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Review Article

Microsporidian *Enterocytozoon hepatopenaei* (EHP) in Shrimp and Its Detection Methods

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ABSTRACT:
In shrimp farming, disease is a serious concern that affects production globally. *Enterocytozoon hepatopenaei* (EHP) was rarely found till 2009 in the black tiger shrimp *Penaeus monodon*. But EHP infection became more widespread during mid-2010 in Asia and affected the most common cultured shrimp species *Penaeus vannamei*. *Enterocytozoon hepatopenaei* (EHP) affects the hepatopancreas of the shrimp and causes Hepatopancreatic Microsporidiosis (HPM). HPM is a disease that delayed host growth and development. HPM is more difficult to manage than other infectious disease due to a lack of sufficient knowledge about its reservoirs and mode of transmission. This study summarized the life cycle of EHP spore and the molecular approaches used by them as an obligate intracellular parasite. It also analyzes existing and novel approaches for the diagnosis, as the majority of the present work on EHP concentrates on that area. We outline the current understanding of EHP infection and transmission dynamics, as well as currently recommended, feasible control strategies being used to restrict its harmful influence on shrimp farming. We also highlight the critical knowledge gaps that must be addressed immediately.

Keywords: *Enterocytozoon hepatopenaei, Penaeus vannamei,* Hepatopancreatic microsporidiosis, Transmission dynamics

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INTRODUCTION

Aquaculture is a rapidly growing business that contributes considerably to the national economy by exporting aquaculture products and providing food security to the country. Until 2009, most brackish water aquaculture growth in India was centered on tiger shrimp and *Penaeus monodon* cultivation. However, tiger shrimp aquaculture has declined since 1995, owing mostly to viral infections (FAO, 2014). In 2009, CAA (Coastal Aquaculture Authority of India) permitted the introduction of exotic species, *P. vannamei*

(Pacific white leg shrimp), as an alternative culture species in India (Gomez-Casado et al., 2011). *P. vannamei* was rapidly accepted by farmers and became the main farmed species because of its SPF status, fast growth rate, and culture feasibility across a wide range of salinity (Alavandi et al., 2017). With the introduction of Pacific white shrimp, the Indian aquaculture sector has seen significant expansion in shrimp output through aquaculture during the last five years. In 2016-17, seafood production amounted to an all-time high of US$ 5.78 billion, up from 9, 45,892 tons and $4.69 billion the previous year (Kumudini, 2017). Shrimp exports climbed by 16.21% in terms of quantity and 20.33% in terms of value. Frozen shrimp is the most important export item, accounting for 38.28% of all exports and 64.50% of total revenues. During 2016-17, total shrimp exports were 4,34,484 MT worth USD 3,726.36 million. *Litopenaeus vannamei* export increased 28.46 percent in quantity from 2,56,699 MT to 3, 29,766 MT in 2016-17 (Namburu and Kunda, 2019) . However, the rising tendency of intensification and commercialization has increased disease outbreaks and is a serious restriction on the industry's sustainability. Aquatic animals are routinely translocated across nations to increase aquaculture productivity and species diversity (Lightner, 2011). The introduction of new illnesses is an inherent concern of such transboundary movement of live aquatic animals. Disease-related losses in aquaculture have made increasing output difficult. Such defeats have occurred in countries all around the world. Diseases in brackish water shrimp aquaculture nations were predicted to cost 3019 million USD between 1987 and 1994. WSD alone is believed to have cost more than $6 billion since its inception in 1992 (Melena et al., 2012). Since before 2009, the newly developing diseases Early Mortality Syndrome, more specifically Acute Hepatopancreatic Necrosis Disease, *Enterocytozoon hepatopenaei*, have resulted in yearly economic losses of more than US$ 1 billion (Sánchez-Paz, 2010). These research studies highlight the mortality and devastation caused by disease outbreaks in the aquaculture industry (Sriurairatana et al., 2014). Due to less treatment options for EHP, only early disease detection and adoption of health management practices will lessen the severity of the problem (Gunalan et al., 2014). This report provides a broad overview of disease incidence in shrimp aquaculture.

MICROSPORIDIOSIS HEPATOPANCREATIC

Enterocytozoon hepatopenaei causes hepatopancreatic microsporidiosis (HPM) (EHP). It was discovered in 2004 as an unidentified microsporidian in growth delayed huge or black tiger shrimp *P. monodon* from Thailand. It was later detailed described and identified in 2009 (Tourtip et al., 2009). It also has substantially smaller spores (about 1 µm long) but eventhough it can affect both *P. vannamei* and *P. monodon*. EHP spores have the potential to transmit directly and immediately from shrimp to shrimp via cannibalism and cohabitation (Walker and Winton, 2010). EHP is restricted to the tubule epithelial cells of the shrimp hepatopancreas (HP) and causes only slowed development (Tang et al., 2007).

