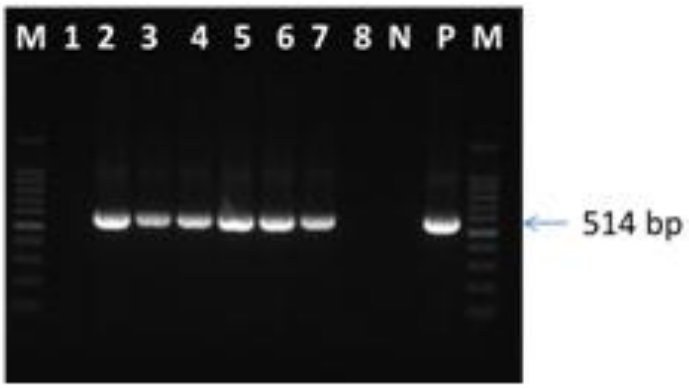

Fig. Gel image of EHP spore wall protein PCR First step.

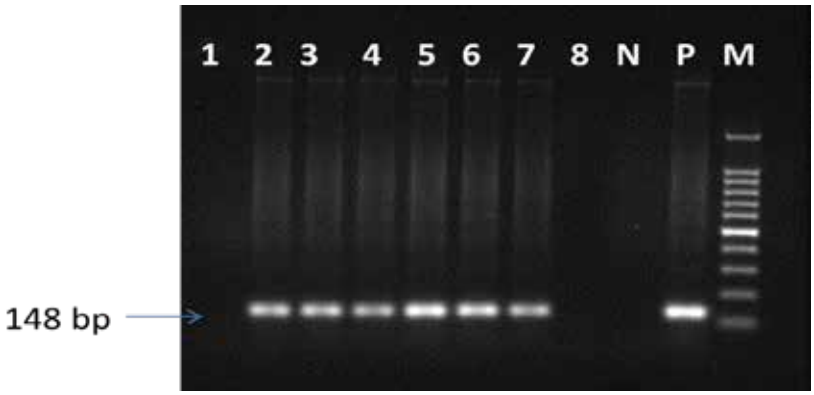

Fig.Gel image of EHP spore wall protein PCR second step

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TaqMan probe based qPCR assay protocol for diagnosis of *Enterocytozoon hepatopenaei* (EHP)

J.J.S.Rajan, T. Bhuvaneswari and M.Poornima

Generally no obvious clinical symptoms are observed in EHP infection. Hence the quantitation of infection in terms of amount of EHP in infected shrimp is essential to adopt the management practices in culture system. The qPCR method is a useful technique for the detection of pathogens qualitatively with relatively higher sensitivity. Among the quantitative detection methods, TaqMan probe based qPCR assay uses a specific probe which can hybridize with the target sequence with a high specificity. As this qPCR tool could effectively prevent nonspecific products in the PCR, it further improves the accuracy of quantitative assay.

Method: The Taqman probe real-time PCR Assay described by Liu et a. (2018) for the detection of EHP infection in shrimp is followed.

Procedure:

Required reagents:

- 2x Master mix
- EHP Primer probe mix
- Internal control Primer probe mix
- Nuclease free water

Primers details:

Primer ID	5′- 3 sequence′
Forward primer F157	AGT AAA CTA TGC CGA CAA
Reverse primer R157	AAT TAA GCA GCA CAA TCC
TaqMan probe	FAM-TCC TGG TAG TGT CCT TCC GT-TAMRA

Master Mix:

Reagents	Vol. x1 rxn (20l)
Water	6.0
Master Mix (2X)	10.0
EHP Primer & Probe Mix*	1.6
ROX 50x	0.4
DNA Template	2.0
Total Volume	20.0

Protocol

A Master Mix is prepared in a tube by combining a 2X Master Mix Reagent and Primer & Probe Mix with nuclease free water (protect from light).

After mixing thoroughly 18µl volumes of the Master Mix are quickly dispensed into the plate.

Add 2µl of DNA test samples and controls

Pulse spin tubes at 3000rpm.

