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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

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A B S T R A C T

Shrimp culture is the most lucrative aquaculture sector in Asia-Pacific region. Recently, shrimp farms in India have been affected by the microsporidian parasite, *Enterocytozoon hepatopenaei* (EHP) associated with retarded growth without other clinical signs, causing significant economic losses to the shrimp industry. The present study was carried out to investigate the incidence of EHP in *Penaeus vannamei* farms of Nellore district of Andhra Pradesh, and to identify the carriers of EHP and the role of management in reducing the incidence of EHP in ponds. PCR analysis by two sets of EHP specific primers indicated the incidence of EHP to be 92.50%. Histopathological studies of the hepatopancreas of affected shrimp showed sloughing of the epithelial cells of hepatopancreatic tubules, lifting and detachment of epithelial layers of hepatopancreatic tubules and heavy damage of hepatopancreas. PCR screening of aquatic macro fauna in the farms showed that samples of crabs, polychaetesand non-penaeids were positive for EHP, implying that they could act as carriers of EHP. On the other hand, fishes were found negative. Strict implementation of Best Management Practices (BMPs) in one selected farm which was confirmed to be infected by EHP clearly showed that practice of BMPs and avoidance of carriers can definitely reduce the incidence of EHP during the subsequent culture in shrimp farms. After implementation of BMPs, significant improvement in growth rate, and Feed Conversion Ratio (FCR) were also observed. In the control pond where BMPs were not implemented, EHP continued to be present, affecting growth and FCR adversely. This is the first report comparing the role of management in EHP infection and identifying carriers of EHP in *P. vannamei* culture in India.

Introduction

Shrimp is considered as one of the most important food item in terms of its rich protein source and value. Shrimp farming in the Asia-Pacific region is one of the most lucrative in aquaculture sectors. In India, the remarkable revival and growth in shrimp culture was brought about by the introduction of non-native species *Penaeus vannamei* during 2009 almost replacing the native species *Penaeus monodon*, the culture of which failed due to disease problems mainly by White Spot Syndrome Virus (WSSV).

Shrimp farms in Asia and Mexico are now facing threat from a microsporidian parasite, *Enterocytozoon hepatopenaei* (EHP) causing significant losses to aquaculture. EHP belongs to microsporidia, an obligate, intracellular parasite related to fungi (Wittner and Weiss, 1999). Stentiford *et al.,* (2013) reported that microsporidians were causing infection in many aquatic animals. EHP was first reported from *Penaeus monodon* in Thailand (2009) and subsequently from China, Indonesia, Thailand, Malaysia, Vietnam and India (Joshi *et al*., 2014; Rajendran *etal*., 2016; Thitamadee *et al*., 2016). Although EHP has not been associated with mortality, recent studies clearly indicate that EHP is associated with growth retardation in *P. vannamei* and *P. monodon* (Tang *et al*., 2016; Giridharan and Uma, 2017).

In India, the first report of incidence of EHP was in 2016 from Andhra Pradesh, one of the south-eastern states of India, where shrimp culture is a major livelihood to the farmers. The economic loss in shrimp production due to EHP appears to be significant. Hence the present study was carried out to investigate the incidence of the microsporidian parasite, *Enterocytozoon hepatopenaei* in Nellore district of Andhra Pradesh (Figure 1), and to identify other aquatic organisms that may carry the parasite and the role of good management in reducing disease incidence and transmission.

Materials and Methods

Survey of *P. vannamei* farms and sample collection

Survey of 120 *P. vannamei* farms in Gudur, Kota, Vakadu, Muthukur, and Kavali in Nellore district of Andhra Pradesh was carried out with the help of a questionnaire to select farms for active targeted surveillance for EHP. The sample collection was carried out as per*World Organisation for Animal Health* (OIE) protocol from 38 farms, where growths of shrimps were sub-optimal, for one year from November, 2015 to October, 2016. A comparison of EHP infection was also made between two crops, before and after implementation of best management practices. The hepatopancreas from shrimp samples were collected aseptically and preserved in 80% ethanol for PCR analysis and in Davidson's fixative for histopathological studies. Genomic DNA was extracted from shrimp hepatopancreas by traditional phenol- chloroform method.

