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High prevalence of *Enterocytozoon hepatopenaei* in shrimps *Penaeus monodon* and *Litopenaeus vannamei* sampled from slow growth ponds in India

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ABSTRACT: Hepatopancreatic microsporidiosis in cultivated *Litopenaeus vannamei* and *Penaeus monodon* is caused by the newly emerged pathogen *Enterocytozoon hepatopenaei* (EHP). It has been detected in shrimp cultured in China, Vietnam and Thailand and is suspected to have occurred in Malaysia and Indonesia and to be associated with severely retarded growth. Due to retarded shrimp growth being reported at farms in the major grow-out states of Tamilnadu, Andhra Pradesh and Odisha in India, shrimp were sampled from a total of 235 affected ponds between March 2014 and April 2015 to identify the presence of EHP. PCR and histology detected a high prevalence of EHP in both *P. monodon* and *L. vannamei*, and infection was confirmed by *in situ* hybridization using an EHP-specific DNA probe. Histology revealed basophilic inclusions in hepatopancreas tubule epithelial cells in which EHP was observed at various developmental stages ranging from plasmodia to mature spores. The sequence of a region of the small subunit rDNA gene amplified by PCR was found to be identical to EHP sequences deposited in GenBank. Bioassays confirmed that EHP infection could be transmitted orally to healthy shrimp. Histology also identified bacterial co-infections in EHP-infected shrimp sampled from slow-growth ponds with low-level mortality. The data confirm that hepatopancreatic microsporidiosis caused by EHP is prevalent in shrimp being cultivated in India. EHP infection control measures thus need to be implemented urgently to limit impacts of slowed shrimp growth.

KEY WORDS: Microsporidian · Slow growth · SSU rDNA · Plasmodia · *Vibrio* · Hepatopancreas · EHP

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INTRODUCTION

Enterocytozoon hepatopenaei (EHP) has been reported in both *Penaeus monodon* (Chayaburakul et al. 2004, Tourtip et al. 2009) and *Litopenaeus vannamei* (Tangprasittipap et al. 2013) farmed in Thailand and also in farmed *P. monodon* exhibiting white faeces syndrome (WFS) in Vietnam (Ha et al. 2010). However, whether EHP is involved directly in causing a similar condition in *L. vannamei* has been questioned due to its presence in both affected and unaffected shrimp, and to the direct oral transmission of EHP to healthy shrimp not resulting in WFS (Tangprasittipap et al. 2013).

Microsporidians have been detected in *P. monodon* described as being affected by monodon slow growth syndrome (MSGS) (Chayaburakul et al. 2004). Based on *in situ* hybridization observations of hepatopancreas histopathology in *L. vannamei* infected with EHP at various severities, EHP has also been suggested to

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have the potential to cause slowed growth in this species (Tangprasittipap et al. 2013). Moreover, the widespread detection of EHP in *L. vannamei* being farmed in China, Indonesia, Malaysia, Vietnam, Thailand and India has implicated it to be the likely cause of the severely retarded growth currently being reported in these countries (Sritunyalucksana et al. 2014).

Pathogen and disease prevalence data are useful for identifying locations in which effective control and management measures have the greatest potential to mitigate production losses. Together with anecdotal reports of EHP being the likely cause of retarded growth in *P. monodon* and *L. vannamei* in the major farming states in India, this motivated us to examine shrimp from ponds displaying slow growth for evidence of EHP infection.

MATERIALS AND METHODS

Shrimp

Between March 2014 and April 2015, shrimp were sampled from farms in Andhra Pradesh, Tamilnadu and Odisha where slow growth was being reported. A total of 235 ponds were sampled. Hepatopancreas (HP) tissue sampled from 5 shrimp from each pond was pooled and preserved in 95% ethanol for PCR analysis. A total of 976 shrimp (4–5 per pond) were fixed in Davidson's AFA fixative for histology (Lightner 1996). Sample details are summarized in Table 1. Shrimp from selected ponds were used in a bioassay. All samples were analysed at the Central Aquaculture Pathology Laboratory, Rajiv Gandhi Centre for Aquaculture.