MICROSPORIDIA

Microsporidia are unicellular obligatory parasites that infect a wide spectrum of eukaryotic hosts. These are spore-forming intracellular parasites that may infect both vertebrates and animals. The polar tube present in the spore utilized to penetrate host cells is a distinguishing feature of microsporidia (Thitamadee et al., 2016). The parasite develops in the host cell's cytoplasm through nuclear proliferation and spore production (sporogony), while certain species have been reported to grow in the nucleoplasm of the host. They are found in abundance in nature, with over 1200 species identified (Stentiford et al., 2013). Microsporidia's involvement in terrestrial hosts has been studied, ranging from infecting nuisance and helpful insects to essential human parasites. However, aquatic hosts are known to infect about half of the documented microsporidian taxa (Keeling and Fast, 2002). The parasite was firstly discovered in *Penaeus monodon* in 2009. It is not possible to detect EHP infection by visual inspection in shrimp because there are no clear symptoms except growth retardation and white feces syndrome (Tourtip et al., 2009). The shrimp hepatopancreas is the EHP's target organ; being the animal's powerhouse, infection in this organ has serious consequences.

TAXONOMY

Phylum: Microspora (Sprague, 1977)
Class: Microsporea (Perrier and Delphy, 1963).
Order: Microsporida (Balbiani, 1882).
Family: Enterocytozoonidae (Cali and Owen, 1990).
Genus: Enterocytozoon (Desportes et al., 1985).

Microsporidia's scientific categorization had changed throughout time. Phylum microspora was once thought to be a protozoan based on traditional identification methods. But advance genetic investigations such as ribosomal RNA sequencing have shown that the phylum microspora should be placed under the Kingdom Fungi (Chayaburakul et al., 2004). The diseases, which are members of the *Enterocytozoonidae* family, infect hosts in a variety of trophic levels in marine, freshwater, and aquatic settings including hyperparasitic copepods, decapods, fish, and humans (Rajendran et al., 2016). Apart from sharing the same ssRNA gene, pathogens in Clade VI have comparable physical characteristics (such as the capacity to invade the host's nucleus) and attack gut epithelial cells. In immune-compromised individuals, *Enterocytozoon bieneusi* is a frequent pathogen (infection in AIDS cases) that was closely linked to the marine crab pathogen *Enterospora canceri* of the family *Nucleospora*,, *Paranucleospora theridion* and also with the marine shrimp pathogen *Enterocytozoon hepatopenaei* (Tourtip et al., 2009). The name *Enterocytozoon hepatopenaei* was proposed based on ultra structural features that is similar to the family Enterocytozoonidae (Stentiford et al., 2013). The intranuclear rather than cytoplasmic development distinguishes them from *Enterospora* and *Nucleospora*. However, because *E. hepatopenaei* shared several characteristics with *E. bieneusi*, such as the mean spore size in a marine habitat (in the case of *E. hepatopenaei*) and a 16% difference in the sequence of 18 SSU rRNA, it was given the name *E. hepatopenaeii* and placed in the Enterocytozoonidae family (Al Rwahnih et al., 2015) .

BIOLOGY

The microsporidian spore germination is one of most remarkable and stunning subcellular events. In most cases, spore germination is triggered by an environmental trigger that varies by species and habitat. Changes in pH, osmolarity, dehydration, the presence of anions or cations, or exposure to UV radiation are all examples of physical and chemical stimuli that can cause spores to germinate in vitro (Keeling and Fast, 2002). The spore germination process began with general spore swelling, followed by specialized swelling of the polaroplast and posterior vacuole, which increased spore osmotic pressure. The anchoring disk ruptures and the polar filament is discharged via eversion as a result of the spore's internal pressure and sporoplasmic membrane disintegration (Stentiford et al., 2013). The length of the discharged polar tube might be between 50 and 500 µm. In less than two seconds, the complete germination process takes place. The discharge tube can impact and puncture the membrane of a prospective host cell if it is nearby. Due to the constant pressure within the spore, the sporoplasm is driven down the polar tube after it has been completely empty (Watson et al., 2015). If a polar tube penetrates a host cell, the parasite's sporoplasm emerges from the tube and enters the host's cytoplasm, infecting the host without the host recognizing the parasite as a foreign intruder as shown in figure 1.

