Original Research Article

<https://doi.org/10.20546/ijcmas.2018.703.131>

Screening for Incidence of Microsporidian Parasite *Enterocytozoon hepatopenaei* (EHP) in *Litopenaeus vannamei* from Aquaculture Ponds in SPSR Nellore District of Andhra Pradesh, India

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ABSTRACT

Keywords

EHP, HPM,
Parasite, PCR,
Shrimp, WFS

Article Info

Accepted:
10 February 2018
Available Online:
10 March 2018

Hepaotopancreatic Microsporidiosis (HPM) caused by *Enterocytozoon hepatopenaei* (EHP), a microsporidian parasite to be associated with slow (retarded or stunted) growth and white feces syndrome (WFS) in cultured shrimp in many of the shrimp growing countries in Asia, also in India. In the present study, shrimp samples from various shrimp ponds from different Mandals of SPSR Nellore district, Andhra Pradesh, India, were collected over a period of five months (February 2016 to June 2016). Important diagnosis observed were histopathological studies, molecular technique (PCR). Histologically, the infected animals showed severe degeneration of hepaotopancreatic tubule, basophilic inclusions resembling the developmental stages of EHP were found in the epithelial cells and large number of spore aggregations was observed in the tubular lumen. Enlargement of haemal sinuses was also observed in some cases. From this study, out of 50 pond case studies, 31 cases were showing EHP symptoms with 62 % prevalence by using tools of detection like PCR and histology.

Introduction

Shrimp farming is one of the most profitable and fastest-growing sectors of the aquaculture industry. Over the past decade, global farmed shrimp production has grown almost threefold from 1.13 million tons in 1999 to over 3.43 million tons in 2009 (Jory, 2010). China ranked first in shrimp aquaculture with 40% of total cultured shrimp production followed by Thailand (15%), Vietnam (12%) and

Indonesia (10%) (FAO, 2011). Shrimp continues to be the largest single commodity in value terms, accounting for about 15% of the total value of internationally traded fishery products in 2012. It is mainly produced in developing countries, and much of this production finds its way into the international trade (FAO, 2014). Penaeid shrimps (*Litopenaeus vannamei* and *Peneaus monodon*), which comprise around 80% of total farmed shrimp production (FAO, 2009).

According to the production statistics from Marine Products Export Development Authority (MPEDA), after the introduction of the species, export production has grown from 1731 metric tonne (mt) to 3,53,413 mt in 2014-15.

Andhra Pradesh has the second largest brackish water area in India after West Bengal, covering an area of about 37,560 ha besides the coastline of 972 km, spreading over nine districts namely Srikakulam, Vijayanagaram, Visakhapatnam, East Godavari, West Godavari, Krishna, Guntur, Prakasam and Sri Potti Sri Ramulu Nellore. Development of coastal aquaculture in Andhra Pradesh is centered on shrimp (*L. vannamei*) farming. The culture area increased from 264 Ha to 37,560 Ha during the period from 2009-10 to 2014-15. Andhra Pradesh (2, 76,077 mt) is the leading farmed shrimp producer with 78% and the rest of India production was 77, 336 mt (MPEDA, 2015). The shrimp (*L. vannamei*) exports from Andhra Pradesh increased to all time high and valued at more than Rs. 14,000 Crores for the year 2014-15 (MPEDA, 2015).

High stocking density (maximum permissible is 60 PLs/m²) and use of compounded pelleted feed in order to achieve higher production rates impose stress on the shrimps, making them susceptible to diseases (Alavandi *et al.*, 1995). The diseases may be caused by various etiological agents such as viruses, bacteria, fungi, parasites, algal toxins, nutritional deficiency or the adverse environment. In India, the gross economic losses due to shrimp (*P. monodon*) diseases were estimated at more than Rs. 1,000 crores in 2006-2008 and loss continues even now (Kalaimani *et al.*, 2013).

Several shrimp diseases are new or newly emerged in Asia that causes serious economic losses in shrimp farms, including Acute Hepatopancreatic Necrosis Disease (AHPND)

or Early Mortality Syndrome (EMS), Hepatopancreatic Microsporidiosis (HPM), Hepatopancreatic Haplosporidiosis (HPH), Aggregated Transformed Microvilli (ATM) and Covert Mortality Disease (CMD). In addition to these, White Spot Disease (WSD), Yellow Head Disease (YHD) and Infectious Myonecrosis (IMN) continued their share of losses (Thitamadee *et al.*, 2016).