Identification of carriers

Aquatic macro fauna (Fishes, crabs, polychaetes and non-penaeids) were collected from EHP infected shrimp farms to determine the path of entry and carriers of the microsporidian parasite.For DNA extraction from carrier organisms, each type of organism were pooled and processed as mentioned above.

PCR analysis for EHP

PCR was performed with two sets of primers, viz., first with primers of Tangprasittipap *et al.,* (2013) and further confirmation was done by a second set of primers of Tang *et al.,*

(2015) that gives an amplicon of 510 bp. Table 1 represents the list of primers used in the study.

The amplification was carried out in 25µl reaction mixture containing 1µM of each primer, 200µM of dNTPs (Promega, USA), 1.25 U of Taq polymerase and 10X buffer (Thermo scientific, USA) and 1 µl of template DNA.

The reaction was performed in thermal cycler (Bio Rad, USA) using the condition: initial denaturation for 94°C for 3 min, followed by 35 cycles of denaturation for 20 sec at 94°C each, annealing for 20 sec at 58°C, extension at 72°C for 45sec with final extension for 5 min at 72°C. For nested PCR, the same master mix was used as in the first step except primers and template.

The first step PCR amplified product served as the template and internal primers as mentioned in Table 1 were used for the nested PCR reaction. The reaction conditions were similar to the first step except that annealing temperature was 64°C for 20 sec.

Agarose gel electrophoresis was carried out at 70-100 volts in 2% agarose gel containing 0.05µg/ml of Ethidium Bromide (EtBr) alongside a 100 bp DNA ladder (Promega, USA) and the amplified DNA was visualized using gel documentation system (Biorad, USA)**.**

PCR for EHP detection was also carried out using second set of primers to rule out false positive amplification with the nested PCR primers. Reaction condition as described by Tang *et al.,* (2015) was followed.

The reaction condition followed was initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing 60°C for 30 sec, extension at 72°C for 30 sec with final extension at 72°C for 5 min. The amplified products were analyzed using a 1.2% agarose gel.

Histopathological examination

The hepatopancreas of *P.vannamei* samples fixed in Davidson's fixative were processed, sectioned and stained for histopathological examination (Bell and Lightner, 1988).

Effect of good management in reducing incidence of EHP

Two ponds in which shrimps were confirmed to be EHP infected were selected and in one of them, the farmer was advised to practice BMPs strictly, from the stage of pond preparation. Both the farms were monitored continuously and samples screened by PCR for EHP.

Results and Discussion

Among the 120 surveyed farms, only 11 farms (9.17%) had license from the Coastal Aquaculture Authority (CAA) for culture of *P. vannamei*. Merely 25 (20.83%) of the farms had implemented biosecurity measures such as bird fencing, bird scares, reservoir pond and fencing of the farms. None of the farms surveyed had Effluent Treatment System (ETS).

Only 8 farms had practiced chlorination and de-chlorination of water in reservoir ponds. 80% of farmers procured tested SPF seed from certified hatcheries. Majority of the farmers (99%) depended on pelleted feed, and in 85-90% of the farms, technicians provided by the feed company themselves monitored the culture every 10-15 days.

PCR analysis for EHP

In the samples collected from 38 farms,

amplified product at the expected size was observed in 35 (92.10%) of 38 samples tested by nested PCR. The results were confirmed with primer set II designed by Tang *et al.*, (2015) which gave an amplicon size of 510 bp. Figures 2 and 3 shows a representative figure of the PCR analyses carried out in our study.

Analysis of Risk factors for EHP

PCR screening of aquatic macro fauna in the farms showed that samples of crabs, polychaetes and non-penaeids (*Acetes sp.*) were positive for EHP, implying that they could act as carriers of EHP. On the other hand, fishes were found negative (Figure 4).

Histopathology

The histological section showed sloughing of epithelial cells of the tubules of hepatopancreas, lifting of epithelial layers, detachment of tubules of hepatopancreas and heavy damage of hepatopancreas (Figure 5).

PCR analysis for EHP after implementation of BMPs

The incidence of EHP was almost absent or less than detectable levels in the pond where BMPs were strictly implemented compared to the control pond in which the farmer continued his usual culture practices (Figure 6).

