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# Epidemiology of Hepatopancreatic Microsporidiosis caused by *Enterocytozoon hepatopenaei* in India

***K.P. JITHENDRAN, A. NAVANEETH KRISHNAN, V. JAGADEESAN, P. EZHIL PRAVEENA AND T. BHUVANESWARI**

## Introduction

Of late, shrimp farming has emerged as one of the most important segments in commercial fishing. This calls for more cautious and informed intervention in handling the threats faced by the industry. *Enterocytozoon hepatopenaei* (EHP), the causative agent of Hepatopancreatic Microsporidiosis (HPM) is one such major threat for the shrimp farming industry. It is an emerging microsporidian parasite for penaeid shrimp, which has been associated with growth retardation and significant losses in several shrimp farming countries in Asia.

More recently, the epizootics of *E. hepatopenaei* have been reported in India with several disease outbreaks. EHP has been found in the tubules of the shrimp's hepatopancreas and damages the organ which eventually may lead to abnormal metabolism and growth retardation in shrimp. This article examines the pathogen, disease, transmission, epidemiology, pathogenesis, diagnosis, treatment and control of Hepatopancreatic Microsporidiosis in penaeid shrimp in India.

## Pathogen

Microsporidia are obligate, intracellular, spore-forming endoparasites known to infect a wide range of eukaryotic hosts, both terrestrial and aquatic. Several microsporidians have been reported as pathogens of penaeid shrimp as well as finfish. *Enterocytozoon hepatopenaei* was first reported as an unnamed microsporidian from growth retarded black tiger shrimp, *P. monodon* from Thailand in 2004. However, this parasite was characterized in detail and taxonomy was elucidated only in 2009. EHP infects *P. monodon*, *P. vannamei* and is suspected to infect *P. japonicus*. The susceptibility of different life cycle stages is also not clear; although post larvae (PL-7 onwards), juveniles, growers and broodstock are observed to be affected by the parasite.

## Geographical distribution

The pathogen is now wide-spread with reported outbreaks in shrimp farming countries in south-east Asia including Vietnam, Thailand, Malaysia, Indonesia, China, India as well as Venezuela in South America (Fig. 1).

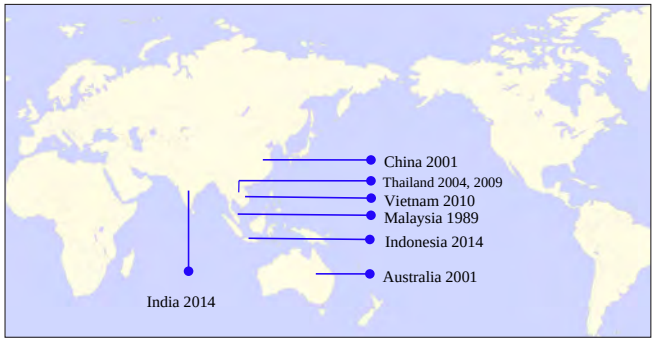


*Fig. 1. Global distribution of microsporidian suspected as EHP in shrimp farming*

In India, the disease emergence has been recorded 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. More recently, the geographical distribution of *E. hepatopenaei* has expanded; with sporadic reports on many shrimp farms in east and west coasts and even in inland aquaculture system being affected. (Fig. 2,

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Table 1). It appears that the disease entered India in the recent past through infected broodstock 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 after a three-fold increase in shrimp production since the introduction of *P. vannamei* in India during 2009. However, EHP has not been taken seriously due to low prevalence, and no substantial loss due to mortality as compared to WSSV. It is also unlikely that EHP has been a pre-existing disease in Indian shrimp aquaculture, as our data on other dominant species *P. monodon* never indicated its presence in India.