Keep the plate/strips/tubes in thermal cycler.

Run PCR program at 95°C for 30 sec, 40cycles of 95°C for 5 sec, 60°C for 30 sec.

After the completion of the reaction observe the Ct value of the samples

Detection channel

EHP -FAM

Decapod internal control- HEX / VIC

Control reactions

No template control (water)

Positive control (target plasmid or DNA dilutions)

Bibliography

Liu, Y.M., Qiu, L., Sheng, A.Z., Wan, X.Y., Cheng, D.Y. and Huang, J., 2018. Quantitative detection method of *Enterocytozoon hepatopenaei* using TaqMan probe real-time PCR. *Journal of Invertebrate Pathology*, 151, 91-196.

Microscopic diagnosis of *Enterocytozoon hepatopenaei* (EHP) - Histopathology

P. Ezhil Praveena, T. Sathish Kumar and N. Jagan Mohan Raj

Microscopical examination of tissues for the presence of any pathological alterations in it is called histopathology. This involves collection of morbid tissues from necropsy, fixation, preparation of sections, staining and finally microscopic interpretation. The various steps involved in histopathology are:

A. Collection of Materials

Collect hepatopancreas from three to five shrimp showing morbid changes along with equal number of normal shrimps. Collect moribund and normal shrimp hepatopancreas separately in containers and label it with all details.

B. Fixation

It is a process by which the cells and tissue constituents are fixed in a physical and partly also in a chemical state so that they will withstand subsequent treatment with various reagents with minimum loss of tissue architecture. This is attained by exposing the tissue to various chemical compounds, called fixatives. 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 shrimp @ 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 shrimp samples should remain in fixative at room temperature for 48-72, which mainly depends on the size of shrimps. The volume of the fixative added should be 10 times more than the volume of the tissues. Thin pieces of various organs of shrimps of 3-5 mm thickness are dissected out from it and are processed.

Common fixatives used for collection of shrimp are:

Davidson Fixative

Ethyl alcohol 95%	330 ml
Formalin	220 ml
Glacial acetic acid	115 ml

Tap/Distilled water	335 ml

Formal Saline

Formalin	100 ml
Sodium Chloride	8.5g
Tap/Distilled water	900 ml

C. Dehydration

This is the process by which the water is removed from the tissues. This is done to prevent undue shrinkage to the tissues. The steps involved in this process are

Ethyl alcohol 70% - 1 hour

Ethyl alcohol 90% - 1 hour

Absolute alcohol I - 1 hour

Absolute alcohol II - 1 hour

D. Clearing

It is process of removal of alcohol from the tissues and prepares it for paraffin penetration for embedding and the steps involved are

Xylene I - 1 hour

Xylene II - 1 hour

E. Embedding

This is the process by which impregnating the tissues completely with paraffin (54-56°C). The steps involved are two changes of paraffin one hour each.

F. Blocking

Melted paraffin is poured into the moulds and the tissues are oriented in such a position that the cutting surface of the tissue faces down. The blocks are removed from the moulds and they are ready for sectioning.

G. Section cutting

The blocks are trimmed off the excess paraffin and the section is cut using a microtome. Then the sections are transferred from the microtome to a tissue flotation bath having warm water. Sections spread out uniformly are then taken on to a clean glass slides coated with Meyer's albumin-glycerin mixture.

H. Staining of sections

Haematoxylin and eosin method of staining (H&E) is the routinely used stain for tissue sections. The steps involved are

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

The processed slides are ready for examination under microscope.

Loop-mediated isothermal amplification (LAMP) protocol for detection of *Enterocytozoon hepatopenaei* (EHP)

T. Sathish Kumar

Loop-mediated isothermal amplification (LAMP) is an alternative nucleic acid amplification technique based on the principal of strand displacement DNA synthesis and production of stem-loop DNA structures under isothermal conditions. The LAMP assay is a simple diagnostic protocol with numerous advantages such as high specificity and sensitivity, working at isothermal conditions, field adaptability and no requirement for sophisticated equipment.