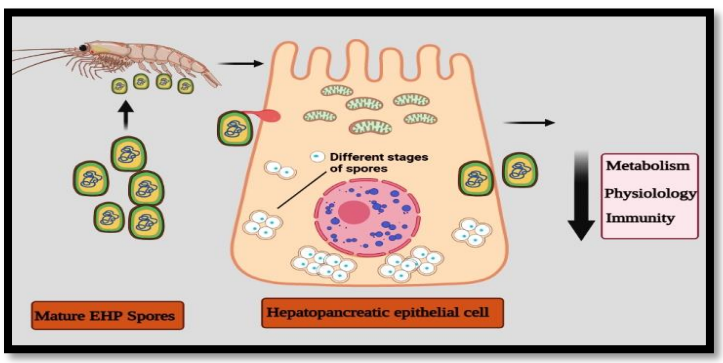

Figure 1: Show germination of EHP spores in hepatopancreatic cell

LIFE CYCLE

Microsporidia have three stages in their life cycle: infective, proliferative, and sporogonic**.** The parasite is known as a meront after it enters the host cell, and it passes through a development and division stage with a lot of interspecies variability. The parasite is frequently surrounded by host organelles such as the endoplasmic reticulum (ER), nuclei, or mitochondria as the host cell reorganizes around it (Arunrut et al., 2016). Host cells can also change shape and size, typically growing as a result of the microsporidian parasite xenoma**.** As a result, the host cell enlarges and undergoes several rounds of nuclear division, resulting in a massive plasmodium loaded with parasites, with mature spores at the core and earlier stages spreading outwards of xenoma (Aldama-Cano et al., 2018). The separation of diplokaryotic nuclei marks the start of sporogony in certain species, whereas meiosis marks the start of sporogony in others. Its stage of the life cycle marked by the number of sporoblasts that may vary from two (bisporous) too many (multisporous) (polysporous) as shown in fig 2. The extrusion apparatus of spore begins to form after cell division. The cells shrink in size and the chitinous endospore layer develops as the extrusion mechanism nears completion and the sporoblasts approach maturity (Edelaar and Bolnick, 2019). The spores of *E. hepatopenaei* are ovoid in shape and have 5–6 visible polar filament coils. The mature spores are expelled after the process is complete. *E. hepatopenaei* spores are introduced into pond water by shrimp through their white excrement, where they might infect other species.

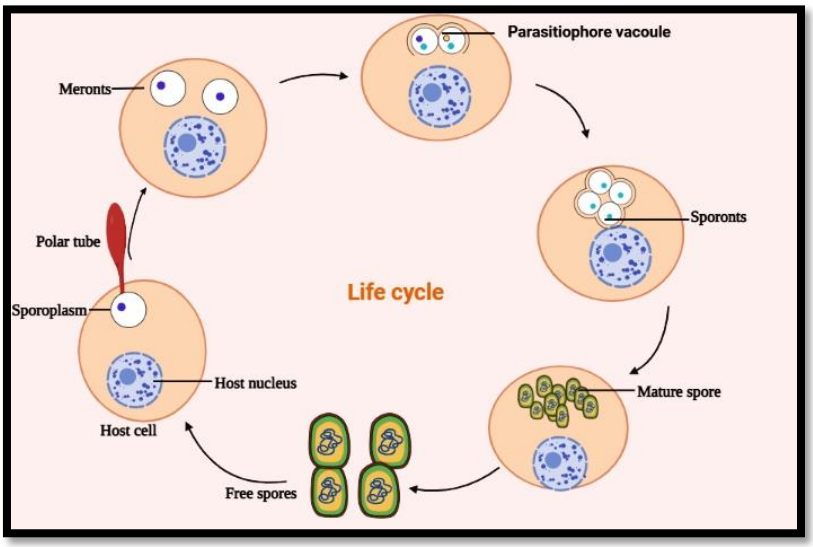

Figure 2: Show life cycle of EHP spores

TRANSMISSION

The microsporidian parasite's virulence is largely determined by the mechanism of transmission. Horizontal transmission relies on a large number of accessible parasites (especially when spores are introduced into water), which leads to parasite proliferation and virulence being selected for Vertical transmission, on the other hand, necessitates host reproduction and so favors virulence reduction (Karthikeyan and Sudhakaran, 2020). Microsporidian parasites such *as Edhazardia aedis* in mosquito host can convert from low-virulence vertical transmission to high-virulence horizontal transmission in well-resourced (reproducing) hosts. Vertical transmission is typically only possible through female hosts (sperm size prevents infection), which favors reproductive manipulation to increase the number of infected animals (Stentiford et al., 2019). Microsporidia are the only eukaryotic parasites that can produce sex ratio distortion by killing or feminizing males (for example, *Amblyospora californica* causes benign infection in female mosquito larvae) (Dunn and Smith, 2001). *Nosema granulosis* disrupts the development of the