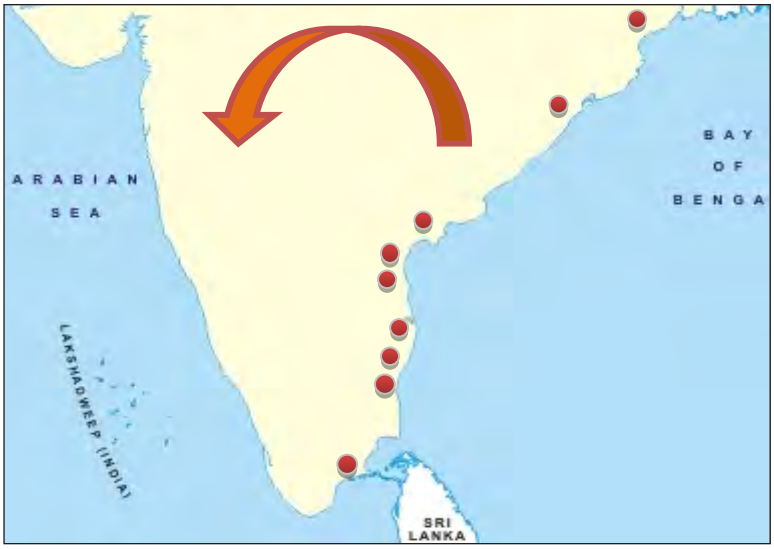


*Fig. 2. Geographical spread of Enterocytozoon hepatopenaei in India*

**Table 1. Published reports of Enterocytozoon hepatopenaei in cultured shrimp in India**

| Sl. No. | Year | Species | Culture system | Clinical signs reported | Geographic location |
|---|---|---|---|---|---|
| 1 | 2014<br>2015 | *P. vannamei* | Farm | Size variation, Slow growth syndrome | Tamil Nadu, Andhra Pradesh, Odisha, and West Bengal |
| 2 | 2014<br>2015 | *P. vannamei*<br>*P. monodon* | Farm | Size variation, Slow growth syndrome | Tamil Nadu, Andhra Pradesh, Odisha |
| 3 | 2014<br>2015 | *P. monodon*<br>*P. vannamei* | Farm | Slow Growth Syndrome,<br>Secondary bacterial infections | Tamil Nadu, Andhra Pradesh, Odisha |
| 4 | 2015 | *P. vannamei* | Farm | White feces syndrome,<br>Growth retardation | Tamil Nadu, Andhra Pradesh |
| 5 | 2016 | *P. vannamei*<br>(post larvae) | Hatch-ery | Black spots on post larvae | Tamil Nadu, Andhra Pradesh |
| 6 | 2016 | *P. vannamei* | Farm | Stunted growth | Tamil Nadu, Andhra Pradesh |
| 7 | 2016 | *P. vannamei* | Farm | NA | Tamil Nadu |
| 8 | 2016<br>2017 | *P. vannamei* | Farm | Size variation,White faeces syndrome,Loose shell | Tamil Nadu, Andhra Pradesh |
| 9 | 2016 | *P. vannamei* | Farm | White feces syndrome, stunted growth | Andhra Pradesh |
| 10 | 2017 | *P. vannamei* | Farm | NA | Maharashtra |
| 11 | 2018 | *P. vannamei* | Farm | Retarded growth | Andhra Pradesh |

### The disease

The parasite does not directly cause mortality in shrimps but known to cause severe growth retardation and hence causes high production losses in many Asian countries. The disease condition caused by the shrimp microsporidian parasite *Enterocytozoon hepatopenaei* is Hepatopancreatic Microsporidiosis (HPM), and is confined to the shrimp hepatopancreas.

The parasite replicates within the cytoplasmic area of tubule epithelial cells in the hepatopancreas, causing severe necrosis of hepatopancreatic tissues and growth retardation and overall reduced farm production. There are no specific clinical signs for EHP infection, but reported to be associated with size variation, stunted growth and/or white faeces syndrome and no mortality. EHP infects the hepatopancreatic tubules limiting the assimilation of nutrients and results in growth retardation.

Size variation may not be apparent during early days of culture (DOC) but farmers report EHP infected shrimp exhibiting white faeces syndrome as early as 23 DOC, often with a phase of recovery (Fig. 3, A&B).