Principle

The LAMP assay occasionally suffers from product cross contamination. The opening of the reaction vial after amplification often results in aerosol contamination. Thus, to overcome such aerosol contamination, we developed a simple and cheap closed tube method using indicator dyes such as phenol red, hydroxyl naphthol blue.

Procedure

The procedure is followed as per method described by Sathish Kumar et al., 2021

Reagents

LAMP Primers stock preparation		
1	FIP (Forward inner primer) and BIP (Backward inner primer)	1.6 µM each
2	F3 (Forward outward primer) and B3 (Forward outward primer)	0.2 µM each
3	LF (Loop forward primer), and LB (Loop backward primer)	0.4 µM each

Reaction mix

Sl no	Reagents	Test sample (µl)	No template control (µl)	Positive control (µl)
1	2X Reaction Mix (RM)	12.5	12.5	12.5
2	Primer Mix (PM)	6	6	6
3	Sample (DNA)	1-2 (100 ng)	0	1(PC)
4	Nuclease free water (H_2O)	Make upto 25	Make upto 25	Make upto 25

Method

The LAMP reaction was performed in a 25 µl assay volume

⬇

Reaction mix should be prepared in ice.

⬇

Once the reaction mix is prepared, tap them gently to mix and spin shortly.

⬇

Keep the reaction tubes at 60ºC in a water bath/heat block, for 45 min. followed by reaction termination at 80ºC for 5 min.

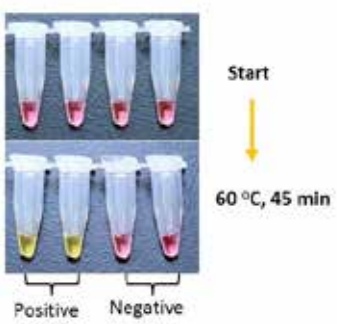
⬇

Observe the PCR tubes for color change under visible light.

⬇

Samples positive for EHP will produce color change from pink to yellow, whereas negative samples remain pink

Result – Color change



Caution in handling the reaction tubes after use

This assay is a highly sensitive detection method for the diagnosis of EHP. Proper measures should be taken to prevent carryover cross-contamination. Pre and Post LAMP reaction handling areas should be exclusively separate. After the assay is complete the reaction tubes should not be opened and should be disposed of in a sealed bag.

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Trouble shooting in PCR and qPCR

M. Poornima, S. K. Otta, T. Bhuvaneswari, J. J. S. Rajan

General trouble shootings in PCR

Observation	Possible Cause	Solution
SEQUENCE ERRORS	Low fidelity polymerase	• Choose a higher fidelity polymerase
	Suboptimal reaction conditions	• Reduce number of cycles • Decrease extension time • Decrease Mg^{++} concentration in the reaction
	Unbalanced nucleotide concentrations	• Prepare fresh deoxynucleotide mixes
	Template DNA has been damaged	• Start with a fresh template • Try repairing good DNA template • Repair Mix • Limit UV exposure time when analyzing or excising PCR product from the gel
	Desired sequence may be toxic to host	• Clone into a non-expression vector • Use a low-copy number cloning vector
INCORRECT PRODUCT SIZE	Incorrect annealing temperature	• Recalculate primer Tm values
	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
	Missing reaction component	• Repeat reaction setup
	Suboptimal reaction conditions	• Optimize Mg^{++} concentration by testing 0.2–1 mM increments • Thoroughly mix Mg^{++} solution and buffer prior to adding to the reaction • Optimize annealing temperature by testing an annealing temperature gradient, starting at 5°C below the lower Tm of the primer pair
	Poor template quality	• Analyze DNA via gel electrophoresis before and after incubation with Mg^{++} • Check 260/280 ratio of DNA template
	Presence of inhibitor in reaction	• Further purify starting template by alcohol precipitation, drop dialysis or commercial clean up kit • Decrease sample volume
	Insufficient number of cycles	• Rerun the reaction with more cycles
	Incorrect thermocycler programming	• Check program, verify times and temperatures
	Inconsistent block temperature	• Test calibration of heating block
	Contamination of reaction tubes or solutions	• Autoclave empty reaction tubes prior to use to eliminate biological inhibitors • Prepare fresh solutions or use new reagents and new tubes
	Complex template	• Use High-Fidelity DNA Polymerases • Include the appropriate GC enhancer. • For longer templates, use specific high fidelity DNA polymerase
MULTIPLE OR NON-	Premature replication	• Use a hot start polymerase • Set up reactions on ice using chilled components and add samples to thermocycler