DNA extraction

DNA was extracted from ~120–150 mg pooled HP tissue using a High Pure PCR Template Preparation Kit (Roche Diagnostics) and quantified using a Bio Photometer Plus spectrophotometer (Eppendorf).

EHP PCR

The EHP nested PCR test targeted a fragment of the EHP SSU rDNA gene and employed PCR (ENF779/ENR779) and nested PCR (ENF176/ENR176) primers and reaction conditions described previously (Tangprasittipap et al. 2013). PCR (25 µl) was conducted using 100 ng DNA and EmeraldAmp GT PCR master mix (Takara Bio) according to the manufacturer's instructions. The thermal cycling conditions used for the PCR were 94°C for 3 min, 35 cycles of 94°C for 20 s, 58°C for 20 s and 72°C for 45 s, followed by 72°C for 5 min, and for the nested PCR were 94°C for 3 min, 35 cycles of 94°C for 20 s, 64°C for 20 s and 72°C for 20 s, followed by 72°C for 5 min. The nested PCR (25 µl) was performed as for the PCR except for the use of 2 µl PCR as template. Following electrophoresis in a 2% agarose gel, amplified DNA was visualized using a UVP UV transilluminator.

Histology

Fixed HP tissue was processed into wax blocks according to standard histology procedures (Bell & Lightner 1988). Sections (4–5 µm thick) were cut

Table 1. Numbers of slow growth ponds sampled from different regions in India in which *Enterocytozoon hepatopenaei* (EHP) was detected by PCR and nested PCR testing of DNA extracted from hepatopancreas tissue pooled from 5 shrimp (*Litopenaeus vannamei* and *Penaeus monodon*)

State	District	Pond number: Total		Pond number: EHP PCR +ve		Pond number: EHP nested PCR +ve (%)	
		L. vannamei	*P. monodon*	*L. vannamei*	*P. monodon*	*L. vannamei*	*P. monodon*
Tamilnadu	Nagapattinam	54	4	13	1	40 (74)	2 (50)
	Thanjavur	7	0	1	0	2 (29)	0
	Cuddalore	5	0	1	0	2 (40)	0
	Thiruvarur	4	0	0	0	2 (50)	0
Andhra Pradesh	Nellore	62	7	12	1	44 (71)	2 (29)
	West Godavari	31	6	14	2	21 (68)	4 (67)
	East Godavari	2	0	0	0	1 (50)	0
	Bapatla	6	0	1	0	3 (50)	0
Odisha	Balasore	21	11	9	1	18 (86)	4 (36)
	Bhadrak	4	11	2	3	3 (75)	7 (64)
Total		196	39	51	8	136 (69)	19 (49)

using a RM2125RTS rotary microtome (Leica), mounted onto glass slides, stained using haematoxylin and eosin (H&E) and observed using a DM750 light microscope (Leica). Digital images were captured using a Leica EC3 camera and the Leica Application suite Version 2.0.0.

