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Assessment of Incidence of *Enterocytozoon hepatopenaei* (EHP) in *Penaeus vannamei* in Maharashtra and Gujarat

Pani Prasad Kurcheti*, Abisha Juliet Mary. S.J. and Dhayanath, M.

Division of Aquatic Environment and Health Management, ICAR- Central Institute of Fisheries Education, Mumbai, INDIA

**Corresponding author: PP Kurcheti; Email : kpaniprasad@cife.edu.in*

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ABSTRACT

The occurrence of *Enterocytozoon hepatopenaei* in *Penaeus vannamei* samples were collected from Maharashtra and Gujarat farms. In the present study, shrimp samples from various shrimp ponds from two districts of Maharashtra and two districts of Gujarat were collected over a period of one year (February 2016 to April 2017). A total of 4513 shrimp samples were assessed for the presence of EHP by molecular characterization. Out of shrimp samples analysed, 31.2% samples were positive for EHP. The screening of EHP was done by single step and nested PCR targeting spore wall protein gene (SWP) of EHP resulting in product size of 514 bp and 148 bp for EHP respectively.

Keywords: *Penaeus vannamei,* EHP, Maharashtra, Gujarat and Nested PCR

Rapid growth in our country has been witnessed in both the production and export, in which shrimp is a major contributor in terms of both quantity and value. This tremendous growth in the aquaculture sector is mainly due to the decline in the capture fisheries. As per the MPEDA release, Andhra Pradesh occupies the first position in India's largest cultured shrimp *(Penaeus vannamei, Penaeus monodon and* scampi*)* production, which is followed by West Bengal, Tamilnadu and Puducherry, Gujarat, and Odisha. Among them, tiger shrimp (*Penaeus monodon*) are farmed in the states of West Bengal, Odisha and Kerala, while the *vannamei* species is preferred in the states of Andhra Pradesh, Gujarat, Tamil Nadu and Odisha. The estimated the productivity of vannamei in Maharashtra was estimated as 6118 tons/ yr whereas the Productivity in Gujarat was estimated as 32946 ton/yr. Although shrimp industry growth in the last five years has been increasing, challenges exist in the form of diseases. In India, the major causes for disease breakout in shrimp aquaculture were due to white spot syndrome virus (WSSV) and infectious hypodermal haematopoietic necrosis virus (IHHNV). Recently a number of disease syndromes such as running mortality syndrome (RMS), white faeces syndrome (WFS) / white gut syndrome and growth retardation have been negatively impacting shrimp aquaculture in India (Otta *et al.*, 2016).

Although, the OIE listed diseases causes a major outbreak in shrimp culture systems, there are also other newly emerging diseases that causes severe production losses to the shrimp industry. Such emerging diseases includes, hepatopancreatic microsporidiosis (HPM), hepatopancreatic haplosporidiosis (HPH), aggregated transformed microvilli (ATM), covert mortality disease (CMD) and a new lethal type of yellow head virus (Thitamadee *et al*., 2016). Hepatopancreatic microsporidiosis caused by *Enterocytozoon hepatopenaei* which is noted as a recently emerging shrimp disease that causes severe growth retardation in shrimps (Anderson *et al*., 1989; Chayaburakal *et al*., 2004; Tourtip *et al*., 2009; Tangprasittipap *et al*., 2013; Tang *et al*., 2017).

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Enterocytozoon hepatopenaei (EHP) is an obligate intracellular pathogen belonging to a group called microsporidia, is yeast like fungus. The pathogens belong to the family *Enterocytozoonidae* known to infect host inhabiting marine, freshwater, and aquatic environments. *Enterocytozoon hepatopenaei* have several features similar to human pathogen, *Enterocytozoon bieneusi*, but it differed in the mean spore size, and has 16% difference in the 18 SSU rRNA gene sequence, and so it was proposed with a new species name *Enterocytozoon hepatopenaei* within the family *Enterocytozoonidae* (Tourtip *et al.*, 2009). In 2010, EHP was reported as being associated with "white faeces syndrome" (WFS) in Vietnam, but later laboratory experiments failed to show association with WFS (Tangprasittipap *et al*., 2013). EHP shows horizontal transmission through cohabitation, cannibalism and through live feed and moreover the possible ways of vertical transmission is poorly understood (Salachan *et al*., 2016). The EHP can be transmitted to a healthy shrimp upon exposure to pond soil up to 15 days. There are no drugs developed for the control of EHP disease. Increasing the pH of the pond to 12 has been recommended as a management measure for the control of EHP (Otta, 2016). Report on EHP in India, was done in 2014 by Sritunyalucksana (Sritunyalucksana *et al*., 2014) and recently Rajendran *et al.,* 2016 reported in *P. vannamei* farm in India (Rajendran *et al.,* 2016; Raveendra *et al.,* 2018).