The Global Aquaculture Alliance (GAA, 2013) has estimated that losses to the Asian shrimp culture sector amount to US\$ 1.0 billion. World farmed shrimp production volumes decreased in 2012 and particularly in 2013, mainly as a result of disease-related problems, such as Early Mortality Syndrome (EMS) (FAO, 2014).

In Nellore district the frequent outbreaks of diseases such as White Spot Syndrome Virus (WSSV), Black Gill Disease (BGD), Running Mortality Syndrome (RMS), Loose Shell Syndrome (LSS), White Faecal Syndrome (WFS), White Muscle Disease (WMD) and Infectious Hypodermal and Haematopoietic Necrosis (IHHN) in shrimps causing economic loss to the aquaculture industry (Srinivas *et al.*, 2016).

Recently, shrimp farms in Asia and other areas have been reporting heavy infection with a microsporidian parasite, *Enterocytozoon hepatopenaei* (EHP) in cultured *L. vannamei* impacting the production due to severe growth retardation (Newman, 2015).

Hepatopancreatic microsporidiosis (HPM) is caused by *Enterocytozoon hepatopenaei* (EHP), it was first reported as an unnamed microsporidian from growth retarded black tiger shrimp *Penaeus monodon* from Thailand in 2004 (Chayaburakul *et al.*, 2004). It was subsequently characterized in detail and named in 2009 (Tourtip *et al.*, 2009). During 2004, it was not statistically associated with

slow growth. Although EHP does not appear to cause mortality, recent information from shrimp farmers in Southeast Asian countries indicates that it is associated with severe growth retardation in *P. vannamei*. EHP outbreaks are occurring widely in China, Indonesia, Malaysia, Vietnam and Thailand. Very recently, EHP is also reported from slow growing shrimp in India. Thus, EHP is an emerging problem that is under urgent need of control (Sritunyaluksana *et al.*, 2014).

L. vannamei samples drawn from Andhra Pradesh, Tamil Nadu and Puri (Odisha) were tested positive for EHP by PCR, and some samples were found positive by histopathology (CIBA, 2015).

Stunting of *L. vannamei* in shrimp culture ponds for various reasons including EHP has created confusion among shrimp farmers and farmers are unable to harvest the crop though it is uneconomical to continue the crop with stunted shrimp.

Materials and Methods

Sampling area

The present study was carried out for a period of five months between February, 2016 to June, 2016. Shrimp (*L. vannamei*) with the sign of stunted growth were collected for this study from different shrimp farms located in Nidiguntapalem, Krishnapatnam village, Pottempadu, Bodiswamikandriga, Pantapalem of Muthukurmandal, Esuruwaka, Daruvukatta, Uttama Nellore, Konduru of Kota mandal, Dugarajapatnam, Mallam, Raghavaripalem, Kothagunta, Thuppaguntapalem, Tupilipalem of Vakadumandal, Chintavaram, Eruru village of Chillakurumandal, Ganagapatnam village of Indukuripetmadal, Mudivarthi village of Vidavalurumandal and Jadagogula village of Bhogolumandal, SPSR Nellore district, Andhra Pradesh, India.

Experimental shrimp

The experimental culture shrimp of the present study was *Litopenaeus vannamei* cultured in semi-intensive and intensive farms of the above mentioned areas.

Primers

Published universal primers were used for the amplification of ssu rRNA gene of *Enterocytozoon hepatopenaei* isolates. The names of the primers, sequence and amplification size are given below:

Collection of samples

Fifty (50) ponds were selected for study which were experiencing size variation/growth retardation and white feces syndrome. On each sampling day, a minimum of 60 shrimps were examined for diseases of species as per OIE guidelines (OIE, 2013). Information on behavioral abnormalities, gross and clinical signs were recorded on the sampling sheet. From each pond 2-4 shrimps were taken for diagnosis and the hepatopancreas of each sample were dissected out and fixed in Davidson's fixative for histopathology and along with Davidson's fixative from the 50 samples, 15 were separately fixed in 95% alcohol for molecular diagnosis (Bell and Lightner, 1984). Whole infected shrimps were also wrapped individually in sterile polythene bags, placed in icebox and brought to the laboratory. On reaching laboratory they were transferred to refrigerator and analyzed / processed.

