Latin American Journal of Aquatic Research, 51(1): 1-11, 2023
DOI: 10.3856/vol51-issue1-fulltext-2693

*Review*

# Identification techniques to prevent the current emerging disease hepatopancreatic microsporidiosis in white shrimp *Penaeus vannamei*: an overview

**José Cuauhtémoc Ibarra-Gámez[1], María Fernanda Rubio-García[1]**
**Ricardo Sánchez-Díaz[1], Ramón Casillas-Hernández[1] & Diana M. Martínez-Ibarra[1]**
[1]Instituto Tecnológico de Sonora, Ciudad Obregón, Sonora, México
Corresponding author: Diana M. Martínez Ibarra (diana.martinez@itson.edu.mx)

**ABSTRACT.** Aquaculture combines techniques for breeding and harvesting aquatic organisms used in shrimp production. It is important as a source of income and for generating foreign exchange in the regions where it is practiced. However, the timely detection of diseases continues to be a great challenge for aquaculture and fisheries production. In recent years, *Enterocytozoon hepatopenaei* (EHP) has emerged as a major pathogen of the Pacific white shrimp *Penaeus vannamei* in many Asian countries (Vietnam, China, Indonesia, Malaysia, Thailand, India, and Korea). In Latin America, only in Venezuela, and to date, there is no report of its presence in Mexico. It is transmitted directly from shrimp to shrimp by oral or fecal means, cannibalism, or exposure to contaminated water. Hepatopancreatic microsporidiosis (HPM) is mainly associated with stunted growth and severe infections that can cause a poor production cycle, mortality, and problems in larva-producing laboratories. This review aims to overview the main microsporidian parasites and diseases found in white shrimp, including the clinical signs, control and prevention measures for EHP infection, and the detection of HPM using different techniques. In order to offer timely detection tools, different techniques are available for the detection and study of microsporidia. Such as optical microscopy, transmission electron microscopy, and histology; however, for diagnostic purposes, molecular methods are preferred due to their sensitivity, specificity, and short-time analysis. Our review suggests that constant monitoring in shrimp hatcheries and farms is essential to avoid the entry or transference of infected organisms, affecting shrimp production and the ideal development of healthy shrimp.

**Keywords:** *Enterocytozoon hepatopenaei*; hepatopancreatic microsporidiosis; *Penaeus vannamei*; white shrimp; diagnosis

## INTRODUCTION

White shrimp *Penaeus vannamei* is native to the Pacific coast, from Central America to the south. It is recognized as the dominant species of farmed shrimp around the world, as it represents more than 99% of shrimp production (Rosenberry 2017). During their first stage of life, they are called nauplii; later, they pass to the stage of protozoa, mysis, and postlarva (Cobo & Pérez 2018).

The external morphology of the penaeid shrimp is elongated and compressed laterally and is divided into cephalothorax (cefalopereion), pleon (abdomen), and telson. Some metabolic activities during nutrient absorption and digestion occur inside the hepato-pancreas (HP) (Boschi & Angelescu 1962, Alday-Sanz 2010). It is important because recent studies have investigated parasites in wild and farmed shrimp that transmit diseases mainly through the ingestion of infected HPs, gonads, or tissues of shrimp (Cuéllar-

Corresponding editor: Sandra Bravo

Ánjel 2013, 2014). Microsporidiosis affects the HP vital functions, which involve excretion, molting, energy storage, lipid and carbohydrate metabolism, and diverse metabolic activities, including the synthesis and secretion of digestive enzymes, absorption of nutrients, synthesis of plasma proteins and their high self-repair ability (Sánchez-Paz et al. 2007, Wang et al. 2019).

Hepatopancreatic microsporidiosis (HPM), whose causative agent is *Enterocytozoon hepatopenaei* (EHP), is a clear example of these novel diseases. HPM was formally described in 2009 in Thailand, where atrophied HPs, empty intestines, malnutrition, and consequent variations in growth were reported in shrimp, which generated fluctuation in production yields and significant economic losses. In 2016, the first report in Latin America was announced in Venezuela (Tang et al. 2017). A bibliographic review was conducted to investigate the clinical signs, prevention measures, and diagnostic techniques for EHP infection. Including DNA analysis by polymerase chain reaction PCR and qPCR (quantitative method), microscopy with hematoxylin-eosin stains, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and *in situ* hybridization techniques. Because EHP is an emerging problem in urgent need of control, this review aims to provide an overview of the main diseases caused by microsporidia found in white shrimp and the methods currently used to detect HPM.

## Main parasites in shrimp

Many shrimp diseases are caused by the presence of parasites from animal or plant origin and need a host to be able to develop. Parasites can infect the host without causing disease or death. The main pathogens in wild and farmed shrimp are gregarines, haplosporidians, epicommensals, metazoans, and microsporidia (Cuéllar 2013) as bacteria, viruses, fungi, protozoans, platyhelminthes, and nematodes. Microsporidia are obligate intracellular parasites infecting eukaryotic cells. The development of these parasites generally occurs within the host cell's cytoplasm through spores' formation and nuclear proliferation (Tourtip et al. 2009, Newman 2018). The main transmission route is by contact with water, and it also can be transmitted by cannibalism or from brood stocks to larvae (Salachan et al. 2017, Kummari et al. 2018). Some of the characteristics of the main microsporidia pathogens are described (Table 1).

## Prevention and control of shrimp diseases

Strict biosecurity protocols and good pond management, such as drying, plowing, chlorination, discoloration of the water, and liming to prevent EHP infection through water and aquatic carriers (Cuéllar-Ánjel 2014, Kummari et al. 2018).

Feeding shrimp with live animals requires freezing and pasteurization before administration to prevent bacterial diseases. A wide variety of microorganisms damage the function and structure of the HP due to the presence of fragile cells that are susceptible to attack by pathogens (Sritunyalucksana et al. 2015, Varela-Mejías 2018). Some diseases that affect the HP are shown (Table 2).