In situ hybridization

To prepare a DIG-labelled DNA for *in situ* hybridization, an EHP SSU rDNA gene fragment was amplified by PCR using the ENF779F/ENF779R primer pair (Tangprasittipap et al. 2013) and the DIG-PCR labelling kit (Roche). Labelled DNA was precipitated in 95% ethanol in the presence of LiCl, washed with 70% ethanol and re-suspended in 30 µl nuclease-free water. HP tissue sections (3–4 µm thick) from 10 shrimp that were PCR-positive for EHP were fixed onto positively charged glass slides and digested with 100 µg ml^{-1} Proteinase K (Invitrogen) in Tris–NaCl–EDTA buffer at 37°C for 15 min. Sections were then pre-hybridized under 500 µl 2× saline sodium citrate containing 50% (v/v) deionized formamide at 42°C for 30 min. The pre-hybridization solution was replaced with 200 µl hybridization solution containing ~20 ng DIG-labelled DNA probe per slide, and the tissue was covered with a coverslip and incubated overnight at 42°C in a Slide Moat humidity chamber (Thermo Scientific). Slides were washed carefully for 5 min in Buffer I (1 M Tris-HCl, 1.5 M NaCl) and tissue sections were covered with 500 µl blocking solution (Roche) at 42°C for 15 min. This solution was removed, remaining traces were blot drained using filter paper and the section was covered with alkaline phosphatase-conjugated anti-DIG antibody diluted 1:1000 in Buffer II (50 ml 1× Buffer I containing 0.25 g Roche Blocking reagent). Slides were washed twice, equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$, pH 9.5) and colour signal was developed by adding NBT-BCIP substrate (Roche) followed by counterstaining using Bismarck Brown Y (Sigma). Tissue sections were observed and photographed using a Leica EC3 camera and Leica Application Suite Version 2.0.0.

Shrimp challenge by feeding HP tissue

Juvenile specific pathogen free (SPF) *L. vannamei* (2–3 g) were stocked into 5 × 50 l plastic tanks (n = 20 per tank). Shrimp in 3 tanks were fed once daily with EHP-infected shrimp HP tissue and once daily with commercial feed pellets for 10 d. Shrimp in the other 2 tanks were fed similarly for 10 d using HP tissue from SPF shrimp. After 10 d, shrimp from both groups were fed twice daily using commercial feed pellets until the challenge trial was terminated on Day 30. During the trial, faecal samples were collected daily from all tanks and frozen for subsequent PCR analysis. At the end of the trial, surviving shrimp were euthanized to sample HP tissue for PCR analysis and histology.

EHP SSU rDNA gene sequence analysis

DNA products amplified by PCR using the ENF779/ENR779 primer pair were purified using a QIAquick Gel Extraction Kit (QIAGEN) and sequenced in both orientations using either ENF779 or ENR779 primers. Sequence chromatograms were analysed using BioEdit version 7.2.5 (Hall 1999) to generate a consensus sequence. BLASTn (NCBI) was used to identify matching sequences in GenBank, and ClustalW (Thompson et al. 1994) was used to generate multiple alignments. The consensus SSU rDNA sequences were deposited in GenBank (accession nos KR021167, KR021168 and KR021169).

RESULTS AND DISCUSSION

Among the pools of HP tissue sampled from 5 shrimp from each of the 235 ponds examined from farms reporting growth retardation, 59 (25%) were identified to be PCR-positive for EHP, and 155 (66%) were identified to be nested PCR-positive for EHP (Fig. 1, Table 1). Through nested PCR, HP pooled from shrimp sampled from 69% of the *L. vannamei* ponds and 49% of the *P. monodon* ponds were EHP-positive.

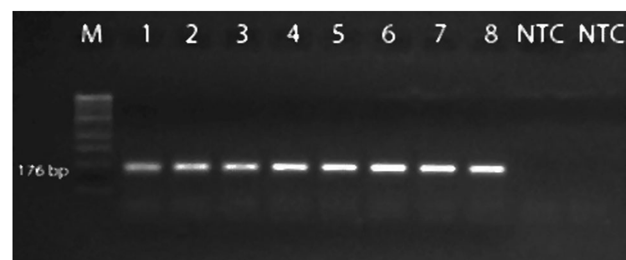

Fig. 1. Representative image showing *Enterocytozoon hepatopenaei* (EHP) SSU rDNA PCR products amplified from hepatopancreas pooled from groups of 5 EHP-infected *Litopenaeus vannamei.* Lane M: 100 bp ladder; Lanes 1–7: EHP-positive samples; Lane 8: EHP-positive control; Lanes 9, 10: no-template controls (NTC)