*Fig. 3. EHP affected shrimp pond showing white faecal threads floating on pond water surface (A); Shrimp showing normal hepatopancreas and floating strands of white faeces hanging from anal portion (B) and, the hepatopancreas wet smears showing developing stages of microsporidian in the hepatopancreatic tubules (C)*

However, white faeces syndrome is not a consistent feature as compared to slow growth and size variation in experimental infections. Infected post larvae maintained in laboratory conditions never had white faecal syndrome but exhibits size variation, slow growth or both. Severe infections by EHP can increase the susceptibility for other bacterial infections like *Vibrio* spp. in shrimp farms and could manifest mild mortality.

EHP infection in pond conditions may happen continuously leading to progressive damage to the hepatopancreas with different degree of infection (Fig. 3, C); hence the signs of EHP appear as size variation, overall slow growth (Fig. 4) and loss during harvest. In hatcheries, if post larvae grow unusually slower and show size variation EHP can be suspected.

*Fig. 4. EHP affected Penaeus vannamei shrimp showing size variation at the time of harvest*

### Biology and transmission

The lifecycle and underlying transmission mechanism of this microsporidian was poorly understood. In general, the life cycles of microsporidia have three phases: infective, proliferative, and sporogonic.

The infective phase is the mature spores, which are ovoid in shape (1.7 x 1 m in fresh preparation) and containing 5-6 coils of the polar filament within, appears the most important diagnostic phase of microsporidia (Fig. 5).

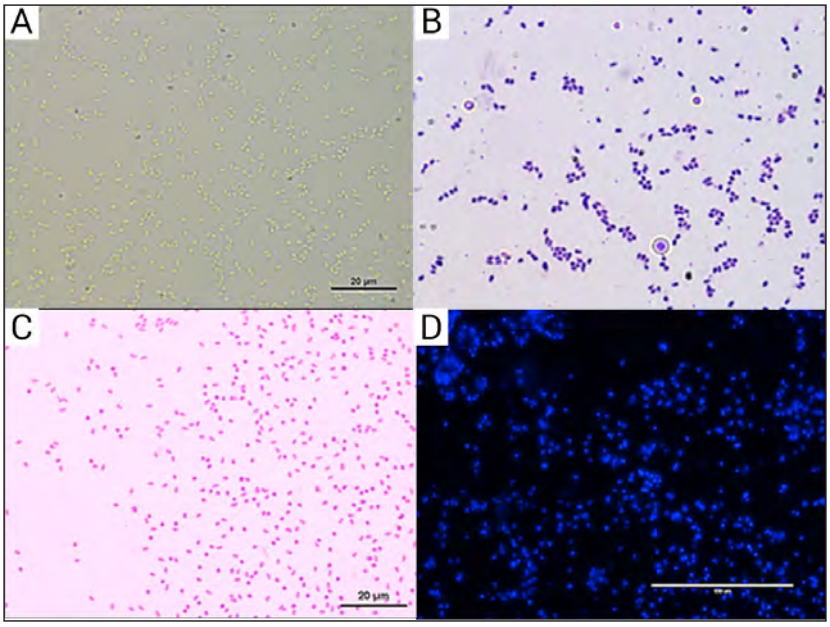


*Fig. 5. Microscopic detection of purified spores of Enterocytozoon hepatopenaei from Penaeus vannamei: A-Fresh spores, B-Giemsa stain, C-Phloxine B stain, D-Calcofluor White stain. 1000 x (digitally enlarged)*

The spores released in the environment are activated when external factors are suitable, and infect host cells by the extrusion of the polar filament containing sporoplasm inside. The proliferative phase includes sporoplasm and meronts in host cells, without chitin and a fixed form, are difficult to observe by light microscopy. The sporogonic phase includes sporonts, sporoblasts and developing spores, in which chitin and proteins gradually accumulate along the spore wall.

Due to the small size and intracellular location, most structures and developmental stages are visualized either by transmission or scanning electron microscopy. Polar filament precursors and other spore organelles formed within the sporogonal plasmodium and packaged into pre-sporoblast units prior to budding of sporoblasts to the host-cell cytoplasm. Once complete, the mature spores are released though faeces to the environment.