## *Enterocytozoon hepatopenaei* (EHP)

From the economic perspective, the Enterocytozoonidae family (suborder Apansporoblastia, phylum Microsporidia, kingdom Fungus) comprises two of the most important microsporidian species: *Enterocytozoon bineusi* and *E. hepatopenaei*, causing significant economic losses in shrimp production by EHP infections (Stentiford et al. 2016, Wiredu-Boakye et al. 2017). This microsporidium infects penaeid shrimp such as *P. vannamei* and is therefore considered a threat to aquaculture. It attacks organs such as the HP and middle intestine and mainly affects the function of nutrient absorption (Tourtip et al. 2009, Han et al. 2018).

Experimental studies showed that EHP is a risk factor for acute hepatopancreatic necrosis and other possible diseases caused by enteric pathogens (Aranguren et al. 2017, Aldama-Cano et al. 2018). EHP has been associated with white feces syndrome. However, studies by Tangprasittipap et al. (2013) reported that the relationship between the agent and pathology had not been confirmed.

Although there are studies on the detection of EHP, the main limitation in the study of EHP is the availability of large amounts of inoculum to carry out experimental studies. For this reason, studies have been complicated by the lack of *in vitro* reproduction methods over short periods, retarding research that could improve *P. vannamei* gene lines and determine possible resistance to EHP (Mai et al. 2020).

## Presence of EHP

In 2004, the first reports of EHP were in crops from Thailand. Here, a new cytoplasmatic microsporidium was reported to have infected the hepatopancreatic tubules of *Penaeus monodon* epithelial cells, causing slow growth syndrome (SGS) (Chayaburakul et al. 2004, Tang et al. 2015). In 2002 the shrimp industry was seriously affected, and the economic losses from

**Table 1.** Characteristics of the main microsporidia that infect shrimp *Penaeus* spp. HP: hepatopancreas, L: length, W: wide.

| Microsporidium | Affected species | Infected tissues | Spore size µm (L×W) | Reference |
|---|---|---|---|---|
| *Perezia* sp. | *P. stylirostris*, *P. vannamei* and *P. monodon* | Muscle, heart, gills, lymphoid organ, and HP | 2.01×1.1 | Han et al. (2016), Sokolova & Hawke (2016) |
| *Agmasona penaei* | *P. vannamei*, *P. merguiensis*, *P. monodon*, *P. semisulcatus*, and *P. setiferus* | Abdominal muscle, gonads, nervous systems, heart, intestines, and HP. | 3.6×2.1 | Sokolova et al. (2015) |
| *Ameson* (*Nosema*) *nelsoni* | *P. vannamei*, *P. aztecus*, *P. duorarum*, *P. esculentus*, *P. latisculcatus*, *P. merguiensis*, *P. semisulcatus* and *P. setiferus* | Striated muscle, hemocytes, and HP | 1.2×2.0 | Cuéllar (2014) |
| *Enterocytozoon hepatopenaei* | *P. vannamei* and *P. monodon* | HP | 0.7×1.1 | Tourtip et al. (2009) |

**Table 2.** Main diseases that affect the hepatopancreas and susceptible life stages of shrimp *Penaeus* spp. ND: not determined, HP: hepatopancreas.

| Disease | Infected species | Susceptible stage | Clinical signs | Reference |
|---|---|---|---|---|
| Nuclear polyhedrosis virus with a single envelope of *Penaeus vannamei* | *P. duorarum*, *P. aztecus*, *P. setiferus*, *P. vannamei*, *P. stylirostris*, *P. marginatus*, *P. penicillatus*, *P. schmitti*, and *P. subtilis*. | Larva and post-larva | ND | Varela & Valverde (2019) |
| Acute hepatopancreatic necrosis | *P. monodon* and *P. vannamei* | Post-larva and juvenile | HP atrophy, erratic swimming, loss of appetite | Dang et al. (2018) |
| Pond bottom mortality virus | *P. vannamei* | Juvenile | HP atrophy, empty stomach and intestine, and retarded growth | Varela-Mejías (2016) |
| Septic necrosis of the hepatopancreas | *P. vannamei* | All stages | HP tubule damage | Varela & Peña (2016) |
| Hepatopancreatic microsporidiosis | *P. monodon* and *P. vannamei* | Juvenile | Delayed growth | Tourtip et al. (2009) |
| Hepatopancreas parvovirus | *P. monodon*, *P. merguiensis*, *P. japonicus*, *P. chinensis*, *P. semisulcatus*, *P. indicus*, *P. schmitti*, *P. vanammei*, and *P. stylirostris*. | Post-larva, juvenile, and adult | HP atrophy and retarded growth | OIE (2007) |
| Haplosporidiosis of the hepatopancreas | *P. vannamei*, *P. stylirostris*, *and P. setiferus* | ND | Loss of appetite, stunted growth, and flabby exoskeleton | Nunan et al. (2007) |
| Hepatopancreactic necrosis | *P. vannamei* | Juvenile and adult | ND | Ibarra-Gámez et al. (2007) |

this disease were significant, approximately USD 300 million (Mai et al. 2020).

Tourtip et al. (2009) reported the presence of mature spores in the HP of *P. monodon* through phylogenetic analysis, which revealed an 84% identity with *E. bineusi*. Histopathology and TEM techniques observed the unique ultrastructural characteristics of the Enterocytozoonidae family. Thus, it was considered a new species called EHP. Similar spores were later reported in China, Indonesia, Malaysia, and Vietnam, infecting the white shrimp *P. vannamei* (Varela-Mejías et al. 2019).

Tang et al. (2017) reported the first case of EHP infecting white shrimp farmed in Venezuela. The study was conducted using histopathology techniques, *in situ* hybridization, and molecular analysis. Histopathology was very similar to EHP from Southeast Asia. However, molecular analysis by phylogeny showed

identity percentages of 99, 93, and 91%, suggesting that EHP from Venezuela was not introduced from Southeast Asia. Along with EHP, white shrimp were infected with Taura syndrome, which had not been reported in Venezuela since 2005. The appearance of EHP and Taura syndrome would harm shrimp production if they spread through different infection routes. Thailand, China, Indonesia, Malaysia, Vietnam, India, Korea, and Venezuela are the countries currently affected by EHP.

**EHP life cycle**

The life cycle of microsporidium consists of three phases, merogony, which is the proliferative phase; sporogony or spore phase; and finally, the infective or mature spore phase (Fig. 1). The first two phases develop intracellularly in host cells (Carpio 2007).