Table.1 List of primers used in the study

Primer	Sequence(5'-3')	Amplicon size	References
Primer set I			
ENF779	CAGCAGGCGCGAAAATTGTCCA	**779bp**	Tangprasittipap*et al.*, (2013).
ENR779	AAGAGATATTGTATTGCGCTTGCTG		
Set I-Nested PCR Primers			
ENF176	CAACGCGGGAAAACTTACCA	**176bp**	
ENR176	ACCTGTTATTGCCTTCTCCCTCC		
Primer set II			
EHP-510F	GCCTGAGAGATGGCTCCCACGT	**510bp**	Tang *et al.*, (2015).
EHP-510R	GCGTACTATCCCCAGAGCCCGA		

Fig.1 Study Area: Nellore District, Andhra Pradesh

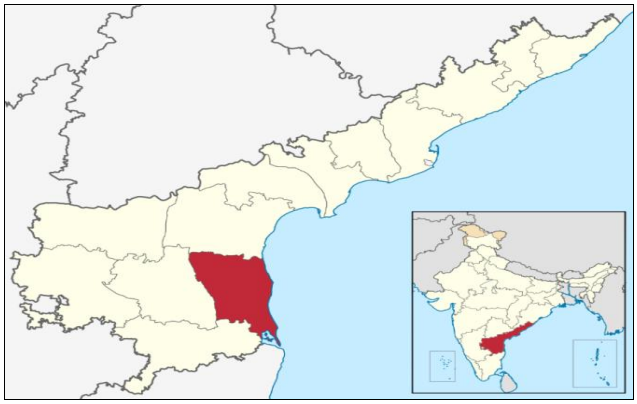


Fig.2 Agarose gel showing PCR for EHP. 2% agarose gel. Lane M- Mol. wt. marker 100bp ladder; lane 2-6: samples; lane P- positive control (176 bp); lane N- negative control

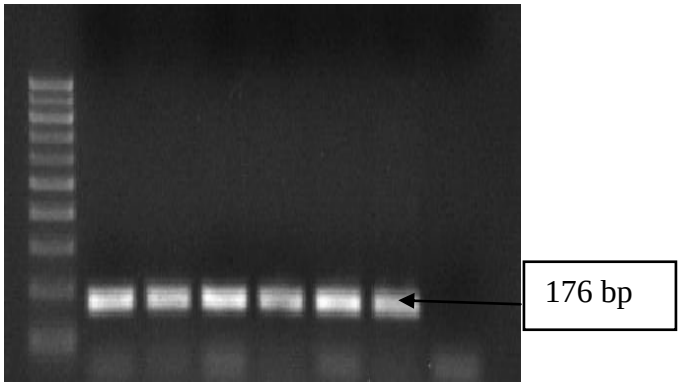


Fig.3 Agarose gel electrophoresis of samples screened for EHP by second set of primers. 1.2% agarose gel. Lane M- Mol. wt. marker 1kb ladder; lane P- positive control (510 bp); lane N- negative control; lane 1- 5 represent samples

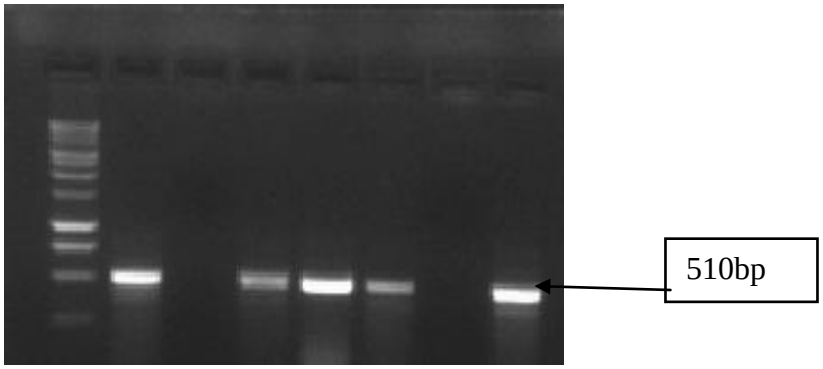


Fig.4 PCR screening for EHP carriers- 2% agarose gel. Lane M- Mol. wt. marker 100bp ladder; lane P- positive control (176 bp); lane N- negative control; lane 4- shrimp; lane 5- fish; lane 6- crab; lane 7- polychaete worm and lane 8- non-penaeids