Molecular diagnostic assays reported for screening of EHP includes single step PCR (Tourtip *et al*., 2009; Tangprasittipap *et al*., 2013), nested PCR (Tangprasittipap *et a*l., 2013; Jaroenlak *et al*., 2016), Loop Mediated Thermal Amplification (LAMP) (Suebsing *et al*., 2013) and Real time PCR (Liu *et al*., 2016). Most of the molecular methods developed targets the 18s rRNA genome of the microsporidian. But recently Jaroenlak *et al*., 2016, reported that the molecular methods targeting 18s rRNA may give false positive results due to the cross reactivity of the primers with other closely related microsporidian such as *Enterospora canceri* which affects *Cancer pagurus* (crabs), fish pathogens with the genus *Nucleospora* and recently with a salmonid fish pathogen the parasite *Paranucleospora theridon*. In order to overcome this problem, Jaroenlak *et al*. (2016) developed a nested PCR assay to avoid false positive detection of the microsporidian *Enterocytozoon hepatopenaei* (EHP) in environmental samples in shrimp farms. This method targets the spore wall protein (SWP) gene of *Enterocytozoon hepatopenaei.* It has been reported that SWP-PCR method is more sensitive than SSU-PCR methods and more specific for the detection of *Enterocytozoon hepatopenaei*.

Since retarded shrimp growths being reported at farms in the major grow out states of Maharashtra and Gujarat, there is a need for the assessment of occurrence of *Enterocytozoon hepatopenaei*, to study the prevalence of EHP. The specific detection of EHP by targeting spore wall protein gene instead of 18s r RNA is necessary as Jaroenlak *et al*. (2016) reported that 18s r RNA gave false positive results.

MATERIALS AND METHODS

Sampling area

The present study was carried out for a period of one year between February 2016 to April 2017. Shrimp (*P. vannamei*) with the sign of stunted growth were collected for this study from different shrimp farms located in Thane and Raigad district of Maharashtra and Navasari, Surat and Valsad districts of Gujarat. In Thane district, Palgar, Bhayender, Vasai, Dhanu, Bordi, Saphale, Naigoan and Murda were covered whereas in Raigad Alibag, Tala, Uran, Roha, Mhasaka, Panvel, murud, Khajni, Mhasala and Srivardhan were covered. The regions selected in Valsad were Hingraj, Pardi, Bhadeli, Kosamba. The total number of 98 samples was done in each sampling covering each farm. About 36 farms were covered in this study.

Experimental shrimps

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

Primers

Published universal primers were used for the amplification of spore wall protein of *Enterocytozoon hepatopenaei* isolates. The names of the primers, sequence and amplification size are given below.

Collection of samples

More than fifty ponds were selected for this study which was experiencing size variation/growth retardation and white faeces syndrome. On each sampling day, a minimum of 20 shrimps were examined for diseases of species. Behavioural signs, gross and clinical signs were recorded. From each pond 4-6 shrimps were taken for diagnosis and the hepatopancreas of each sample were dissected out and fixed in 95% alcohol for molecular diagnosis. 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 -20 ºC and analyzed.

Molecular diagnosis

Molecular diagnosis has done at Animal Health Laboratory, CIFE, Mumbai, India.

DNA extraction from the samples

For the DNA isolation from the samples gills, pleopods, gut and hepatopancreas were collected aseptically and analysed. The collected tissue samples weigh not more than 50 mg. The collected tissue samples were added to the 1ml of DNA extraction solution (Himedia, India). The mixture was homogenized using a homogenizer (Roche, India) and kept for 15 min incubation at the room temperature of 28 ºC. The mixture was centrifuged at 10,000 g for 10 min and the supernatant was collected in fresh sterile tube. To the supernatant, equal volume of absolute alcohol was added and centrifuged at 10000 g for 5 min. The supernatant was discarded and the DNA pellet was washed twice with 1ml of 95% alcohol by centrifugation at 8000 g for 5 min. The supernatant was removed, the DNA pellet was allowed to air dry for 5 min and 100 µl of deionised water was added and stored at 4 ˚C, for further use.

PCR screening of samples for EHP

For the nested SWP PCR method (SWP-PCR), primers from the literature Jaroenlak, *et al.* (2016) was used. The PCR reaction mixture for both steps (25 µl) contained 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.2 µM of each primer, 0.5 unit of *Taq* DNA polymerase. For the first step PCR, added templates consisted of either 100 ng of total DNA extracted from EHP-infected, shrimp hepatopancreatic tissue and gill tissue. The PCR protocol for the first PCR reaction used primers SWP_1F and SWP_1R (Table 1) and consisted of a 5-min initial denaturation at 95°C followed by 30 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 58 °C and extension for 45 s at 68 °C with a final 5-min extension step at 68°C. The expected PCR product was 514 bp. For the second (nested) PCR step, the template consisted of 1 µl of the final reaction solution from the first PCR step. The PCR protocol for the second, nested PCR reaction used primers SWP_2F and SWP_2R (Table 1), with an initial denaturation at 95°C for 5 min followed by 20 cycles of 30 s denaturation at 95 °C, 30 s annealing at 64 °C and 20 s extension at 68 °C with a final extension for 5-min at 68°C. The expected PCR product was 148 bp. The amplicons were analyzed by 1.2% agarose gel electrophoresis with ethidium bromide staining and using a DNA ladder marker (100 bp).