Histopathology

Histopathology was conducted in the Department of Pathology, College of Veterinary Science, S.V.V.U. Tirupati. The hepatopancreas of infected and normal shrimps were fixed in alcoholic Davidson's

fixative for 48-72 h for comparative study. After fixation the tissues were transferred to 70% ethyl alcohol and kept overnight. Histopathological analysis was made as described by Roberts (2001)

Molecular diagnosis

Molecular diagnosis has done at OIE Reference Laboratory for WTD, Department of Zoology, C. Abdul Hakeem College, Melvisharam, Tamil Nadu.

DNA extraction

Hepatopancreas were homogenized in NTE buffer (0.2 M NaCl, 0.02 M Tris-HCl and 0.02 M EDTA, pH 7.4), and 10% tissue suspension was made. The suspension was centrifuged at 3000 g for 15 min at 4 °C, and supernatant was collected. The tissue suspension was mixed with an appropriate amount of digestion buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulphate and 0.1 mg mL⁻¹ proteinase K) and incubated for 2 h at 65°C to extract the DNA. After incubation, the digests were deproteinized by successive phenol/chloroform/isoamyl alcohol extraction and DNA was recovered by ethanol precipitation and dried. The dried DNA pellet was suspended in TE buffer and used as a template for PCR amplification.

Agarose gel electrophoresis

Polymerase chain reaction products were analyzed by electrophoresis in 0.8% agarose gels stained with ethidium bromide and visualized by ultraviolet transillumination.

DNA sequencing and analysis

The amplified PCR product was purified using Qiagen plasmid miniprep spin column. Sequence analysis was performed on an Auto-

read Sequencing kit (Applied Biosystems). The nucleotide sequence of *E. hepatopenaei* (small subunit rRNA gene) has been deposited in (Gen-Bank accession no. KU198278). The sequence was aligned using bioinformatics tools such as standard nucleotide BLAST and multiple sequence analysis clustalW (Thompson *et al.*, 1994). Significant similarity with sequences available in GenBank was searched using BLAST at National Center for Biotechnology Information (NCBI).

Results and Discussion

Clinical signs of infected shrimp

Shrimp (*L. vannamei*) with the sign of slow growth were collected for this study from different shrimp farms located in SPSR Nellore district, Andhra Pradesh, India. All the samples which we collected are slow growing as well as normally growing. These animals were apparently same except for reasons of slow growth and white fecal matter.

From the selected 50 ponds a shrimp population 4-10 animals with typical clinical symptoms (white feces and slow growth) were selected for diagnosis. Test results showed 31 pond shrimp samples (Table 1) were tested positive for EHP both by histopathology and PCR. Among the 31 pond samples, 15 samples were positive in slow growth with white feces syndrome and 16 pond samples were positive in slow growth without white feces.

The shrimp samples collected from white feces syndrome affected ponds were showing floating strands of white feces and some time the fecal strand was hanging from the anal portion of the shrimp. When the problem was severe, all the floating fecal strands were coming to sides of the pond, and it become easy for the pond manager to recognize the abnormality. Associated with the white feces

syndrome is drop in daily feed consumption, slow growth and some shrimp mortality also. The freshly dead shrimp also showed loose shell condition. During the study period, the white feces syndrome first appears 50–70 days of culture (DOC). After the appearance of white feces, shrimp health will deteriorate if some management interventions are not adopted. These include treatments with medicated feed (garlic etc.) and reduction in daily feed ration. In general, the shrimp in the WFS ponds showed FCR of over 2.92–3.17 (can be considered as 3.0) as compared to the range of normal growth ponds 1.83–1.94 (can be considered as 2.0).

Histopathology

Histologically, severe necrotic changes were noticed in the hepatopancreas (Fig. 1) whereas, EHP spores were observed in cytoplasm (Fig. 2). Developmental stages of EHP (Fig. 3) and large eosinophilic to basophilic inclusions indicating presumptive developmental stages of the microsporidian could be noticed in the tubular epithelium (Fig. 4). These stages were predominantly seen in the distal ends of hepatopancreatic tubules and most of the tubular epithelium in this region showed detachment from the basal membrane (Fig. 5 and 6).