androgenic gland in juvenile *Gammarus duebeni*, resulting in feminization and subsequent transmission (Yang et al., 2011). *E. hepatopenaei* is easily spread by cohabitation and by cannibalizing those that were moribund or dead from infection as shown in fig 3.The vertical transmission pathway (trans-ovum) of *E. hepatopenaei* is poorly characterized. So far, no secondary hosts have been identified as being involved in *E. hepatopenaei* transmission (Jithendran et al., 2019).

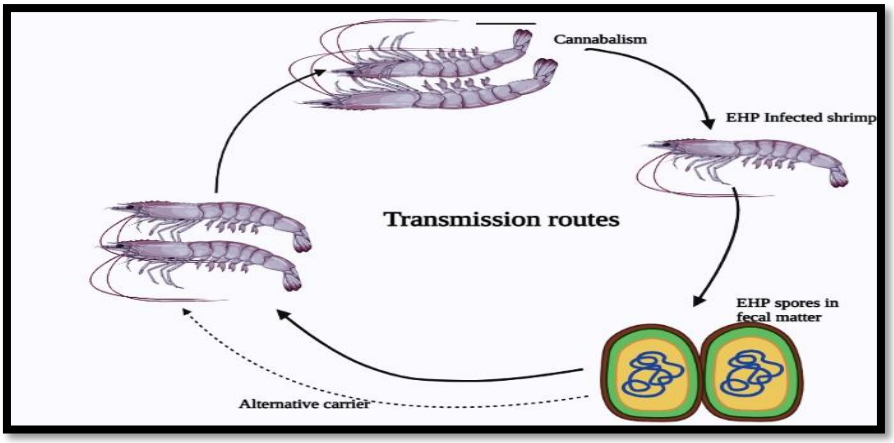

Figure 3: Show different transmission routes of EHP spores

METHODS OF DETECTION

Clinical manifestations: There are no distinguishing gross indications of EHP infection. In the absence of other disorders, the infection may be indicated by the appearance of particularly slowed growth with a lowered feed conversion ratio (FCR). Microscopic and/or molecular approaches must be used to confirm EHP.

Microscopy: the mounts of Hepatopancreatic tissue and fecal strands are studied under light microscopy by Geimsa staining (Tourtip et al., 2009; Tang et al., 2007). The presence of distinctive spores under oil immersion (100 x) under a light microscope is used to make the diagnosis. The spores are quite tiny (1.1±0.2 x 0.6-0.7±0.1 µm) and have a polar filament with 4-5 coils (Alavandi et al., 2017). This approach may not be effective in identifying spores that are present in small numbers. However, the relationship between parasite burden and growth retardation must be determined.

Histopathology: Histopathology, above all other diagnostic procedures, is highly important in proving the true infection, i.e., the presence of a pathogen with successive pathological alterations in the cells and tissues. HP has been identified as the target organ for HPM in shrimp (Bell and Lightner, 1988). As a result, HP is collected in ten times the volume of Davidson's fixative. Spore development is only detected in the cytoplasm of B cells. In the absence of spores, extensive infection of the HP medial and proximal tubule epithelial cells is seen. Free spores produced by lysed cells can occasionally be visible in tubule lumens (Alavandi et al., 2017). In certain cases, spore production is limited or has not yet begun, making conclusive identification difficult or impossible. In such circumstances, PCR detection is advised (Hou et al., 2018).

Serologic tests: Human microsporidiosis is diagnosed by serological techniques such as immunoperoxidase, immunofluorescence, and enzyme-linked immunosorbent assay (ELISA) and Western blots (Joseph et al., 2005). However, because no comparable assessments have been reported, the sensitivity and specificity of these approaches for detecting antimicrosporidial antibodies remain unknown. These approaches are not directly relevant to shrimp since they lack an antibody-producing defense system.