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

General trouble shootings in Real Time –PCR

Problem	Possible Causes	Solution
The assay is insensitive and the amplification plots look abnormal	Inappropriate assay design The probe inadequately labeled.	The assay requires optimization. Re-design the assay if possible.
The Cq data for dilutions of the standard curve are irregularly spaced	Inappropriate assay design leading to inefficient priming. Errors in the dilutions of the template for the standard	Proper dilutions of template. Purify sample template

	curve. The sample contains an inhibitor.	
The positive control amplifies but there are no amplification results from a sample known to contain target	Sample concentration inappropriate. Inhibition in the test sample.	Add up to 0.3% BSA to the PCR or purify the input nucleic acid
Low or absent fluorescence in both the test sample and in the positive control. The correct PCR product is visible on the gel and the design is verified	A. SYBR Green Dye-Based Detection – Bad SYBR Green I binding dye	Purchase new SYBR Green I binding dye or a new qPCR mix with SYBR Green binding dye
	B. Probe Detection	
	a. High/low background fluorescence. Incorrect probe concentration	Use correct concentration. Purchase a new probe.
	b. Degraded probe. Poor probe labeling. Incorrect collection of fluorescent data.	Purchase a new probe.
The fluorescence plots suddenly spike upwards	Bad reference dye or instrument error (e.g., door was opened during run).	Smooth ΔR amplification plots: Purchase a new reference dye. Always protect dye from light during storage. Spike persists in ΔR: Repeat run, if the problem persists seek engineering help for instrument.
Multiple Tm peaks in melt profile data	Multiple Products on a Gel The primers are non- specific and are producing multiple products. The Mg 2+ concentration in the reaction is too high or the annealing temperature is too low.	Titrate the Mg 2+ to determine the optimum concentration. Perform annealing temperature gradient to select the optimum annealing temperature for the PCR. Design unique primers.

Artificial germination of EHP spore

T. Sathish Kumar

Spore germination is a rapid strategy carried out by spores to infect the host cell without its knowledge. Spore germination starts with swelling of the polaroplast, and expansion of the posterior vacuole resulting in swelling of the spore. Swelling of the spore ultimately increases the osmotic pressure. Increasing pressure inside the spore ruptures the thinner apex of the spore case and expels the polar tube/injection tube by eversion. Expansion of the posterior vacuole forces the cytoplasm and nucleus through the tube. The ejected polar tube length may range from 50–500 µm in length. The whole event of spore germination can be completed within a few seconds. The ejected polar tube/injection tube can penetrate any obstacle, including other microsporidian spores, and inject the sporoplasm like a needle into the host cell cytoplasm. Spore germination is induced by different environmental triggers specific to species and habitat. In vitro germination of spores is achieved by many physical and chemical inducing factors such as changes in pH, dehydration followed by rehydration, hyperosmotic settings, presence of anions or cations, and exposure to ultraviolet light or peroxides.

Induced germination of EHP spore protocol:

10µl of purified EHP spores (1.0×10^4/ml)

⬇

Add 20 µl of 1% KOH (pH > 10.5)

⬇

Incubate at room temperature for 15 min

⬇

Observeunder a lightmicroscope (100× objective lens)for spore germination

References

CIBA (Central Institute of Brackishwater Aquaculture), 2019, CIBA, Annual report 2019, https://krishi.icar.gov.in/jspui/bitstream/123456789/43559/1/CIBA%20Annual%20Report%202019.pdf.