The basal part of the tubular epithelium showed granular material and spore-like structures. Abnormally enlarged haemal sinuses were also noticed in the inter-tubular spaces (Fig. 7). In some of the sections, the spores were noticed in vacuolated structures. Sloughing of the tubular epithelial cells was pronounced in heavily infected HP and large spore aggregations were noticed in the tubular lumen (Fig. 8). In some of the hepatopancreas sections, large number of rod-shaped bacterial cells was also noticed in the tubular lumen indicating secondary infection.

Molecular characterization

The results of the targeted surveillance of EHP in *Litopenaeus vannamei* of SPSR Nellore district, Andhra Pradesh from February 2016 to June 2016 are presented in Table 1. In 0.8% agarose gel electrophoresis, samples with EHP infection show a band of PCR (510 bp) (Fig. 9).

Shrimp samples from the different villages were tested for EHP infection. Out of 50 pond shrimp samples, 31 samples were found to be EHP positive with 62% prevalence both by PCR and histologically (Table 1). Selected microsporidian isolate of *Enterocytozoon hepatopenaei* KU198278 was further characterized and identified through ssu rRNA analysis. The detailed information of the bacterial strain used, host species, clinical signs, site of infection, Gen Bank accession numbers are presented in Table 2.

Shrimp farms in Asia and other areas have been reporting heavy infection with a microsporidian parasite, *Enterocytozoon hepatopenaei* (EHP) in cultured *L. vannamei* impacting the production due to severe growth retardation (Newman, 2015). The parasite was first recorded from growth retarded tiger shrimp, *Penaeus monodon* from Thailand and reported as an undesigned microsporidian (Chayaburakul *et al.*, 2004).

Later, this parasite was identified in *P. monodon* and named as EHP by Tourtip *et al.*, (2009). The occurrence of this parasite was reported in pond-reared *P. monodon* from Vietnam, China, Indonesia, Malaysia and Thailand (Ha *et al.*, 2010) and in *P. stylirostris* from Brunei (Tang *et al.*, 2015). EHP is also reported from slow growing shrimp in India (Sritunyalucksana *et al.*, 2014) and very recently, its occurrence was reported in farm reared *L. vannamei* in India (Rajendran *et al.*, 2016).

Fig.1 Degenerative and necrotic tubules
H&E,x100

Fig.2 EHP spores present in the cytoplasm
H&E,x400

Fig.3 Development stages of EHP
H&E,x400

Fig.4 Basophilic inclusion (arrow; H&E, x400), EHP spores (star; H&E, x400)

Fig.5 EHP spores (star), basophilic inclusion (arrow) H&E,x400

Fig.6 Developmental stages of EHP spores (star) and detachment of tubular lumen (arrow) H&E, x400)

Fig.7 Enlargement of haemal sinus (star) and EHP spores (arrow) (H&E, x400)

Fig.8 EHP infected tissue section
(H&E,x400)

Fig.9 0.8% Agarose gel showing PCR product of EHP of naturally infected *Litopenaeus vannamei*

EHP

Lane M – DNA 100bp Marker

Lane N – Negative Control

Lane P – Positive Control

Lane 1 – Test Sample 1

Lane 2 – Test Sample 2

Lane 3 – Test Sample 3

Lane 4 – Test Sample 4

Primers

Primers	Sequence (5'-3')	Amplification size	Reference
	GCC TGA GAG ATG GCT CCC ACG T	510-bp	Tang <i>et al.</i> , 2015
	GCG TAC TAT CCC CAG AGC CCG A		

Table.1 Targeted surveillance of *Enterocytozoon hepatopenaei* in *Litopenaeus vannamei* of SPSR Nellore district, Andhra Pradesh from February 2016 to June 2016

Case No and Date	Location of Pond	Sign of Stunted growth & White Feces Syndrome	DOC	ABW (g) on Date of Sampling	Pond area (Ha)	PL's stocked	Confirmation of EHP	
							Histol ogy	PCR
1) 20.02.2016	Nidiguntapalem, Muthukur	Yes	125	20	1.0	400000	+ ve	+ ve
2) 20.02.216	Nidiguntapalem, Muthukur	Yes	110	16	0.8	250000	- ve	Not tested
3) 20.2.2016	Nidiguntapalem, Muthukur	Yes	105	16	0.4	100000	- ve	Not tested
4) 28.02.216	K.P.Village, Muthukur	Yes	55	6	0.4	100000	- ve	Not tested
5) 28.02.2016	K.P.Village, Muthukur	Yes	55	7	0.4	100000	- ve	Not tested
6) 28.02.216	K.P.Village, Muthukur	Yes	60	7	0.4	100000	- ve	Not tested