General characteristics of the life cycle start with the ingestion or inhalation of spores. Germination results from the injection of the sporoplasm into the host cell's cytoplasm through the polar tube's extrusion apparatus, consecutively passing to the merogony phase where meronts are produced that will form sporonts. These spores, when dividing, will give rise to sporoblasts that become mature spores (Han & Weiss 2017). The infected epithelial cells of the HP swell and subsequently rupture to release mature spores, which facilitates the autoinfection of other HP cells or the release of mature spores into the environment through feces; in this way, they can infect other shrimp (Chaijarasphong et al. 2020), and the infection process is repeated.

The spore is considered the focal point of infection. It comprises three thick layers: the exospore, endospore, and plasmalemma. The exospore is the first layer; it is electrodense as it already has a dense electron and protein layer. It is generally uniform on the surface. The endospore carries a smaller layer of electrons, which is why it is called electrolucent. It is internal and consists of alpha-chitin and glycoproteins. The third is the plasma membrane, or plasmalemma, which contains the cytoplasm or sporoplasm, the infectious material of the microsporidia. The sporoplasm contains a nucleus, ribosomes, and invasion organelle or extrusion apparatus that consists of a polar tube attached to one end of the spore by an anchoring disk and a series of membranes (Keeling & Fast 2002, Weiss & Becnel 2014).

The microsporidia spores are the only ones with a polar tube wrapped around the nucleus and can have up to 30 coils depending on the species. It is in charge of injecting the spore parasite into a new host cell. The process is very fast, and the conditions that favor it vary. Conditions that promote infection are changes in pH, dehydration followed by hydration, hydrogen peroxide, ultraviolet irradiation, and calcium flux (Han & Weiss 2017, Han et al. 2022).

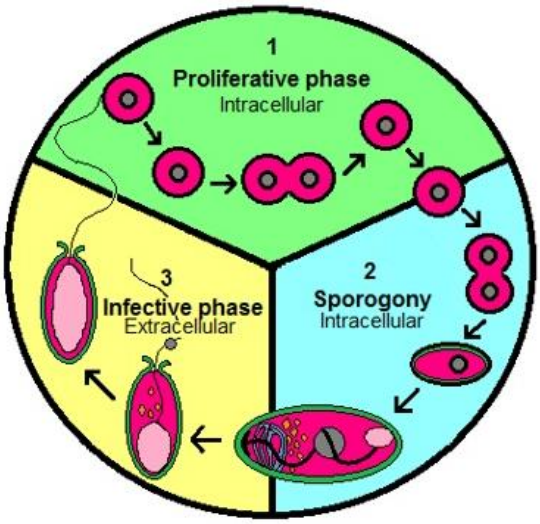


**Figure 1.** Microsporidia life cycle.

EHP spores are oval-shaped and measure approximately 0.7 to 1.1 µm. Mature spores are made up of an electron-dense wall containing a single nucleus, a posterior vacuole of five to six coils of polar filament, and an anchoring disk connected to the polar filament that is used to inoculate sporoplasm into host cells for germination (Tourtip et al. 2009, Jaroenlak et al. 2018, Varela-Mejías et al. 2019).

The main parts that comprise the spore are the following: a rigid wall composed of exospore and endospore, polar filament, anchor disk, polaroplast, posterior vacuole, nucleus, and plasma membrane (Fig. 2) (Stentiford et al. 2016).

**EHP mechanism of infection**

Unlike other microsporidia, EHP does not need an intermediate host. It can complete its life cycle in the gastrointestinal tract of the shrimp. It is transmitted directly from shrimp to shrimp by oral and fecal means, cannibalism, or exposure to contaminated water (Tangprasittipap et al. 2013, Salachan et al. 2017). Research by Tang et al. (2016) showed that infections could progressively spread as shrimp farming developed.

EHP replicates within the cytoplasmic area of the epithelial cells of the hepatopancreatic tubules. The polar filament inoculates the host cells and binds to the shrimp heparin receptor using the cell wall protein EhSWP1.

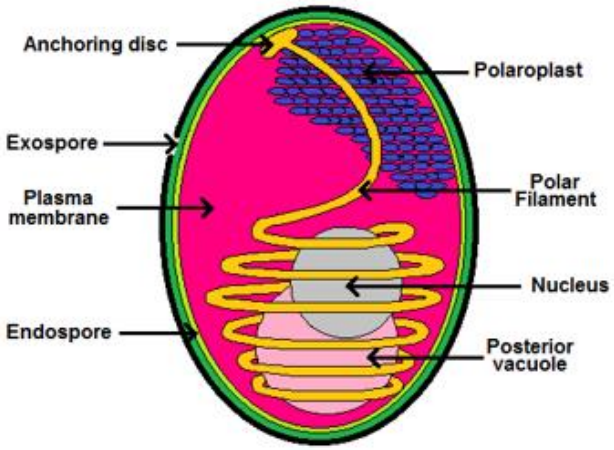


**Figure 2.** Graphic representation of the main parts of the spore *Enterocytozoon hepatopenaei*.

The spore is the infectious stage of EHP; it is surrounded by a wall subdivided into exospores and endospores, which confers resistance to external agents (Tourtip et al. 2009, Jaroenlak et al. 2018).

## Clinical signs of hepatopancreatic microsporidiosis (HPM)

HPM, whose causative agent is the microsporidium EHP, is characterized by retardation of the shrimp's development and increased susceptibility to outbreaks of other diseases, such as Taura syndrome, acute hepatopancreatic necrosis disease, and white stool syndrome (Varela-Mejías et al. 2019).

Infected shrimp show similar clinical signs to those of a chronic infection. They have a pale and stunted hepatopancreas and empty or choppy intestines and therefore show weakness caused by malnutrition. Consequently, they become vulnerable to other bacterial infections of the genus *Vibrio* that could lead to mortality (Rajendran et al. 2016, Kummari et al. 2018).