*Enterocytozoon hepatopenaei* morphologically resembles other microsporidian, but is transmitted directly from shrimp to shrimp by the oral route. Transmission occurs readily among individuals through cohabitation (spores released into the water through shrimp faeces) and as well as through healthy shrimp cannibalizing those that were moribund or dead due to infection. Other transmission route (trans-ovum) is poorly understood though suspected. So far, no secondary hosts are known to be involved in transmission of *E. Hepatopenaei* but carrier roles for live feeds (polychaete, mussel, clams etc.) are suspected. Laboratory infection has been successful through cohabitation and through oral route by consumption of infected tissue (cannibalism, predation etc.) and there exist no sufficient data to prove possible vertical transmission.

**Laboratory diagnosis**

Shrimp hepatopancreas is the target organ for the detection of EHP infection, primarily because of development within the cytoplasm of hepatopacreatic tubular cells. Mature spores are released through faeces, and hence faecal threads can also be used for non-lethal screening of precious SPF broodstock.

EHP infection can be detected by demonstrating spores (1.7 by 1 µm) in light microscopy of fresh or stained faecal and hepatopancreas tissue smears (Fig. 5, A-D) or by histology of hepatopancreas tissue sections (Fig. 6, A&B). The microscopic techniques may be enhanced by concentration and differential centrifugation of spores to detect light infections or even by special staining techniques.

Molecular based methods, such as polymerase chain reaction (Fig. 7) targeting 18s small sub unit rRNA (SSU-PCR), spore wall protein (SWP-PCR) and EHP-specific -tubulin gene, using DNA extracted from hepatopancreas tissue, faeces and whole post larva (PL) are the most commonly used diagnostic method for EHP detection in shrimps. Other molecular methods such as in situ-hybridisation, real time PCR and LAMP may also be the choice available for EHP detection in specialised laboratories.

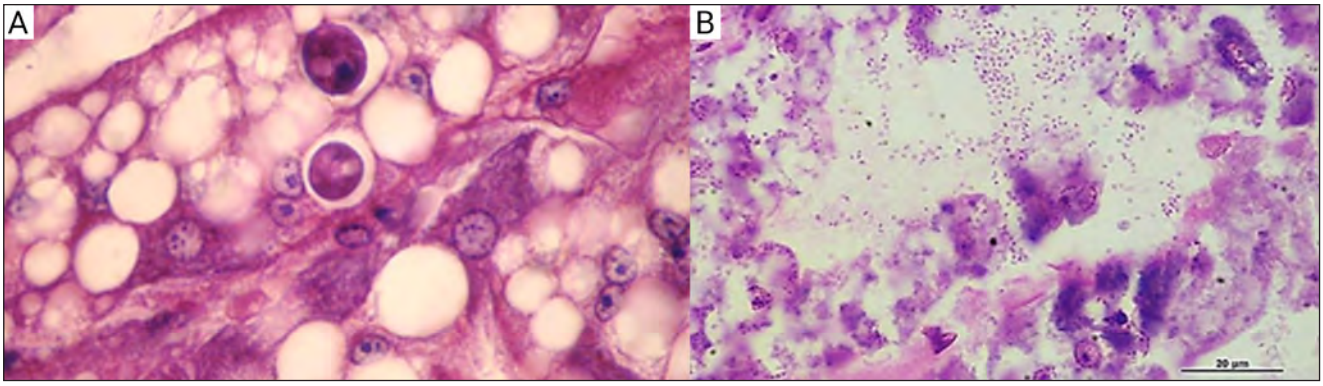


*Fig.6. Histological section of shrimp hepatopancreas showing early and late plasmodia stages (A), and the necrosis of hepatopancreas and packed microsporidian spores in the lumen (B)*