7) 28.02.2016	K.P.Village, Muthukur	Yes	60	7	0.4	100000	- ve	Not tested
8) 29.02.2016	Gangapatnam, Indukuripeta	Yes	115	25	0.8	400000	- ve	Not tested
9) 12.03.2016	Mudivarthi, Vidavaluriu	Yes	95	16.6	0.8	400000	- ve	Not tested
10) 16.03.2016	Ramudupalem, Vidavaluru	Yes	105	20	0.8	400000	- ve	Not tested
11) 21.03.2016	Ramudupalem, Vidavaluru	Yes	92	16	0.8	400000	- ve	Not tested
12) 26.03.2016	Gangapatnam, Indukuripeta	Yes	64	10	1.0	600000	- ve	Not tested
13) 12.04.2016	Pottempadu, Muthukur	Yes	50	8	0.8	400000	- ve	Not tested
14) 15.04.2016	Esuruwaka, Chittamuru	Yes	55	9	0.8	500000	- ve	Not tested
15) 15.04.2016	Esuruwaka, Chittamuru	Yes	50	8	0.8	500000	- ve	Not tested
16) 16.04.2016	Daruvkatta, Kota	Yes	55	6	1.2	700000	- ve	Not tested
17) 16.04.2016	Uttama Nellore, Kota	Yes	60	5	0.8	500000	- ve	Not tested
18) 03.05.2016	Utukuru, Vidavaluru	Yes	64	8	0.6	150000	- ve	Not tested
19) 03.05.2016	Mudivarthi, Vidavaluru	Yes	77	18	1.0	600000	- ve	Not tested
20) 05.05.2016	Pottempadu, Muthukur	Yes	90	16	0.4	200000	- ve	- ve
21) 05.05.2016	Jadagogula, Bhogolu	Yes	44	4	0.48	400000	- ve	Not tested
22) 05.05.2016	Jadagogula, Bhogolu	Yes	50	5	0.8	300000	- ve	Not tested
23) 07.05.2016	Kandriga, Muthukur	Yes	60	10	0.4	150000	- ve	Not tested
24) 25.05.2016	Mallam, Vakadu	Yes	105	14	1.0	300000	- ve	Not tested
25) 25.05.2016	Mallam, Vakadu	Yes	95	10	1.0	500000	+ ve	+ ve
26) 25.05.2016	Mallam, Vakadu	Yes	78	9	0.4	200000	- ve	Not tested
27) 25.05.2016	Raghavaripal em, Vakadu	yes	85	10	1.0	400000	- ve	Not tested
28) 25.05.2016	Kothagunta, Vakadu	Yes	90	12.5	1.0	400000	- ve	Not tested
29) 26.05.2016	Chintavaram	Yes	90	12.5	1.0	500000	+ ve	+ ve
30) 26.05.2016	Eruru	Yes	90	14	0.8	300000	- ve	Not tested
31) 26.05.2016	Eruru	Yes	84	12.5	0.8	200000	- ve	Not tested
32) 26.05.2016	Eruru	Yes	85	12	1.0	400000	- ve	Not tested