Polymerase chain reaction (PCR): Polymerase chain reaction is a method that selectively amplifies a specific targeted region of DNA by using specified primers. The word PCR comes from one of its major components, a DNA polymerase that is utilized to amplify a

fragment of DNA by in vitro enzymatic replication (Liu et al. 2018). As the PCR process advances, the DNA produced serves as a template for replication, which is then exponentially amplified as shown in fig 4 (Kono and Arakawa, 2019). PCR has become a crucial technology in medical and biological research in recent years. Representative shrimp samples are obtained in 95% ethanol for PCR detection of EHP. PCR targeting the SSU rRNA gene (SSU-PCR) is used to diagnose EHP, due to cross-reactivity of the SSU-PCR primers with DNA (Tourtip et al., 2009; Tang et al., 2007). A nested PCR technique targeting spore wall protein of EHP gene is selected to solve this problem. It yields no false positives from closely similar microsporidia (Chen et al., 2013). The new SWP-PCR method is 100 times more sensitive than SSU-PCR. This method may be utilized for EHP in HP, feed, feces, and ambient materials because of its higher specificity and sensitivity as summarized in table 1 below.

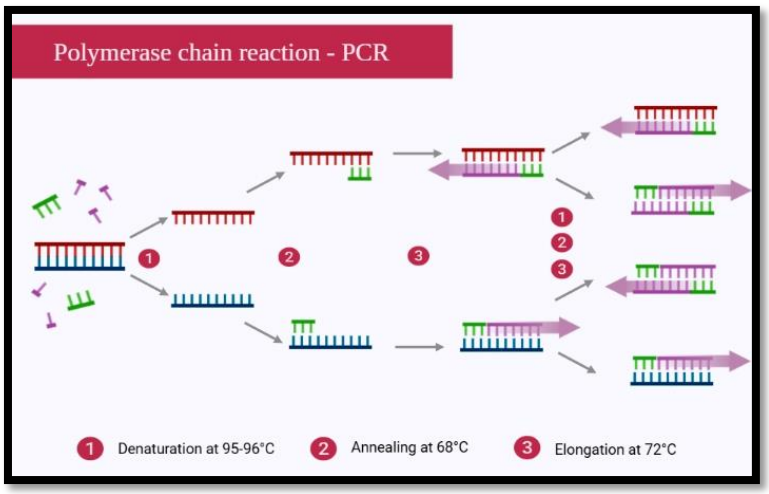

Figure 4: Show PCR reaction

Table 1: Comparison of detection methods via their accessibility, sensitivity, specificity, and quantitative aspects A-D (A = Best; D = Worst)

S. N.	Method	Accessibility	Sensitivity	Specificity	Quantitative	References
1.	**Clinical methods**					
a.	Light microscopy	B	D	C	C	Tourtip et al., 2009; Tang et al., 2007
b.	In situ hybridization	D	C	B	B	Sanguanrut et al., 2018
2.	**Molecular Methods**					
a.	qPCR	D	A	B	A	Liu et al., 2018
b.	One-step PCR	C	B	B	C	Koiwai et al., 2018
c.	LAMP	D	A	B	A	Kanitchinda et al., 2020
d.	Nested PCR	A	A	B	C	Jaroenlak et al., 2016
e.	RPA	A	A	B	C	Zhou et al., 2020

PCR: Polymerase Chain Reaction; LAMP: Loop-Mediated Isothermal Amplification; RPA: Recombinase Polymerase Amplification.

CONCLUSIONS AND RESEARCH DIRECTIONS FOR THE FUTURE

EHP has evolved as one of the most serious infections in Asia for the exotic, *Penaeus vannamei*. This is in contrast to its prior record of low-level infections and cryptic nature in cultured giant tiger shrimp *P. monodon*. It can create significant infections, which can lead to development slowdown and perhaps paralysis, as well as other ailments. As with other major shrimp diseases, the greatest line of defense against EHP is prevention.

We outline the following suggestion, based on key issues raised in this study that significantly lower the frequency of EHP infections in shrimp farming systems:

- Live feeds should be avoided or if used they should be pasteurized or frozen for 48 hours at 20 °C to kill EHP spores infective stages.
- To reduce the risk of defecation, female broodstock should be starved for 4–6 hours before being moved to spawning tanks.
- To eradicate EHP life stages that may have developed from broodstock faeces in spawning tanks, eggs or nauplii should be adequately washed with clean hatchery water before stage N6.
- Before stocking ponds (especially those previously infected with EHP), quick lime (CaO) treatment is recommended to stimulate spore germination and inactivation, lowering infection pressure.
- After stocking EHP-negative PL, the pond's shrimp should be checked and tested for EHP on a regular basis utilizing molecular methods.
- If pond sampling confirms early detection of EHP pond management should be changed to reduce horizontal spread.

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