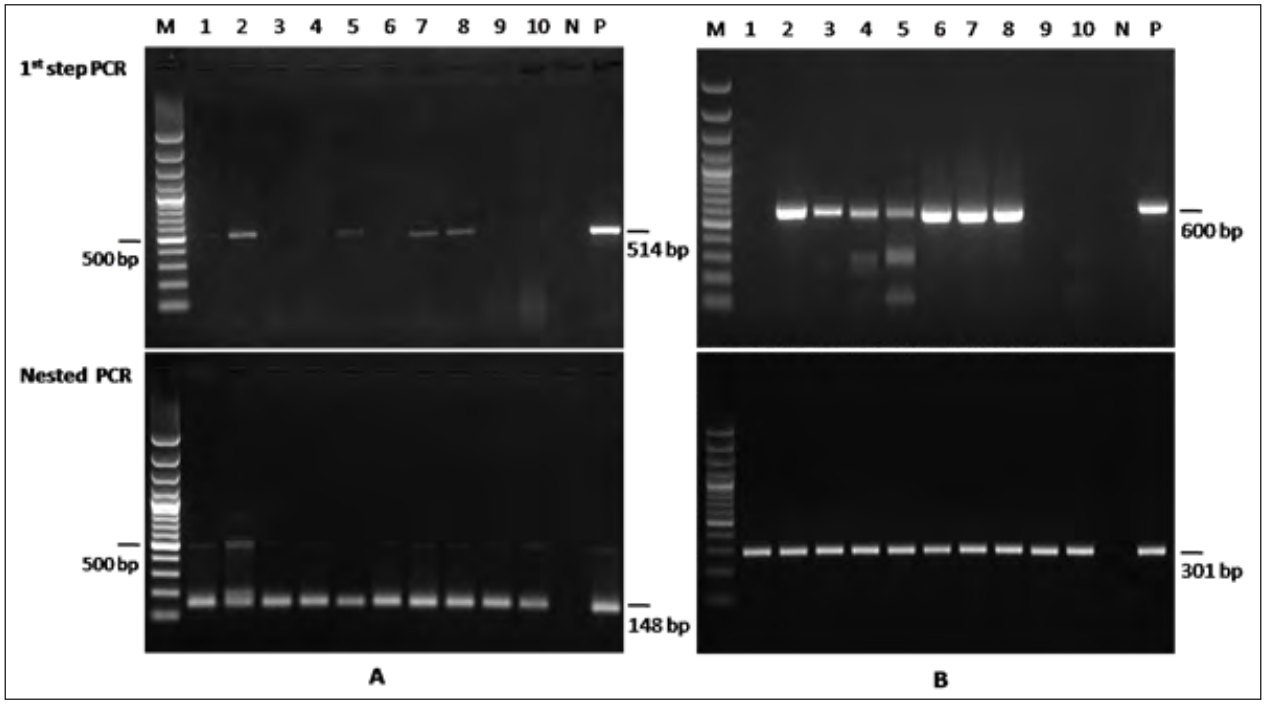


*Fig. 7. Nested PCR detection of Enterocytozoon hepatopenaei in shrimp samples A- PCR targeting spore wall protein gene, B- PCR targeting 18s subunit (SSU) rDNA gene*

**Prevention and control**

It has been found that EHP can be transmitted directly from shrimp to shrimp through feeding of EHP-infected hepatopancreas and cohabitation making control a difficult preposition in the culture ponds. Further, the microsporidian spores seem to be resistant to environmental condition and can persist in shrimp ponds and carry-over infection to next culture. This could lead to severe growth retardation in shrimp and massive production loss.

Today, there are no efficient treatments available for the control of EHP infection, as is the case with humans and animals. Spore activity was found to be inhibited by freezing at -20°C for at least 2 h and storage at 4°C. Low doses of chlorine, $KMnO_4$ and ethanol was found to be inhibitory to spore activity. The ponds having history of disease should be adequately dried (for 3-4 weeks) after harvest and residual spores in the soil may be inactivated by physical and chemical methods. In grow-out system, stocking EHP-free seeds and proper pond preparation between subsequent crops is of paramount importance to ensure that EHP spores along with the carriers were destroyed. Pond-drying followed by application of burnt lime or shell lime (CaO) on the pond bottom will help get rid of EHP spores from the pond sediment.

Better management practices (BMPs) are the only way to prevent the epidemics of EHP. However, farm level biosecurity measures are inadequate due to dominance of small and marginal farms in this sector.

Hatcheries may monitor the incoming shrimp brood stock for EHP by using faecal sample. Use of EHP-free live feeds and by following complete disinfection of hatchery facility with 2.5% sodium hydroxide solution (with minimum contact time-3 hrs) followed by drying the facility for a week, then rinsing with acidified chlorine (200 ppm) in between the production cycle may be ensured.