## HPM control

There are currently no reports suggesting suitable treatments for HPM. However, some shrimp farmers use garlic paste (30-40 g $kg^{-1}$ feed) as an alternative treatment due to its antimicrobial activity. In Indonesia, producers have applied probiotics and garlic paste supplemented with vitamin C (Peña-Navarro et al. 2013, Tang et al. 2016). The antimicrobial properties of garlic have been extensively studied since ancient times. Research indicates that raw garlic extract, due to its main antioxidant components, allyl cysteine, alliin, allicin, and allyl disulfide (Mohammad 2017), has antimicrobial activity against yeast, fungi, and a broad spectrum of gram-positive and negative microorganisms. Such as *Staphylococcus aureus*, *Streptococcus viridans*, *S. hemolyticus*, *Klebsiella pneumonae*, *Proteus vulgaris*, *Escherichia coli*, *Bacillus subtilis*, *Salmonella enteritidis*, *Bacillus mycoides* and *Serratia marcescens* (Santhosha et al. 2013). In their molecular structure, these components have amino groups. They could interact with the components of the cell membranes of pathogenic microorganisms, consequently causing a cellular breakdown and the inactivation of the microorganism.

Other successful studies include the inhibition of polar filament extrusion by purified spores of microsporidium. A study by Aldama et al. (2018) showed that potassium permanganate ($KMnO_4$) at a concentration of 15 ppm for 15 min, active chlorine at 40 ppm for 15 min, ethanol at 20% (v/v) for 15 min, and formalin at 200 ppm for 24 h inhibited almost 100% of the spores.

Currently, there are no specific medications for treating EHP; therefore, an early diagnosis is the only way to avoid production losses (Zhou et al. 2020).

## HPM diagnostic

### Polymerase chain reaction (PCR)

Molecular techniques (PCR, RT-PCR, and qPCR) are widely used tools for the detection of pathogens in shrimp due to their sensitivity and constitute protocols recommended by the World Organization for Animal Health (OIE by its Spanish acronym) (Cruz-Flores et al. 2019).

PCR aims to obtain multiple copies of the DNA region of interest using a Taq polymerase, primers, nucleotides, a buffer, and cofactors, among other high-purity compounds such as water. The conditions and proportions can vary depending on the purpose. For EHP detection, sample preparation is a crucial step. To avoid false negative results and underestimate the severity of the infection, it is recommended to homogenize the entire HP, followed by the removal of a subsample for the extraction of the DNA template that will be used in the subsequent analysis (Chaijarasphong et al. 2020).

Few PCR methods have been described for the detection of EHP. One-step PCR is a simple, easy method and provides a detection range of 1000-10,000 copies per reaction. It is not sensitive enough to detect organisms carrying the infection. However, Tang et al. (2015) selected primers EHP-510F/R from the 18S rRNA gene of the EHP genome, and PCR analysis was carried out with DNA extracted from EHP-infected shrimp. The results showed that the method detected EHP in infected shrimp. The primers did not react with

*Pleistophora*, a microsporidium associated with cotton shrimp disease, nor to an amoeba that infected shrimp gills.

Kummari et al. (2018) observed that in the white shrimp HP samples collected from 38 different farms, 35 tested positive for EHP by nested PCR analysis using the primers designed by Tang et al. (2015) with an amplicon size of 510 pb. The incidence of EHP in India was 92.5%.

Han et al. (2020) reported that Southeast Asian shrimp were infected with two emerging pathogens, EHP and *Vibrio parahaemolyticus*, positive for pirA and pirB toxins associated with acute hepatopancreatic necrosis disease. The study shows that using qPCR analysis, around 28% of the 60 samples analyzed were confirmed positive for EHP and only 2% for acute hepatopancreatic necrosis disease.

The SSU-rRNA genes have been widely used as PCR targets for species identification and disease diagnosis. Although PCR-based methods are sufficiently specific for detecting species, some misinterpretation could arise if the chosen gene of the target species is very similar to those of other closely related organisms. However, a comparative study by Jaroenlak et al. (2016) to investigate whether EHP SSU-rRNA primers could amplify homologous regions from other highly related microsporidia revealed that homologous regions were highly conserved. The sequences showed 67-90% similarity to other microsporidia, indicating that it could lead to a false positive result.

Although SSU-rRNA primers have been effectively used to diagnose other diseases in shrimp, they may not be an appropriate alternative to detect EHP. The development of a discriminatory PCR method is recommended. Jaroenlak et al. (2016) used EHP's sequence of the spore wall protein (SWP) gene. The method demonstrated low similarity between the sequences, indicating that the SWP regions amplicons are better at distinguishing EHP from other microsporidia in PCR assays.

The detection limits of the protocols designed for EHP are 10 copies per reaction, and it is a semi-quantitative method as well (Chaijarasphong et al. 2020). However, these methods require appropriate training, sample preparation, equipment, and reaction time. A fast method such as real-time RPA with good specificity, simplicity, and reliability would be appropriate for detecting EHP in remote areas. Ma et al. (2021) performed a comparative test of real-time RPA for detecting EHP with the nested-PCR method proposed by Jaroenlak et al. (2016). It reported speed (3-7 min), simple procedure, and detection sensitivity, comparable with the nested PCR method, the detection limit was 13 copies per reaction in 95% of the cases. Compared with the one-step PCR method (Table 3), the nested-PCR method is more elaborate and uses two sets of primers, but it is also 10 times more sensitive and detects low infection levels.

The reported methods are highly sensitive for the detection of EHP. However, selecting the method as a diagnostic tool depends on the conditions and resources for proper analysis.

**Transmission electron microscopy (TEM)**

This tool makes diagnosing different pathogens possible, allowing the ultrastructural characteristics to be observed on a fluorescent screen through a leaded protection glass (Nin 2000, Jurado & Petruccelli 2005). This technology allows the visualization of organelles and their description, enabling follow-ups to the microsporidium phases. Diverse authors have been able to describe the spores of EHP using this method (Tourtip et al. 2009, Newman 2018, Cruz-Flores et al. 2019).

Tourtip et al. (2009) observed the ultrastructure of EHP through TEM, as the micrograph revealed various stages of the microsporidium in the cytoplasm of the epithelial cells of the HP tubules. Although the initial stages of the meronts were not observed, the early and late stages of sporogony, typical of members of the Enterocytozoonidae family, were observed.

Using TEM, Cruz-Flores et al. (2019) detected EHP spores in HP biopsy samples. The observed spores showed a width of 1.0 ± 0.8 µm and a length of 0.7 ± 0.4 µm.