HP tissue from individual shrimp analysed in parallel by histology identified 468/976 (50%) with evidence of EHP infection (Figs. 2–4). EHP-infected shrimp were characterized by HP tubules containing numerous microsporidian spores appearing as acidophilic structures confined to vacuoles within the cytoplasm of reserve, blister, fibrillar and embryonic cells. No spores were observed in midgut and hindgut epithelial cells or cells of the other tissues and organs. Various microsporidian development stages, including early and late plasmodia and mature spores, were observed in the cytoplasm of the HP tubules. Mature spores were also observed free in the lumen of HP tubules. EHP-infected HP tubule cells were also often surrounded by aggregated haemocytes.

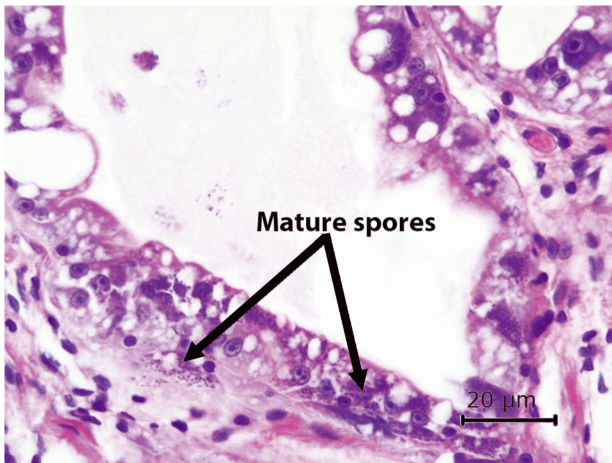

Fig. 2. H&E-stained hepatopancreas tissue section showing acidophilic, granular inclusions in the cytoplasm of tubule epithelial cells of a *Litopenaeus vannamei* infected with *Enterocytozoon hepatopenaei* (40× magnification)

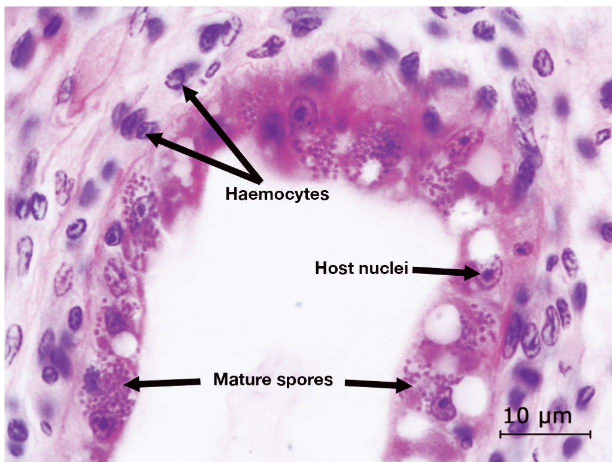

Fig. 3. H&E-stained hepatopancreas tissue section showing acidophilic, granular inclusions in the cytoplasm of the tubule epithelial cells with surrounded haemocytic aggregation, host nuclei and mature spores (100× magnification)

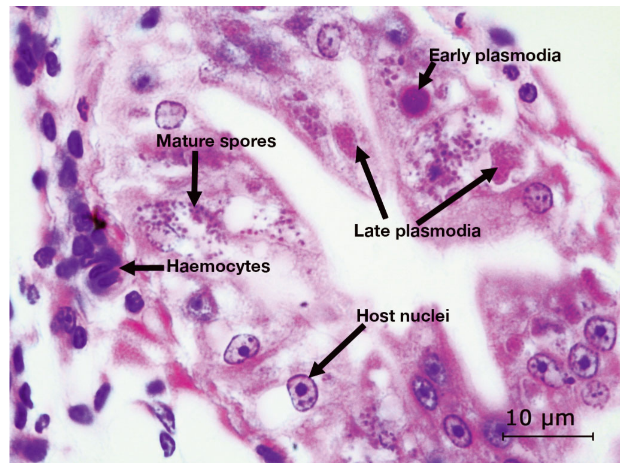

Fig. 4. H&E-stained hepatopancreas tissue section showing several development stages of *Enterocytozoon hepatopenaei* (early plasmodia, late plasmodia and mature spores) in the cytoplasm of tubule epithelial cells. The image also shows aggregated haemocytes surrounding the tubules (100× magnification)