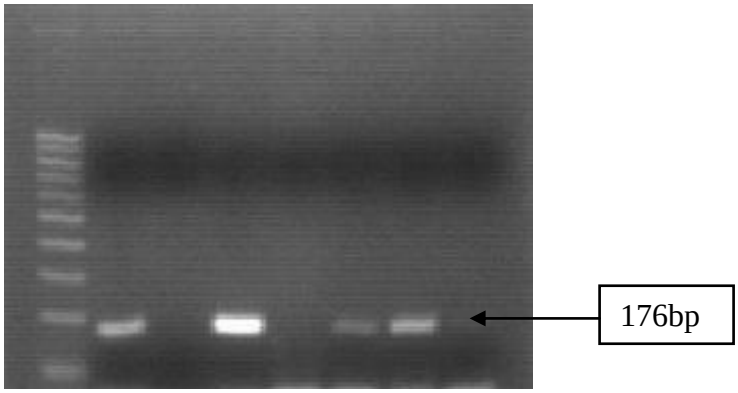


Fig.5 Histopathology in the hepatopancreatic tissue of EHP infected *P. vannamei.* (HandE, 10X). A. Shows the normal cells of hepatopancreas; B. sloughing of hepatopancreas; C. Heavy cellular damage; D. Lifting of epithelial layers; E. Detachment of tubules of hepatopancreas. Note: arrows show the significant changes in hepatopancreas of infected tissues

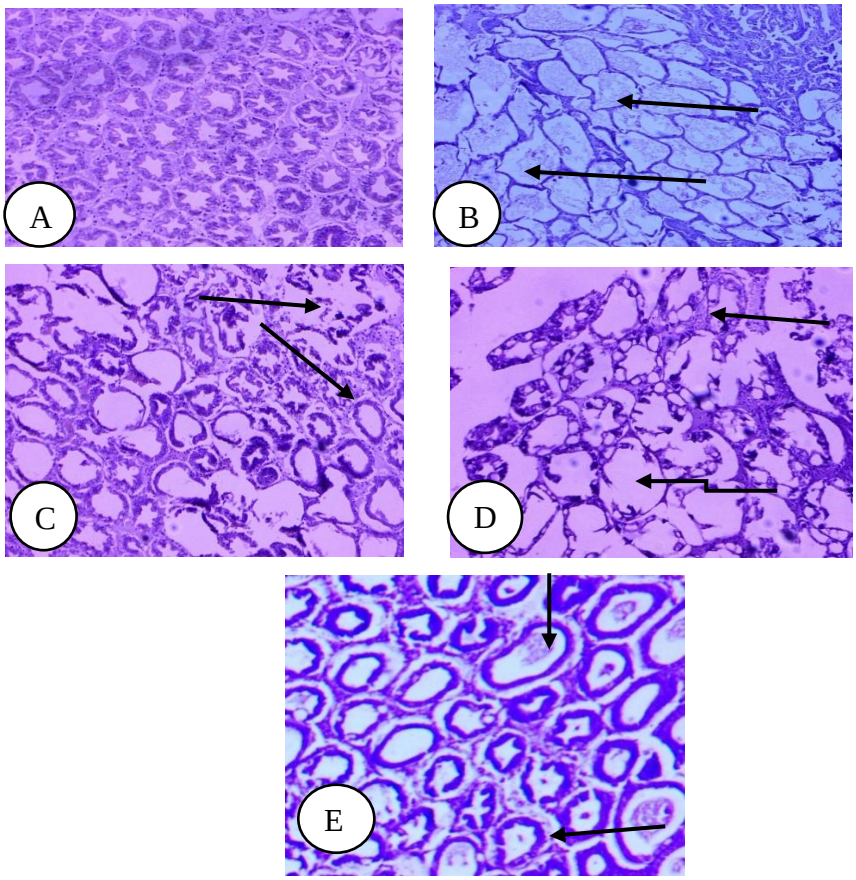
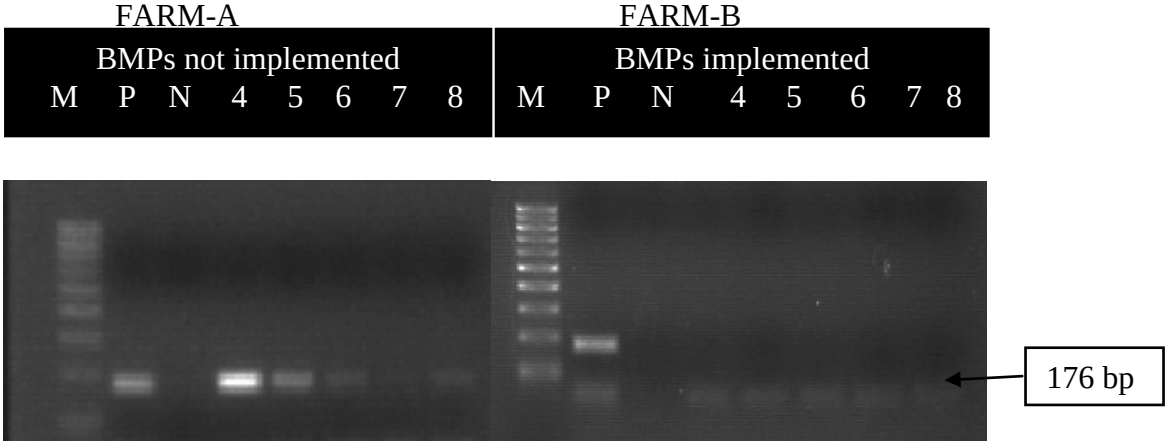