**Histopathology**

It is a diagnostic tool that identifies changes at a cellular level through tissue cuts subjected to a series of physical processes and special staining (Cuéllar-Ánjel 2014).

The methodology of Bell & Lightner (1988) proposes that the organisms are fixed in Davidson alcohol-formalin-acetic acid. The application must be made immediately after the death of the animal. In order to preserve the organism in the exact conditions of its death, the process involves cuts, alcohol dehydration, paraffin embedding, microtome cuts, and hematoxylin-eosin (H&E) staining.

Through this diagnostic technique, it is possible to observe EHP spores that infect the HP epithelial cells. In the study by Tourtip et al. (2009), the histological sections of the HP revealed various stages of the development of EHP, represented by staining of cyto-

**Table 3.** Specific primers for the identification of *Enterocytozoon hepatopenaei*.

| Reference | Primer name | Sequence (5'-3') | Amplicon size (pb) | Observations |
|---|---|---|---|---|
| Tang et al. (2015) | EHP- 510 F | GCCTGAGAGATGGCTCCCACGT | 510 | One step PCR |
| | EHP-510 R | GCGTACTATCCCCAGAGCCCGA | | |
| Han et al. (2018) | EHP-947F | GGTAATAATTGGGCTAAAGGT | 947 | First step PCR |
| | EHP-947R | GCTTCAGCCTCAGTAAATTC | | |
| | EHP-618F | GGTAATAATTGGGCTAAAGGT | 618 | Second step (nested)-PCR |
| | EHP-618R | GCTTCAGCCTCAGTAAATTC | | |
| Jaroenlak et al. (2016) | SWP_1F | TTGCAGAGTGTTGTTAAGGGTTT | 514 | First step (nested)-PCR |
| | SWP_1R | CACGATGTGTCTTTGCAATTTTC | | |
| | SWP_2F | TTGGCGGCACAATTCTCAAACA | 147 | Second step (nested)-PCR |
| | SWP_2R | GCTGTTTGTCTCCAACTGTATTTGA | | |
| Zhou et al. (2020) | F2 | CATTGAGTTTGTTGAGAGTAGCGGAACGGAT | 111 | RPA |
| | R2 | CTAAGCATCGCTTTCGCCTCCGTTGGTC | | |
| Ma et al. (2021) | RPA-F | ACAATTTCAAACACTGTAAACCTTAAAGCA | 176 | RPA |
| | RPA-P | TAAAAAGAGACGATATTTACACAGACACAG[FAM-dT][THF] | | |

plasmic inclusions. In addition, they observed mature spores, which coincided with the spores observed using TEM. Tang et al. (2015), in their histological examinations of HPs of *P. vannamei*, showed basophilic inclusions within the cytoplasm of epithelial cells of hepatopancreatic tubules and mature spores.

### *In situ* hybridization (ISH)

ISH is a tool used to determine the location of the pathogen's nucleic acid in sections of animal tissue with positive PCR. It employs fluorochrome-labeled oligonucleotide probes that target specific rRNA sequences and enables rapid and specific identification of microbial cells (Martínez 2011, Jaroenlak et al. 2016). However, it is recommended that other methods complement this method.

The study by Tang et al. (2015) showed that *Penaeus stylirostris* and *P. monodon* were infected with EHP using histology and *in situ* hybridization techniques; however, those samples without spore detection by histology were found positive by ISH. Thus, ISH has greater sensitivity in the detection of pathogens.

Biju et al. (2016) observed positive hybridization in parallel sections of HP cells in histological sections and found that 32% of EHP-positive shrimps had a bacterial coinfection with *Vibrio* species, which is described as septic hepatopancreatic necrosis.

White shrimp farming constitutes more than 50% of the world's crustacean production. Although Asian countries dominate the global production of this and other species, Latin American countries such as Ecuador, Brazil, and Mexico have the natural conditions to be potential leaders in shrimp aquaculture.

Although EHP does not appear to cause high mortalities in *P. vannamei* cultures, it is a latent and critical threat. Signs of infection directly impact shrimp development and are reflected by severe growth retardation and possible coinfections with other pathogens. With appropriate diagnostic tools and monitoring programs for the prevention of EHP, it is possible to control and guarantee the availability of healthy shrimp and sustainable aquaculture.

When shrimp do not present any clinical symptoms, EHP is mainly diagnosed by two methods. The first is DNA analysis by PCR and qPCR, the gold standard tool for diagnosing many shrimp diseases. The second is the detection of spores by microscopy with hematoxylin-eosin stains (histology), scanning electron microscopy, transmission electron microscopy, and *in situ* hybridization techniques with a digoxigenin-labeled probe (qualitative methods) (Karthikeyan & Sudhakaran 2016, Liu et al. 2018).

In the present study, the comparison by review of diagnostic techniques indicates that only severe infection by EHP shows the presence of spores in a smear of HP. In contrast, mild infection cannot be detected using only microscopy as a detection tool. On the other hand, the histopathological features of EHP infection can be observed, which include the presence

of different stages of the microsporidian in the epithelial cells of the HP tubules, as well as free spores from lysed or detached epithelial cells. Tourtip et al. (2009) , Tangprasittipap et al. (2013) and Tang et al. (2015) found that these findings can be observed using the Bell & Lightner (1988) methodology of standard H&E. However, the initial stages and spores are very small, requiring an oil immersion lens to distinguish from normal host cell content without specific staining methods, especially in low-level signs of infection. For certainty of the EHP infection diagnosis, it is recommended to carry out molecular techniques and histological inspections.

## CONCLUSIONS

HPM is transmitted by ingesting infected tissue, ingesting fecal matter, or exposure to contaminated water. Consequently, the vital functions of the HP are affected. EHP is a new disease that can generate significant economic losses in the aquaculture industry. It is recommended that sanitary authorities regulate strict biosecurity protocols and surveillance programs to avoid the appearance of pathogens in countries where EHP has not occurred. Because there is no treatment to counteract HPM infection, it is recommended to use molecular techniques (PCR) to detect these pathogens in shrimp due to their high sensitivity. Due to the similarity of the homologous regions to be amplified (67-90%) with other microsporidia, it is advisable to use specific primers that reduce the incidence of false positive results.