In situ hybridization analysis of histology sections using the DIG-labelled EHP SSU rDNA probe showed strong positive hybridization signal in parallel sections of EHP-infected HP tubule cells (Fig. 5). No signal occurred in other tissues.

Of the 468 shrimp showing histological evidence of EHP infection, 148 (32%) also possessed bacterial co-infections, most likely *Vibrio* species, described generally as septic hepatopancreatic necrosis (SHPN)

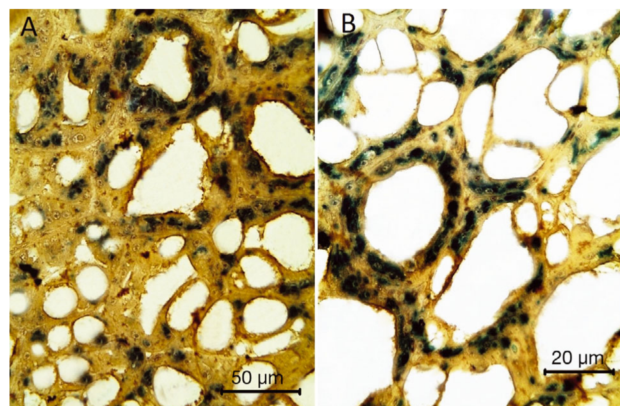

Fig. 5. *Enterocytozoon hepatopenaei* (EHP) *in situ* hybridization signal (dark blue/green colour) in hepatopancreas tubule epithelial cells of *Litopenaeus vannamei* infected with *Enterocytozoon hepatopenaei*. (A) 10× magnification; (B) 40× magnification

Table 2. Prevalence of *Enterocytozoon hepatopenaei* (EHP) infection, and of bacterial co-infection as evidenced by septic hepatopancreatic necrosis (SHPN), based on histological examination of 4–5 shrimp (*Litopenaeus vannamei* and *Penaeus monodon*) from each slow-growth pond

State	District	Pond number		Shrimp number			
		L. vannamei	*P. monodon*	Total	EHP +ve [no. (%)] Without SHPN	EHP +ve [no. (%)] With SHPN	EHP and SHPN +ve (%)
Tamilnadu	Nagapattinam	54	4	236	94 (40)	41 (17)	30
	Thanjavur	7	0	27	1 (14)	0	
	Cuddalore	5	0	21	0	0	
	Thiruvarur	4	0	16	0	0	
Andhra Pradesh	Nellore	62	7	278	93 (11)	47 (17)	34
	West Godavari	31	6	161	53 (33)	27 (17)	34
	East Godavari	2	0	09	0	0	
	Bapatla	6	0	26	2 (8)	0	
Odisha	Balasore	21	11	141	47 (33)	23 (16)	33
	Bhadrak	4	11	61	30 (49)	10 (16)	25
Total		196	39	976	320 (33)	148 (15)	32