**Way forward**

The rapid spread of HPM in India within a span of three years is mainly due to lack of awareness, as the disease does not cause mortality compared to viral diseases. High density of susceptible host and serial infections of individual animals resulted in 'mass cultivation' of microsporidian spores in farms. Incipient nature and persistence of microsporidian spores in environment along with faeco-oral route of infection eventually favours this parasite to multiply in rearing ponds.

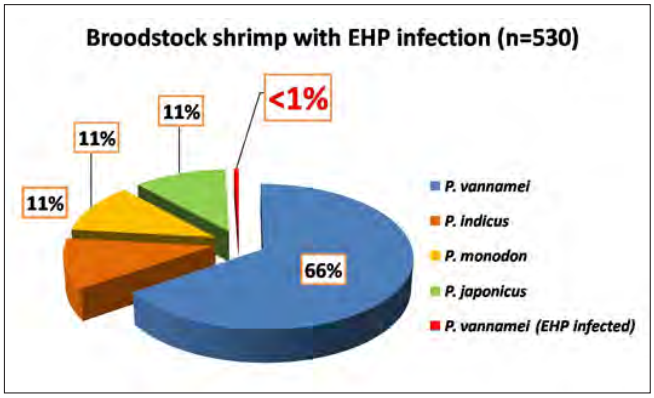


*Fig. 8. Enterocytozoon hepatopenaei infection pattern in shrimp broodstock samples by nested PCR targeting spore wall protein gene [Only < 1% of the SPF P. vannamei tested were found positive, while all indigenous species were negative for EHP by non-lethal screening using faecal samples]*

The exact source and nature of microsporidian transmission in Indian scenario is still not clear, though imported brood stock of *P. vannamei*, artemia cysts and post-larvae meant for stocking has been found positive for *E. hepatopenaei* during 2016 onwards (unpublished data).

Infection in hatchery may be through SPF brood stock or life cycle stages or live feeds. Rearing of SPF brood stock in non-bio secured facility and use of pond-raised brood stock may also facilitate massive infection. Hence a pre-screening of post-larvae for EHP is one option for farmers before stocking in ponds.

Care should be taken while sourcing seeds for introduction to newer areas, high density culture systems, viz., biofloc technology as the infections get magnified as the culture advances. Clear understanding on prevalence of EHP in wild caught broodstock of indigenous species of shrimp such as *P. indicus, P. monodon* and *P. japonicus* (Fig. 8) are needed. Though *P. monodon* is reported to be susceptible to EHP in India, this appears to be acquired at farm level.

No cases of EHP have been reported in traditional farming areas of western coast till date and we do not have the data on pre-existence of this infection prior to 2014 (Table 1). Constraints in dealing with this disease include, limitation of diagnostic techniques, lack of proper diagnostic facility and skilled man power.

Existence of cryptic carrier hosts in the system and availability of wide host range in new environment also could pose new issues in farming to deal this newly emerging problem in Indian aquaculture. Diagnosis of EHP in SPF brood stock of *P. vannamei* and post larvae in local hatcheries and other imported aquaculture feed viz., Artemia cysts (unpublished data) is a matter of great concern in shrimp aquaculture sector and warrant close look and policy interventions.

**Conclusion**

In shrimp aquaculture, a microsporidian parasite *E. hepatopenaei*, the causative agent of Hepatopancreatic Microsporidiosis had resulted in significant economic losses, in many shrimp farming nations.

The infection is not associated with any visible clinical signs or mortality in shrimp, it causes growth retardation to the extent of 15-30%. The EHP infection may be suspected with the occurrence of unusually inconsistent or retarded growth, or unusually high FCR in the absence of other gross signs of disease. Shrimp hepatopancreas is the target organ for EHP infection.

EHP infection can be transmitted horizontally through cohabitation and oral route and possibly vertical transmission. Geographic spread of EHP in shrimp farms and increasing incidences in brood stock, post larvae and other aquaculture input is a matter of great concern for future. The studies on the host range, biology, mode of transmission and control methods are needed on priority basis.