## ACKNOWLEDGMENTS

The second author is grateful to CONACYT (923296). This work was supported by the aquaculture health committee of the state of Sonora (COSAES) and the Sinaloa state aquaculture health committee (CESASIN). This research also was financed by Project N°D3B33 2020102 "shrimp disease study" and PROFAPI N°2020-0579 from Instituto Tecnológico de Sonora.

## REFERENCES

Aldama-Cano, D.J., Sanguanrut, P., Munkongwongsiri, N., Ibarra-Gámez, J.C., Itsathitphaisarn, O., Vanichviriyakit, R. & Thitamadee, S. 2018. Bioassay for spore polar tube extrusion of shrimp *Enterocytozoon hepatopenaei* (EHP). Aquaculture, 490: 156-161. doi: 10.1016/ j.aquaculture.2018.02.039

Alday-Sanz, V. 2010. The shrimp book. Nottingham University Press, Nottingham.

Aranguren, L.F., Han, J.E. & Tang, K.F. 2017. *Enterocytozoon hepatopenaei* (EHP) is a risk factor for acute hepatopancreatic necrosis disease (AHPND) and septic hepatopancreatic necrosis (SHPN) in the Pacific white shrimp *Penaeus vannamei*. Aquaculture, 471: 37-42. doi: 10.1016/j.aquaculture.2016.12.038

Bell, T.A. & Lightner, D.V. 1988. A handbook of normal penaeid shrimp histology. World Aquaculture Society, Arizona.

Biju, N., Sathiyaraj, G., Raj, M., Shanmugam, V., Baskaran, B., Govindan, U. & Chellamma, T.S.R.Y. 2016. High prevalence of *Enterocytozoon hepatopenaei* in shrimps *Penaeus monodon* and *Litopenaeus vannamei* sampled from slow growth ponds in India. Diseases of Aquatic Organisms, 120: 225-230. doi: 10.3354/dao03036

Boschi, E.E. & Angelescu, V.A. 1962. Descripción de la morfología externa e interna del langostino con algunas aplicaciones de índole taxonómica y biológica. Boletín del Instituto de Biología Marina, 1: 1-72.

Carpio, B. 2007. Enfermedades en camarones penaeidos: microsporidiosis en *Penaeus* (*Litopenaeus*) *vannamei* (parte ii). Boletín informativo 146, CENAIM, Guayaquil.

Chaijarasphong, T., Munkongwongsiri, N., Stentiford, G.D., Aldama-Cano, D.J., Thansa K., Flegel, T.W., et al. 2020. The shrimp microsporidian *Enterocytozoon hepatopenaei* (EHP): biology, pathology, diagnostics and control. Journal of Invertebrate Pathology, 186: 107458. doi: 10.1016 /j.jip.2020.107458

Chayaburakul, K., Nash, G., Pratanpipat, P., Sriurairatana, S. & Withyachumnarnkul, B. 2004. Multiple pathogens found in growth-retarded black tiger shrimp *Penaeus monodon* cultivated in Thailand. Diseases of Aquatic Organisms, 60: 89-96. doi: 10.3354/dao 060089

Cobo, R. & Pérez, L. 2018. Aspectos generales del cultivo y la genética del camarón blanco del Pacífico *Litopenaeus vannamei* (Boone, 1931). Revista Cubana de Investigaciones Pesqueras, 35: 0138-8452.

Cruz-Flores, R., Mai, H.N., Noble, B.L., Schofield, P.J. & Dhar, A.K. 2019. Detection of *Enterocytozoon hepatopenaei* using an invasive but non-lethal sampling method in shrimp (*Penaeus vannamei*). Journal of Microbiological Methods, 162: 38-41. doi: 10.1016/j.mimet.2019.05.008

Cuellar-Ánjel, J. 2013. Enfermedades parasitarias en camarones. The Center for Food Security & Public

Health/Institute for International Cooperation in Animal Biologics, Iowa State University, Iowa.

Cuellar-Ánjel, J. 2014. Haplosporidios. In: Morales, V. & Cuéllar-Anjel, J. (Eds.). Guía técnica - Patología e inmunología de camarones penaeidos. OIRSA, 2: 209-210.

Dang, T.L., Pham, A.T. & Phan, T.V. 2018. Acute hepatopancreatic necrosis disease (AHPND) in Vietnam. Journal of Asian Fisheries Society, 31: 274-282.

Han, B. & Weiss, L.M. 2017. Microsporidia: obligate intracellular pathogens within the fungal kingdom. Microbiology Spectrum, 5. doi: 10.1128/microbiolspec.FUNK-0018-2016

Han, B., Takvorian, P.M. & Weiss, L.M. 2022. The function and structure of the microsporidia polar tube. In: Weiss, L.M. & Reinke, A.W. (Eds.). Microsporidia. Experientia Supplementum, 114. Springer, Cham.

Han, J.E., Tang, K.F. & Kim, J.H. 2018. The use of beta-tubulin gene for phylogenetic analysis of the microsporidian parasite *Enterocytozoon hepatopenaei* (EHP) and in the development of a nested PCR as its diagnostic tool. Aquaculture, 495: 899-902.

Han, J.E., Tang, K.F., Pantoja, C.R., Lightner, D.V., Redman, R.M. & Le Groumellec, M. 2016. Detection of a new microsporidium *Perezia* sp. in shrimps *Penaeus monodon* and *P. indicus* by histopathology, *in situ* hybridization and PCR. Diseases of Aquatic Organisms, 120: 165-171. doi: 10.3354/ dao03022

Han, J.E., Lee, S.C., Park, S.C., Jeon, H.J., Kim, K.Y., Lee, Y.S. & Choi, S.K. 2020. Molecular detection of *Enterocytozoon hepatopenaei* and *Vibrio parahaemolyticus*-associated acute hepatopancreatic necrosis disease in Southeast Asian *Penaeus vannamei* shrimp imported into Korea. Aquaculture, 517: 734812. doi: 10.1016/j.aquaculture.2019.734812

Ibarra-Gámez, J.C., Galaviz-Silva, L. & Molina-Garza, Z.J. 2007. Distribución de la bacteria causante de la necrosis hepatopancreática (NHPB) en cultivos de camarón blanco, *Litopenaeus vannamei*, en México. Ciencias marinas, 33: 1-9.