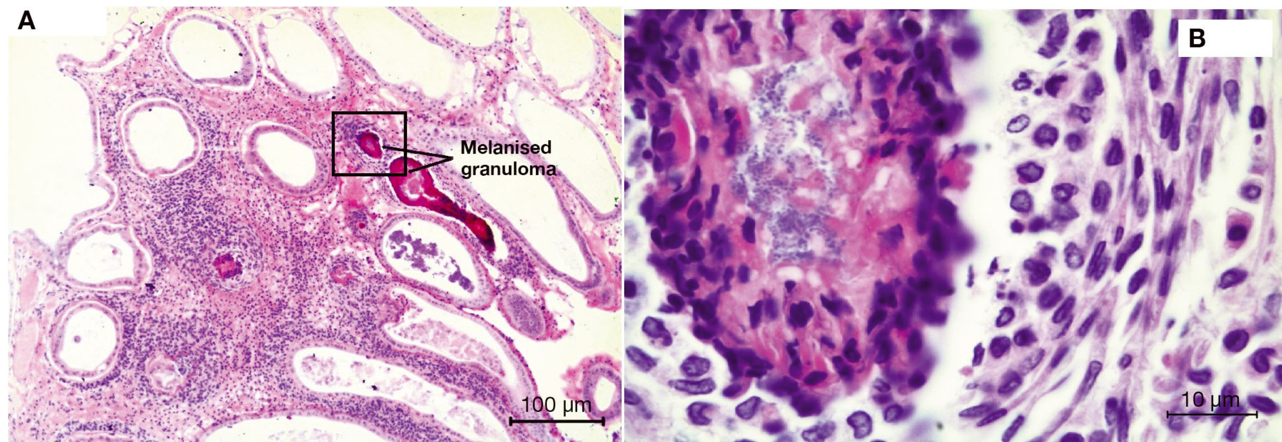

Fig. 6. H&E-stained hepatopancreas tissue section of a *Litopenaeus vannamei* infected with *Enterocytozoon hepatopenaei* showing melanised granulomatous lesions and severe haemocytic aggregation associated with bacterial co-infection. (A) 10× magnification; (B) 100× magnification

(Table 2, Fig. 6). SHPN was also found in some shrimp displaying no evidence of EHP infection (data not shown). Whether the bacteria represented opportunistic secondary infections that assisted in contributing to low-level mortality often noted in EHP-affected ponds is not known. However, shrimp sampled from affected ponds with low-level mortality tended to show EHP infection together with bacterial lesions. Shrimp infected with EHP were generally smaller than non-infected shrimp of the same age, and the severity of growth retardation was generally correlated with increased infection severity as graded by histology (Table 3).

PCR and histology were used to show that EHP infection could be readily transmitted to naive SPF *L. vannamei* by feeding them HP tissues sampled from EHP-infected shrimp, as reported previously (Tangprasittipap et al. 2013). Using histology, all challenged shrimp displayed characteristic features of EHP microsporidian infection in HP tubules, and nested PCR also detected the presence of EHP DNA in faeces collected from tanks containing EHP-challenged shrimp.

BLASTn analysis of the SSU rDNA sequence amplified by PCR from 3 independent shrimp showed them to be identical to that of an *Enterocytozoon hepatopenaei* strain from Thailand (GenBank KF362129) and to be 89 % and 88 % identical, respectively, to the cognate SSU rDNA sequences of *Enterocytozoon salmonis* and *Enterocytozoon bieneusi*. This finding suggests a close phylogenetic and potential epidemiological relationship between EHP strains affecting shrimp being farmed in Thailand and India.

Table 3. Representative data on shrimp size relative to *Enterocytozoon hepatopenaei* (EHP) infection severity as graded by histology. Sample code relates to location of sampling: NGP: Nagapattinam; TN: Tamilnadu; NLR: Nellore; AP: Andhra Pradesh; WGD: West Godavari District; EGD: East Godavari District; BLS: Balasore; OD: Odisha; BHK: Bhadrak. EHP infection severity as graded by histology: G0 < G1 < G2 < G3 < G4, where G0 represents no signs of infection and G4 represents a high number of parasites or severe infection