Figure.6 Comparison of two farms where (1) BMPs were not implemented (FARM -A) and (2) where BMPs were implemented (FARM –B). Agarose gel electrophoresis of samples screened for EHP by PCR. 2% agarose gel. Lane1(M)- Mol. wt. marker 100bp ladder; lane 2(P)- positive control (176 bp); lane 3(N)- negative control; lane 4-8 samples (Both cases)



Recently, growth retardation/slow growth due to EHP has become a matter of concern in shrimp farms across the world. It has now become one of the most serious emerging pathogen, leading to significant economic losses in shrimp culture industry. However there was no published data on the incidence of EHP in India until recently. In 2016, Rajendran *et al.*, reported the first incidence of EHP from shrimp farms of Andhra Pradesh and Tamil Nadu and estimated the overall incidence of EHP to be 96.50%. In the present investigation, the incidence was found to be 92.50% from the farms surveyed in the Nellore district, Andhra Pradesh.

During this study, the incidence of EHP infection was identified by both histopathology and PCR. The changes observed by histopathological examinations in the hepatopancreatic tissue were in agreement with the previous reports (Tourtip *et al*., 2009; Tangprasittipap *et al*., 2013; Rajendran *et al.*, 2016; Tang *et al*., 2016). The damage caused to the hepatopancreas affects the metabolic activities and growth of the shrimp, accounting for the retarded growth observed in shrimp ponds with high EHP incidence.

Risk factors in disease prevention

Among the sampled farms only 11 farms (9.17%) had obtained license from the Coastal Aquaculture Authority (CAA) of India. Generally CAA gives clear guidelines on the practices to be followed during *P. vannamei* culture. Reluctance to take the required permission from CAA clearly indicates that there were no recommended activities during pond preparation and culture days. Out of 120 farms, only 25 (20.83%) of the farms had implemented biosecurity. Adopting these measures is highly essential to prevent the entry of unknown or new pathogens and other aquatic species that may act as carriers for emerging diseases into cultured ponds and it is difficult to eradicate once it is established in a new location.

During sampling we could not observe Effluent Treatment System (ETS) installed in any of the culture farms. Discharge of effluent water without any treatment will eventually lead to contamination of surrounding environment and rapid spread of infectious pathogens. Among the farms surveyed, only 9.6% of farms had practiced chlorination and de-chlorination of water in reservoir ponds. Earlier studies have clearly shown the advantages of treating the water in reservoir ponds in farms before use in culture ponds (Emberson *et al*., 1999; Gunalan *et al*., 2011; Suresh *et al*., 2018). It is one of the most essential biosecurity measures to be implemented to prevent diseases. It was also observed that while 80% of farms procured tested Specific Pathogen Free (SPF) seed from certified hatcheries, many hatcheries sold seed produced from farm reared brood stock and the size of Post-Larvae (PL) was often very small (PL_{7-8}), increasing the chances of diseases and consequently, lower survival.