General laboratory biosafety

In view of rapid development, in both basic and applied research in the field of aquaculture and related disciplines, there are concerns with respect risks to research staff during laboratory operations and overall risks due to the waste generated during the research activities. All the general laboratory safety regulations outlined for any R&D lab also apply to the shared PCR laboratories as well. The purpose of this chapter is to provide specific guidance and protocols for the protection of the users of the shared PCR laboratories. The plan proposed here will help to optimize use of equipment, space and safety of the workers at large.

Biosafety requirements in shared PCR laboratories

Biosafety for research staff

Personal protection

1. Laboratory coveralls, gowns or uniforms and appropriate gloves must be worn for all procedures that may involve direct or accidental contact with hazardous chemicals and other potentially infectious materials
2. After use, gloves should be removed aseptically and hands must then be washed.
3. Personnel must wash their hands after handling infectious materials and animals, and before they leave the laboratory working areas.
4. Safety glasses, face shields (visors) or other protective devices must be worn when it is necessary to protect the eyes and face from splashes, impacting objects and sources of artificial ultraviolet radiation.
5. It is prohibited to wear protective laboratory clothing outside the laboratory, e.g. in canteens, coffee rooms, offices, libraries, staff rooms and toilets.
6. Open-toed footwear must not be worn in laboratories.
7. Eating, drinking, smoking, applying cosmetics and handling contact lenses is prohibited in the laboratory working areas.
8. Storing human foods or drinks anywhere in the laboratory working areas is prohibited.
9. Protective laboratory clothing that has been used in the laboratory must not be stored in the same lockers or cupboards as street clothing.

Procedures

1. Pipetting by mouth must be strictly forbidden.
2. Materials must not be placed in the mouth. Labels must not be licked.
3. All technical procedures should be performed in a way that minimizes the formation of aerosols and droplets.
4. The use of hypodermic needles and syringes should be limited. They must not be used as substitutes for pipetting devices or for any purpose other than injection or aspiration of fluids from laboratory animals.
5. All spills, accidents and overt or potential exposures to infectious materials must be reported to the laboratory supervisor. A written record of such accidents and incidents should be maintained.
6. A written procedure for the clean-up of all spills must be developed and followed.
7. Contaminated liquids must be decontaminated (chemically or physically) before discharge to the sanitary sewer. An effluent treatment system may be required, depending on the risk assessment for the agent(s) being handled.
8. Written documents that are expected to be removed from the laboratory need to be protected from contamination while in the laboratory.

Guidelines for biosafety - laboratory operations

1. 'Learners' in the lab should be taught the 'dos and don'ts' of lab practices before they start the work.
2. Laboratories should be clean and items arranged in an orderly manner, free from materials that are not pertinent to the work.
3. Work surfaces must be decontaminated after any spill of potentially dangerous material and at the end of the working day.
4. All contaminated materials, specimens and cultures must be decontaminated before disposal or cleaning for reuse.
5. The cupboards/cabinets in the laboratory should be labeled /categorized properly for easy availability/identification of materials (chemicals /glassware /plasticware/or any other items) and items should be replaced properly.
6. Laboratory items/furniture (glassware/equipments/fumehood/ balances) have to be used for the identified purpose only.
7. Beverages and other food items should never be served / consumed inside the lab.

8. Inflammable liquids should be kept in separate containers with red paint marking (danger mark) and should be stored in fire- proof cabinets.
9. Acids and volatile solvents and digestion of biological samples should be handled strictly under fume hood.
10. Every lab should have first aid box with emergency eye washer. Researchers/staff should use aprons whenever/whereverthey handle hazardous materials.
11. Corrosive liquids should be handled properly using corrosive-resistant hand gloves.
12. Gas cylinders containing explosive gases should be kept outside the laboratories.