Jaroenlak, P., Boakye, D.W., Vanichviriyakit, R., Williams, B.A., Sritunyalucksana, K. & Itsathitphaisarn, O. 2018. Identification, characterization, and heparin binding capacity of a spore-wall, virulence protein from the shrimp microsporidian, *Enterocytozoon hepatopenaei* (EHP). Parasites Vectors, 11: 177. doi: 10.1186/ s13071-018-2758-z

Jaroenlak, P., Sanguanrut, P., Williams, B.A., Stentiford, G.D., Flegel, T.W., Sritunyalucksana, K. & Itsathitphaisarn, O. 2016. A nested PCR assay to avoid false positive detection of the microsporidian *Enterocytozoon hepatopenaei* (EHP) in environmental samples in shrimp farms. Plos One, 11: 166320. doi: 10.1371/journal.pone.0166320

Jurado, S.B. & Petruccelli, M.Á. 2005. Aplicaciones de la microscopía electrónica de transmisión en el diagnóstico microbiológico. Analecta Veterinaria, 25: 18-24.

Karthikeyan, K. & Sudhakaran, R. 2016. Shrimp microsporidian parasite *Enterocytozoon hepatopenaei* (EHP): a threat for cultivated penaeid shrimp. South Indian Journal of Biological Sciences, 2: 388-394. doi: 10.22205/sijbs/2016/v2/i4/103444

Keeling, P.J. & Fast, N.M. 2002. Microsporidia: biology and evolution of highly reduced intracellular parasites. Annual Review of Microbiology, 56: 93-116. doi: 10.1146/annurev.micro.56.012302. 160854

Kummari, S., Rathlavath, S., Pillai, D. & Rajesh, G. 2018. Hepatopancreatic microsporidiasis (HPM) in shrimp culture: a review. International Journal of Current Microbiology and Applied of Sciences, 7: 3208-3215. doi: 10.20546/ijcmas.2018.701.383

Kummari, S., Haridas, D.V., Handique, S., Peter, S., Rakesh, C.G., Sneha, K.G. & Pillai, D. 2018. Incidence of hepatopancreatic microsporidiasis, by *Enterocytozoon hepatopenaei* (EHP) in *Penaeus vannamei* culture in Nellore District, Andhra Pradesh, India and the role of management in its prevention and transmission. Journal of Current Microbiology and Applied of Sciences, 7: 2125-2134. doi: 10.20546/ ijcmas.2018.702.254

Liu, Y.M., Qiu, L., Sheng, A.Z., Wan, X.Y., Cheng, D.Y. & Huang, J. 2018. Quantitative detection method of *Enterocytozoon hepatopenaei* using TaqMan probe real-time PCR. Journal of Invertebrate Pathology, 151: 191-196. doi: 10.1016/j.jip.2017.12.006

Ma, C., Fan, S., Wang, Y., Yang, H., Qiao, Y., Jiang, G., et al. 2021. Rapid detection of *Enterocytozoon hepatopenaei* infection in shrimp with a real-time isothermal recombinase polymerase amplification assay. Frontiers in Cellular and Infection Microbiology, 11: 631960. doi: 10.3389/fcimb.2021.631960

Mai, H.N., Cruz-Flores, R., Caro, L.F.A., White, B.N. & Dhar, A.K. 2020. A comparative study of *Enterocytozoon hepatopenaei* (EHP) challenge methods in *Penaeus vannamei*. Journal of Invertebrate Pathology, 171: 107336. doi: 10.1016/j.jip.2020.107336

Martínez, R.R. 2011. Empleo de la técnica hibridación *in situ* fluorescente para visualizar microorganismos. Revista Salud UIS, 43.

Mohammad, M. 2017. Medicinal herbs, strong sources of antioxidants in aquaculture: a mini review. Modern Applications in Pharmacy & Pharmacology, 1: 000504.

Newman, S.G. 2018. Microsporidian impacts shrimp production. Global Seafood Alliance, New Hampshire.

Nin, G.V. 2000. Introducción a la microscopía electrónica aplicada a las ciencias biológicas. UNAM, Ciudad de México.

Peña-Navarro, N., Vargas-Cordero, R. & Varela-Mejías, A. 2013. Productos naturales como estimuladores del sistema inmunológico de *Litopenaeus vannamei*, infectado con *Vibrio parahaemolyticus*. Agronomía Mesoamericana, 24: 133-147.

Rajendran, K.V., Shivam, S., Praveena, P.E., Rajan, J.J.S., Kumar, T.S., Avunje, S. & Alavandi, S.V. 2016. Emergence of *Enterocytozoon hepatopenaei* (EHP) in farmed *Penaeus* (*Litopenaeus*) *vannamei* in India. Aquaculture, 454: 272-280.

Rosenberry, Bob. 2017. Farmed species. Shrimp News International. [https://www.shrimpnews.com/FreeReportsFolder/GeneralInformationFolder/FarmedSpecies.html#Introduction]. Reviewed: February 19, 2020.

Salachan, P.V., Jaroenlak, P., Thitamadee, S., Itsathitphaisam, O. & Sritunyalucksana, K. 2017. Laboratory cohabitation challenge model for shrimp hepatopancreatic microsporidiosis (HPM) caused by *Enterocytozoon hepatopenaei* (EHP). BMC Veterinary Research, 13: 1-7. doi: 10.1186/s12917-016-0923-1

Sánchez-Paz, A., García-Carreño, F., Hernández-López, J., Muhlia-Almazán, A. & Yepiz-Plascencia, G. 2007. Effect of short-term starvation on hepatopancreas and plasma energy reserves of the Pacífic white shrimp (*Litopenaeus vannamei*). Journal Experimental Marine Biology and Ecology, 340: 184-193. doi: 10.1016/j.jembe.2006.09.006

Santhosha, S.G., Prakash-Jamuna, S.N. & Prabhavathi, S.N. 2013. Bioactive components of garlic and their physiological role in health maintenance: a review. Food Bioscience, 3: 59-74. doi: 10.1016/j.fbio.2013.07.001

Sokolova, Y. & Hawke, J. 2016. *Perezia nelsoni* (Microsporidia) in *Agmasoma penaei* infected Atlantic white shrimp *Litopenaeus setiferus* (Paenaidae, Decapoda) and phylogenetic analysis of *Perezia* spp. Complex. Protistology, 10: 3.