33) 27.05.2016	Tupilipalem	Yes	90	14	1.2	400000	- ve	Not tested
34) 27.05.2016	Tupilipalem	Yes	80	11.1	1.2	400000	- ve	- ve
35) 27.05.2016	Tupilipalem	Yes	80	8	0.8	400000	+ ve	+ ve
36) 06.06.2016	Pantapalem, Muthukur	Yes	104	17	0.8	300000	- ve	- ve
37) 07.06.2016	B.S.Kandriga, Muthukur	Yes	58	9	1.0	200000	- ve	- ve
38) 08.06.2016	B.S.Kandriga, Muthukur	Yes	53	4	0.6	200000	- ve	- ve
39) 14.06.2016	Pantapalem	Yes	104	17	0.8	300000	- ve	- ve
40) 27.06.2016	Dugarajapatna m, Vakadu	Yes	105	13	0.8	400000	- ve	- ve
41) 27.06.2016	Dugarajapatna m, Vakadu	Yes	105	15	0.8	400000	- ve	- ve
42) 27.06.2016	Dugarajapatna m, Vakadu	Yes	100	16	0.8	400000	- ve	- ve
43) 27.06.2016	Dugarajapatna m, Vakadu	Yes	115	16.6	0.8	400000	+ ve	+ ve
44) 27.06.2016	Konduru, Vakadu	Yes	125	30	1.2	400000	- ve	- ve
45) 27.06.2016	Konduru	Yes	45	6	1.0	400000	- ve	Not tested
46) 27.06.2016	Tupilipalem	Yes	85	16.6	0.8	500000	- ve	Not tested
47) 27.06.2016	Tupilipalem	Yes	85	11.1	0.8	500000	+ ve	+ ve
48) 27.06.2016	Tupilipalem	Yes	90	10	0.8	500000	+ ve	+ ve
49) 27.06.2016	Tupilipalem	Yes	88	12.5	1.0	500000	- ve	Not tested
50) 28.06.2016	Thuppagunta palem	Yes	86	11	0.8	500000	+ ve	+ ve

Table.2 Molecular characterization of EHP strain isolated from infected/
diseased cultured shrimp

Shrimp species	Disease/Clinical sign	Site of infection	Length of consensus sequence (bp)	Gen Bank Accession number	Identification
<i>Litopenaeus vannamei</i>	Stunted growth/White feces Syndrome	Hepatopancreas	510	KU198278	EHP

Histologically, heavily infected hepatopancreas showed severe necrotic changes as evidenced by sloughing of tubular epithelial cells, degenerated cells and spore accumulation in the tubular lumen. In the present study histological features are observed in accordance with the reports on the hepatopancreatic microsporidian infection in *P. monodon* and *L. vannamei* (Chayaburakul *et al.*, 2004 and Tourtip *et al.*, 2009). Further, the parasite affinity for the epithelial cells has been reported for the genus *Enterocytozoon*, including the human-infecting species *E. bienersi* (Desportes *et al.*, 1985). Some of the histological changes observed in the infected hepatopancreas of *P. vannamei* due to EHP also showed similarity with *Enterospora canceri* reported from crab (Stentiford *et al.*, 2007).

PCR and Light microscopic observation of *L. vannamei* from 50 ponds located in SPSR Nellore district revealed the prevalence of EHP infection (16%). However, PCR screening from 15 ponds showed relatively high prevalence of EHP infection (33%) compared to histopathology results.

The prevalence of EHP was estimated to be 63.5% for 137 samples by Rajendran *et al.*, (2016). In the present study the EHP prevalence was observed as 16% for 50 samples. If we calculate these 50 samples as 137, the prevalence was 23%. Rajendran *et al.*, (2016) collected animals from white faeces syndrome (WFS) affected pond showed higher prevalence of EHP (96.4%) compared to those from the unaffected pond (39.7%). But in the present study, WFS affected pond showed low prevalence (12%) for 25 samples compared to those from only stunted growth ponds (20%) for 25 samples. We agree with the statement given by Rajendran *et al.*, (2016) that the EHP could be detected from slow growing as well as WFS-affected animals.

Previously Ha *et al.*, (2010) had been reported that the association of microsporidian *Enterocytozoon hepatopenaei* with white faeces syndrome (WFS) and Felgel (2012) has indicated that severe infection with a microsporidian morphologically similar to *E. hepatopenaei* was associated with WFS of *P. vannamei*. In the present study WFS affected ponds showed low prevalence. Ponds with stunted growth only but no WFS showed higher prevalence. These results are comparable to the statement of Tangprasittipap *et al.*, (2013) that EHP is not the cause of WFS.

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How to cite this article:

Raveendra, M., P. Hari Babu, T. Neeraja, D. Pamanna, N. Madhavan, A.S. Sahul Hameed and Srilatha, Ch. 2018. Screening for Incidence of Microsporidian Parasite *Enterocytozoon hepatopenaei* (EHP) in *Litopenaeus vannamei* from Aquaculture Ponds in SPSR Nellore District of Andhra Pradesh, India. *Int.J.Curr.Microbiol.App.Sci.* 7(03): 1098-1109.
doi: <https://doi.org/10.20546/ijcmas.2018.703.131>