Guidelines for bio-safety- disposal of wastegenerated during research activities

Hazardous laboratory wastes need to be monitored and disposed properly. There is a need for bio-safety guidelines for proper disposal of waste generated in research activities. Materials for decontamination and disposal should be pre-sorted and placed in autoclavable plastic bags that are colour-coded- according to whether the contents are bio-degradable, non-biodegradable or sharps. Materials to be autoclaved or incinerated should be separated. No pre-cleaning should be attempted of any contaminated materials before it is decontaminated by autoclaving or disinfection. The procedure for handling/disposal for contaminated materials/wastes along with separation system and categories to be followed are listed as below:

1. Non-contaminated (non-infectious) waste can be disposed of as general waste.
2. Contaminated "sharps" – hypodermic needles, scalpels, knives and broken glass; these should always be collected in puncture-proof containers fitted with covers and treated as infectious. Disposable syringes, used alone or with needles, should be placed in sharps disposal containers and incinerated, with prior autoclaving if required. Sharps disposal containers must be disposed off before filled to the capacity. Sharps disposal containers must not be discarded in landfills.

   Contaminated material should be decontaminated by autoclaving and thereafter washing and reuse or recycling should be done (such as glass petri-plates, test tubes, glass wares used for microbiological work.
3. Biodegradable contaminated material (agar, culture broth, tissue culture slants, cotton and swabs, protein and fats) should be segregated in colour coded bags and decontaminated by autoclaving beforedisposal.

4. Non-biodegradable (plastic tubes and tips,organic recalcitrant chemicals, gloves, gel wastes, glass, media tins and bottles, rubber, aluminium foils etc.) should be segregated in colour coded bags before decontamination and disposal.
5. The research division/section/unit heads should train personnel working in various labs to manage the waste generated as per these guidelines. Extra sensitization is required for those working with disease pathogens and molecular biology work.
6. Concentrated stock solution of toxic chemicals such as heavy metals, nitrogenous, sulfurous and phosphorous compounds, reagents used for routine analysis and ethidium bromide should not be disposed in the wash basin. They should be deactivated before disposal. To avoid the need for deactivation, minimum quantity of stock solution as per requirement should be prepared, preserved properly and also be used fully without any disposal. Some examples of hazardous chemicals and their deactivation procedures are listed in Table 1.
7. All lab workers should wear appropriate safety gears while handling waste.
8. Research workers should handle gel containing ethidium bromide only with gloves. Such gels should be biosorbed with activated charcoal before disposal. The gloves used for the molecular work, should never be exposed outside the lab or allowed to contaminate other areas.
9. Glassware / plasticware used for micro- organisms should properly be sterilized. Hazards of microbial/other pathogen waste generated should properly be disposed by adopting good lab practices, for whichpersons involved should be trained.
10. Dead and post-experimental animals should be disposed of by incinerating them in pits covered with soil.
11. Based on the activity of hazardous waste, they can be identified as corrosive, inflammable, ignitable, reactive, toxic, infectious and radioactive. If such wastes are identified and their hazardous nature cannot be eliminated, special precautions as mentioned in the Table-1 need to be taken and if required separate guidelines would need to be developed.
12. Gloves (contaminated with ethidium bromide or other toxic substances) shouldnot be worn to answer the telephone or use other equipment in the lab.
13. Ethidium bromide solutions should be disposed of in the red waste containerlocated in the fume hood/staining bench of PCR lab. If hazardous waste other than ethidium bromide is put into these containers itshould be clearly marked

on the label attached.

14. Ethidium bromide spills should be cleaned up immediately, using properprecautions (wear gloves, put material used to clean up in bio-hazardous wastebins, wipe contaminated area down well with ethanol or detergent).
15. Used gels and gel waste should be discarded in the bio- hazardous waste bin, which is located beneath the gel electrophoresis benches.
16. Used gels and gel waste should not be put down any of the sinks.
17. Packing and transportation must followapplicable national and/or international regulations.

Laboratory Rules for shared laboratory

Each laboratory should develop a system of regulations most suited for managing smooth and ambient work flow.