During sampling for EHP, white feces floating on pond surface was noticed in many of the ponds where reduced shrimp growth occurred. Tangprasittipap *et al.*, (2013) studied EHP and its causal relationship with White Feces Syndrome (WFS). They concluded that EHP is not the cause of WFS in *P. vannamei* culture. Later, Tang *et al.*, (2015) observed densely packed spores of the microsporidian EHP and relatively fewer numbers of rod-shaped bacteria within the white feces and stated that EHP is a cause of WFS in *P. vannamei*. Rajendran *et al.*, (2016) studied the incidence of EHP in Andhra Pradesh, India and reported an incidence of 39.7% in ponds without White Feces Syndrome (WFS) and very high incidence

(96.4%) in the ponds which experienced WFS. The usage of live feeds poses significant threat to shrimp culture; the infected live feeds can infect and spread EHP through feces (Newman, 2015). Tangprasittpap *et al.,* (2013) and Tang *et al.,* (2016) performed the feeding and cohabitation bioassay, and stated that EHP can be transmitted horizontally through cannibalism. Salachan*et al.,* (2017) stated that EHP can be directly transmitted to other shrimps via water. In the present study, aquatic macro fauna screened for EHP by nested PCR were positive, indicating that the shrimps, polychaetes, crabs and non-penaeids may act as carriers of EHP. Similar studies conducted by Tang *et al.,* (2015) and Chiyansuvata *et al.,* (2015) revealed that Artemia and Grapsidae family crabs were infected by EHP.

Role of Management in Disease Prevention

There is a need to develop control measures for Hepatopancreatic microsporidiasis caused by EHP. The present study was undertaken to analyze the role of management in disease prevention in aquaculture. After the active targeted surveillance in growth retarded shrimp farms, some important BMPs were implemented in shrimp farms where EHP was positive by histology and PCR analysis. The BMPs included proper pond preparation (Sludge removal, drying, ploughing, weed removal, application of lime @ 6000kg/Ha), stocking EHP negative tested high health seed at recommended stocking densities, crab fencing and using reservoir pond.

In the present study, after 85 days of culture, samples were collected and screened for EHP by PCR from both the farms. The samples tested for EHP were found negative in the BMP implemented farm compared to positive controls (Figure 6). The results clearly suggest that good management plays an important role in disease prevention. Significant improvement in growth and FCR were also observed in the pond which adopted BMPs (Data not shown).

With respect to management of shrimp farms against Hepatopancreatic microsporidiasis, farmers of Nellore district, Andhra Pradesh, used natural remedies for EHP and WFS control such as garlic paste, bitter gourd paste and onions paste with turmeric powder (freshly prepared) @10-20g/kg feed. Natural remedies are environment friendly. However, their activity to prevent/reduce EHP infection is still uncertain.

Tang *et al.,* (2016) found that usage of Fumagillin-B, an antimicrobial agent is ineffective in reducing or eliminating EHP spores in infected shrimps and reported that, farmers of Indonesia followed two types of management strategies to prevent EHP and WFS. 1. Application of probiotic to reduce *Vibrio spp*. populations in shrimp ponds, 2. Application of feed additives such as garlic (fresh or processed powder) @ 10-30g/kg feed to reduce the pathogens in shrimp digestive system; Vitamin C (2g/kg feed); and antiprotozoal such as Metronidazole.

Pond management alone is not sufficient to control diseases. We need to strictly implement other management measures, such as screening of seed and use of disease free high health seed alone for stocking, maintenance of good quality live feeds, proper pond preparation with biosecurity measures, regular health monitoring and proper disposal of infected animal from shrimp farms. Till date there is no known treatment for EHP. Studies need to be undertaken to find or develop effective but environment friendly control measures and treatments for EHP to achieve sustainability of shrimp culture.

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References

Bell, T.A., and Lightner, D.V. 1988. A handbook of normal penaeids shrimp histology. World Aquaculture Society, Baton Rouge, LA.

Chiyansuvata, P., Chantangsi, C., Chutmongkonkul, M., Tangtrongpiros, J., and Chansue, N. 2015. *Proceedings of the 14th Chulalongkom University Veterinary Conference.* Bangkok, Thailand.