Sokolova, Y., Pelin, A., Hawke, J. & Corradi, N. 2015. Morphology and phylogeny of *Agmasoma penaei* (Microsporidia) from the type host, *Litopenaeus setiferus*, and the type locality, Louisiana, USA. International Journal for Parasitology, 45: 1-16.

Sritunyalucksana, K., Sanguanrut, P., Salachan, P.V., Thitamadee, S. & Flegel, T.W. 2015. Urgent appeal to control spread of the shrimp microsporidian parasite *Enterocytozoon hepatopenaei* (EHP). Network of Aquaculture Centres in Asia-Pacific (NACA). [https://enaca.org/?id=101]. Reviewed: January 20, 2022.

Stentiford, G.D., Becnel, J.J., Weiss, L.M., Keeling, P.J., Didier, E.S., Williams, B.A.P., et al. 2016. Microsporidia - emergent pathogens in the global food chain. Trends in Parasitology, 32: 336-348. doi: 10.1016/j.pt.2015.12.004

Tang, K.F., Aranguren, L.F., Piamsomboon, P., Han, J.E., Maskaykina, I.Y. & Schmidt, M.M. 2017. Detection of the microsporidian *Enterocytozoon hepatopenaei* (EHP) and Taura syndrome virus in *Penaeus vannamei* cultured in Venezuela. Aquaculture, 480: 17-21.

Tang, K.F., Pantoja, C.R., Redman, R.M., Han, J.E., Tran, L.H. & Lightner, D.V. 2015. Development of *in situ* hybridization and PCR assays for the detection of *Enterocytozoon hepatopenaei* (EHP), a microsporidian parasite infecting penaeid shrimp. Journal of Invertebrate Pathology, 130: 37-41. doi: 10.1016/j.jip.2015.06.009

Tang, K.F., Han, J.E., Aranguren, L.F., White-Noble, B., Schmidt, M.M., Piamsomboon, P. & Hanggono, B. 2016. Dense populations of the microsporidian *Enterocytozoon hepatopenaei* (EHP) in feces of *Penaeus vannamei* exhibiting white feces syndrome and pathways of their transmission to healthy shrimp. Journal of Invertebrate Pathology, 140: 1-7.

Tangprasittipap, A., Srisala, J., Chouwdee, S., Somboon, M., Chuchird, N., Limsuwan, C., et al. 2013. The microsporidian *Enterocytozoon hepatopenaei* is not the cause of white feces syndrome in whiteleg shrimp *Penaeus* (*Litopenaeus*) *vannamei*. BMC Veterinary Research, 9: 139.

Tourtip, S., Wongtripop, S., Stentiford, G.D., Bateman, K.S., Sriurairatana, S., Chavadej, J., et al. 2009. *Enterocytozoon hepatopenaei* sp. nov. (Microsporidia: Enterocytozoonidae), a parasite of the black tiger shrimp *Penaeus monodon* (Decapoda: Penaeidae): Fine structure and phylogenetic relationships. Journal

of Invertebrate Pathology, 102: 21-29. doi: 10.1016/j.jip.2009.06.004

Varela-Mejías, A. 2016. Nodavirus de la mortalidad encubierta (CMNV) en camarones marinos de cultivo. Nota técnica. Universidad Estatal a Distancia, 19: 33-40.

Varela-Mejías, A. 2018. Patologías del hepatopáncreas en camarones marinos cultivados en América y su diagnóstico diferencial mediante histopatología. Revista AquaTIC, 50: 13-30.

Varela-Mejías, A. & Peña-Navarro, N. 2016. Histopatología diferencial de tres enfermedades bacterianas que afectan la hepatopáncreas de camarones peneidos. Agronomía Mesoamericana, 27: 73-80. doi: 10.15517/am.v27i1.21887

Varela-Mejías, A. & Valverde-Moya, J. 2019. *Baculovirus penaei* como factor de riesgo para infecciones bacterianas en hepatopáncreas de *Penaeus vannamei*. Revista de Investigaciones Veterinarias del Perú, 30: 377-386. doi: 10.15381/rivep.v30i1.14961

Varela-Mejías, A., Peña-Navarro, N. & Aranguren-Caro, L.F. 2019. Microsporidiosis hepatopancreática causada por *Enterocytozoon hepatopenaei* (EHP) en camarones de cultivo. Revista Panorama Acuícola, 24: 41-46.

*Received: January 14, 2021; Accepted: October 28, 2022*

Wang, Z., Qu, Y., Yan, M., Li, J., Zou, J. & Fan, L. 2019. Physiological responses of Pacific white shrimp *Litopenaeus vannamei* to temperature fluctuation in low-salinity water. Frontiers in Physiology, 10: 1025. doi: 10.3389/fphys.2019.01025

Weiss, L.M. & Becnel, J.J. 2014. Microsporidia: pathogens of opportunity. Wiley-Blackwell, Oxford.

Wiredu-Boakye, D., Jaroenlak, P., Prachumwat, A., Williams, T.A., Bateman, K.S., Itsathitphaisarn, O., et al. 2017. Decay of the glycolytic pathway and adaptation to intranuclear parasitism within *Enterocytozoonidae microsporidia*. Environmental Microbiology, 19: 2077-2089.

World Organization for Animal Health (OIE). 2007. Hepatopancreatic parvovirus disease. OIE Aquatic Animal Disease Cards. [https://www.oie.int/fileadmin/Home/eng/International_Standard_Setting/docs/pdf/HepatopancreaticParvovirusCard2007_Revised92707_pdf]. Reviewed: February 19, 2020.

Zhou, S., Wang, M., Liu, M., Jiang, K., Wang, B. & Wang, L., 2020. Rapid detection of *Enterocytozoon hepatopenaei* in shrimp through an isothermal recombinase polymerase amplification assay. Aquaculture, 521: 734987. doi: 10.1016/j.aquaculture.2020.734987