1. Users should be provided with their own bench space when possible. It is the responsibility of the user to keep their bench space clean. When bench space is shared users should be respectful of the other user's space.
4. Users are responsible for their own dishes and spills. Clean up is NOT the responsibilityof the laboratory supervisors.
5. Dishes should NOT be left in the gel electrophoresis tank.
6. Broken or malfunctioning equipment should be reported to a laboratory supervisor IMMEDIATELY.
7. Instructions on how to use the thermocyclers, spectrophotometer, Imaging System and centrifuge are kept beside them.
8. All users must make entry and sign in the logbook prior to use.
9. Pipettes labeled for PCR use should be usedfor PCR only. **NO EXCEPTIONS.**

Table 1- Disposal of chemical wastes in a PCRlaboratory

Chemical	Method of deactivation
Ethidiumbromide	Filter the ethidium bromide solution through charcoal filtration. Pour filtrate down the drain. Place charcoal filter in sealed bag and place in bio hazardous waste box for incineration

Recombinant DNA	Deactivate recombinant DNA using DEPC or UV radiation or by autoclaving
Heavy metals	Minimum quantity of stock solution as per requirement should be prepared, preserved properly and also be used fully without any disposal because heavy metals cannot be biodegraded or eliminated
Nitrogenous, sulfurous and phosphorous compounds	Minimum quantity of stock solution as per requirement should be prepared, preserved properly and also be used fully without any disposal.
Reagents for routine analysis of chemical and biochemical parameters	Minimum quantity of reagents as per requirement should be prepared and should be used fully.
Volatile solvents and acids	Volatile solvents and acids should strictly be handled under fume hood. In the case of any leakage or spillage, all the doors and windows should be opened and the entry of persons should be restricted in the lab till complete escape of fumes. In the case of person having any accident with the spillage of acid, only water should be applied (please avoid the use of alkali) and should immediately be taken to nearby hospital. All the labs should have medical aids.

Guidelines for ethidium bromide waste management and disposal

Ethidium bromide is a compound used in many PCR laboratories for visualization of nucleic acid during gel electrophoresis applications. Ethidium bromide solution is incorporated into the electrophoresis gel as a dye for the DNA, RNA, or other molecules to be visualized. Ethidium bromide is mutagenic and moderately toxic and must be handled with care.

The powder form is considered an irritant to the upper respiratory tract, eyes, and skin, hence preparation of stock solutions and any operations capable of generating ethidium bromide dust or aerosols should be done in a fume hood to prevent inhalation. Nitrile

gloves, a lab coat and eye protection should be worn at all times, as with working with any hazardous material.

When working with ethidium bromide, minimize the potential for spills. Where practical, purchase pre-mixed stock solutions from chemical manufacturers *in lieu* of preparing solutions. If solutions of ethidium bromide must be prepared, consider performing this process in a fume hood.

- Spills must be addressed immediately; for small spills (<20 ml) of concentrations up to 10 mg/ml; absorb with paper towel and then treat the area with 70-95% ethanol/isopropanol and wipe up-all to be collected as hazardous waste. Repeat as necessary (if still fluorescing). Final rinse with soap and water (paper towels for this step were discarded into regular trash). DO NOT clean ethidium bromide spills with bleach solutions.
- When an ultraviolet light source is used in work with ethidium bromide, added caution is required. As a general rule, avoid exposing unprotected skin and eyes to intense UV sources.
- Ethidium bromide waste should not be poured down the drain, or thrown in the trash, unless the waste has been deactivated or filtered. The following are the recommended disposal procedures for ethidium bromide:
- Dry the gels under the hood in an open container before bringing to the waste room for disposal. Dilute aqueous solutions containing <0.5 mg/ml ethidium bromide can be filtered or deactivated. Concentrated solutions (>0.5 mg/ml) should be collected for disposal as hazardous waste. Similarly, solutions that contain heavy metals, organics, cyanides or sulfides should be collected and disposed of as hazardous waste. Filtering the aqueous ethidium bromide waste solutions, free of other contaminants, through a bed of activated charcoal is a relatively simple and effective method for removal of ethidium bromide. The filtrate may then be poured down the drain.
- Autoclaving chemical waste creates exposure to the chemicals as they heat and volatilizes; hence should not be attempted.
- Consider switching to less-toxic alternatives to ethidium bromide (SYBR Safe DNA gelstain) to reduce potential hazardous exposures in the lab.

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