Emberson, C. R., Samocha, T. M., and Wood, G. F. (1999). Use of ground saline water for commercial production of Litopenaeusvannamei in the Sonora Desert, Arizona, USA. *World Aquaculture*, *99*.

Giridharan, M., and Uma, A. 2017. A Report on the Hepatopancreatic Microsporidiosis Caused by *Enterocytozoon hepatopenaei* (EHP) in Penaeus vannamei (Pacific White Shrimp) Farms in Thiruvallur District, Tamilnadu, India. *International Journal of Current Microbiology and Applied Sciences. 6*(6): 147-152.

Gunalan, B., Soundarapandian, P., Kumaran, R., Anand, T., Kotiya, A.S., Maheswaran, C., and Pushparaj, N. (2011). Growth of Cultured White Leg Shrimp *LitopenaeusVannamei* (Boone 1931) In Different Stocking Density. *Advances in Applied Science Research*. 2 (3): 107-113.

Joshi, J., Srisala, J., Truong, V.H., Chen, I.T., Nuangsaeng, B., Suthienkul, O., Lo, C.F., Flegel, T.W., Sritunyalucksana, K., and Thitamadee, S. (2014). Variation in *Vibrio parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). *Aquaculture.* 428–429, 297–302.

Newman, S.G. (2015). Microsporidian Impacts shrimp production – industry efforts address control, not eradication. *Global Aquaculture Advocate*, 16-17(March/April).

Rajendran, K.V., Shivam, S., EzhilPraveena, P., SahayaRajan, J.J, Sathish Kumar, T, SatheeshaAvunje, Jagadeesan V., Prasad Babu SVANV, AshishPande, Navaneeth Krishnan A., Alavandi S.V., and Vijavan K.K. (2016). Emergence of *Enterocytozoon hepatopenaei* (EHP) in farmed *Penaeus* (*Litopenaeus) vannamei*in India. *Aquaculture*. 454: 272-280.

Salachan, P.V., Jaroenlak, P., Thitamadee, S., Itsathitphaisam, O., and Sritunyalucksana, K. (2017). Laboratory cohabitation challenge model for shrimp hepatopancreatic microsporidiosis (HPM) caused by *Enterocytozoon hepatopenaei* (EHP). *BMC Veterinary Research*. 13:9.

Stentiford, G.D., Feist, S.W., Stone, D.M., Bateman, K.S., and Dunn, A.M. (2013). Microsporidia: diverse, dynamic and emergent pathogens in aquatic systems. *Trends Parasitology*. 29: 567-578.

Suresh, K., Srinu, R., Pillai, D. and Rajesh, G. (2018). Hepatopancreatic Microsporidiasis (HPM) in shrimp culture: A Review. *International journal of current microbiology and applied sciences*. 7(01): 3208-3215.

Tang, K. F., Pantoja, C. R., Redman, R. M., Han, J. E., Tran, L. H., and 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.

Tang, K. F., Han, J. E., Aranguren, L. F., White-Noble, B., Schmidt, M. M., Piamsomboon, P and 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. and Sritunyalucksana, K. (2013). The microsporidian *Enterocytozoon hepatopenaei* is not the cause of white feces syndrome in whiteleg shrimp Penaeus (Litopenaeus) vannamei. *BMC veterinary research*. *9*(1): 139.

Thitamadee, S., Prachumwat, A., Srisala, J., Jaroenlak, P., Salachan, P.V, Sritunyalucksana, K., Flegel, T.W., and Itsathitphaisarn, O. (2016). Review of current disease threats for cultivated penaeid shrimp in Asia. *Aquaculture*. 452:69–87.

Tourtip, S., Wongtripop, S., Stentiford, G. D., Bateman, K. S., Sriurairatana, S., Chavadej, J., and Withyachumnarnkul, B. (2009). Enterocytozoonhepatopenaei sp. nov. (Microsporida: Enterocytozoonidae), a parasite of the black tiger shrimp *Penaeusmonodon* (Decapoda: Penaeidae): Fine structure and phylogenetic relationships. *Journal of Invertebrate Pathology*. *102*(1): 21-29.

Wittner, M., and Weiss, L. M. (1999). *The microsporidia and microsporidiosis*. ASM Press, Washington: D.C.

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