Number	Sample code	Days of culture	Body mass range (g)	Histology grade
1	NGP-TN-01	50	2–3	G2
2	NGP-TN-02	65	4–5	G3
3	NGP-TN-03	59	3–4	G3
4	NGP-TN-04	60	3–4	G3
5	NGP-TN-05	67	7–8	G2
6	NGP-TN-06	67	7–8	G2
7	NGP-TN-07	93	9–10	G1
8	NGP-TN-08	73	7–8	G1
9	NGP-TN-09	48	2–3	G2
10	NGP-TN-10	70	8–9	G1
11	NLR-AP-01	57	4–5	G2
12	NLR-AP-02	57	3–4	G3
13	NLR-AP-03	58	3–4	G3
14	NLR-AP-04	58	3–4	G3
15	WGD-AP-01	65	6–7	G2
16	WGD-AP-02	90	8–9	G2
17	WGD-AP-03	125	17–18	G2
18	EGD-AP-01	63	4–5	G2
19	EGD-AP-02	58	4–5	G2
20	EGD-AP-03	65	5–6	G2
21	BLS-OD-01	49	2–3	G2
22	BLS-OD-02	62	5–6	G3
23	BHK-OD-01	57	3–4	G3
24	BHK-OD-02	58	4–5	G3

Taken together, the data presented here indicate that EHP is prevalent in shrimp ponds showing slow growth in the Indian coastal states of Andhra Pradesh, Tamilnadu and Odisha. As EHP is an emerging pathogen of growing concern to shrimp aquaculture (Sritunyalucksana et al. 2014), targeted management systems and biosecurity measures in hatcheries and farms are required to limit its impact and spread. Nested PCR screening of broodstock and postlarvae needs to be examined for its effectiveness. As EHP is a spore-forming parasite, water disinfection (Sritunyalucksana et al. 2014) and the identification of potential carrier species will also be critical to such measures.

The severity of *E. hepatopenaei* infection in many of the shrimp examined suggests that the developing parasite might impose a high energy demand on the host, which may impact growth (Tangprasittipap et al. 2013). While slowed growth appears to be the primary impact of EHP infection, evidence of bacterial co-infections in affected shrimp sampled from ponds with low-level mortality suggests that it could be useful to examine its potential role in mortality. Moreover, while the number of shrimp examined here from each pond was low, the reasons for retarded shrimp growth in ponds in which EHP was not detected also need to be investigated.

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LITERATURE CITED

Bell TA, Lightner DV (1988) A handbook of normal penaeid shrimp histology. The World Aquaculture Society, Baton Rouge, LA

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. Dis Aquat Org 60:89–96

Ha NTH, Ha DT, Thuy NT, Lien VTK (2010) *Enterocytozoon hepatopenaei* parasitizing on tiger shrimp (*Penaeus monodon*) infected by white feces culture in Vietnam, has been detected. Agric Rural Develop Sci Technol 12: 45–50 (In Vietnamese with English abstract)

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Lightner DV (1996) A handbook of shrimp pathology and diagnosis procedures for diseases of cultured penaeid shrimp. World Aquaculture Society, Baton Rouge, LA

Sritunyalucksana K, Sanguanrut P, Vinu Salachan P, Thitamadee S, Flegel TW (2014) Urgent appeal to control spread of the shrimp microsporidian parasite *Enterocytozoon hepatopenaei* (EHP). Available at www.enaca.org. News posted on 24/11/2014

Tangprasittipap A, Srisala J, Chouwdee S, Somboon M and others (2013) The microsporidian *Enterocytozoon hepatopenaei* is not the cause of white feces syndrome in white leg shrimp *Penaeus* (*Litopenaeus*) *vannamei.* BMC Vet Res 9:139

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Tourtip S, Wongtripop S, Stentiford GD, Bateman KS and others (2009) *Enterocytozoon hepatopenaei* sp. nov. (Microsporida: Enterocytozoonidae), a parasite of the black tiger shrimp *Penaeus monodon* (Decapoda: Penaeidae): fine structure and phylogenetic relationships. J Invertebr Pathol 102:21–29

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