Agarose gel electrophoresis

The amplified products from the 1st step and nested PCR were analyzed by electrophoresis in 1.2% agarose gels stained with ethidium bromide and visualized by ultraviolet transillumination.

Table 1: Primer used in this study

Primer name	Sequence	Amplification size	Reference
	First PCR		
SWP_1F	TTGCAGAGTGTTGTTAAGGGTTT	514-bp	Jaroenlak, Pattana *et al*., 2016
SWP_1R	CACGATGTGTCTTTGCAATTTTC		
	Nested PCR		
SWP_2F	TTGGCGGCACAATTCTCAAACA	148-bp	Jaroenlak, Pattana *et al*., 2016
SWP_2R	GCTGTTTGTCTCCAACTGTATTTGA		

RESULTS AND DISCUSSION

Prevalence of EHP

EHP was found to be endemic in Australia (Tourtip *et al*., 2009), and also reported in Thailand (Chayaburakul *et al.,* 2004), Vietnam (Ha *et al*., 2010), Malaysia, China, and Venezuela (Tang *et al*., 2017). The first Indian report of EHP was recorded in south east coast of India in the year of 2016. Nowadays, there has been a well-documented awareness among the farmers regarding the occurrence of WSSV, but the shrimp farmers are giving less importance for the incidence of the parasitic disease, and suffered severe losses due to EHP associated with white faeces (WFS). Rajendran *et al*. (2016) conducted a prevalence study on EHP at south east coast of India and the results showed high prevalence of EHP infection of about 63.5%. Following this, Studies were carried out in the states of Tamil Nadu, Andhra Pradesh and Orissa and reported 66% prevalence with nested PCR method. Prathisha *et al*. (2019) assessed the occurrence of EHP in South Tamil Nadu shows 36.98% prevalence. The above studies confirmed the incidence of EHP in the states of Tamil Nadu, Andhra Pradesh and Orissa. In our study, sampling was carried out to assess the occurrence of EHP in Maharashtra and Gujarat. The results of the targeted surveillance of EHP in *Penaeus vannamei* of Gujarat and Maharashtra from February 2016 to April 2017 are presented in Table 2. In 1.2 % agarose gel electrophoresis, samples with EHP infection show a band of PCR (510 bp and 148bp) (Fig. 2).

In the present investigation, shrimp samples from the different farms were tested for EHP infection. The clinical signs noted in the affected shrimps were, pale gills and retarded growth with size variation. Out of

Table 2: Positive samples of *Enterocytozoon hepatopenaei* in *Penaeus vannamei* from February 2016 to April 2017 of Maharashtra and Gujarat

Date of collection	Location of the Farm	Stage	Tissue collected	ABW at the date of sampling	Confirmation of EHP
10.08.16	Murud	*P.vannamei* Adult (20) 150 DOC	HP, Gills	27g	SS and Nested PCR +ve
16.08.16	Murud	*P.vannamei* PL-18 (60)	HP, Gills	3.4g	SS and Nested PCR +ve
16.09.18	Murud	*P.vannamei* Adult (30) 60 days	HP, Gills	9.3 g	SS and Nested PCR +ve
1.10.16	Murud	*P.vannamei* Adult (30) 120DOC	HP, Gills	20.1g	SS and Nested PCR +ve
6.05.17	Mahaghar	*P.vannamei* Adult (30) 70DOC	HP, Gills	12.2g	SS and Nested PCR +ve
12.7.17	Shrivardhan	*P.vannamei* Adult (30) 80days	HP, Gills	14.3g	SS and Nested PCR +ve
20.06.16	Saphale	*P.vannamei* Adult (30) 57 DOC	HP, Gills	8-9g	SS and Nested PCR +ve
15.07.16	Saphale	*P.vannamei* Adult (30) 60 DOC	HP, Gills	8-9g	SS and Nested PCR +ve
15.07.16	Bhayendhar	*P.vannamei* Adult (24) 46DOC	HP, Gills	5-6 g	SS and Nested PCR +ve
17.08.16	Bordi	*P.vannamei* PL- 10 (60)	HP, Gills	2g	Nested PCR +ve
20.08.16	Palghar	*P.vannamei* PL- 10 (60)	HP, Gills	2.3g	SS and Nested PCR +ve
29.08.16	Palghar	*P.vannamei* PL- 10 (60)	HP, Gills	1.3g	SS and Nested PCR +ve
09.09.16	Bordi	*P.vannamei* PL- 18 (60)	HP, Gills	2.6g	SS and Nested PCR +ve

14.09.16	Niagoan	*P.vannamei* Adult (60) 120 DOC	HP, Gills	19.8g	Nested PCR +ve
26.09.16	Dhanu	*P.vannamei* Adult (30) 105 DOC	HP, Gills	17.9g	SS and Nested PCR +ve
7.11.16	Saphale	*P.vannamei* Adult (30) 68DOC	HP, Gills	10.5g	Nested PCR +ve
10.11.16	Saphale	*P.vannamei* Adult (20) 75DOC	HP, Gills	13.4g	Nested PCR +ve
14.12.16	Dhanu	P.monodon Adult (30) 120 DOC	HP, Gills	19.5g	SS and Nested PCR +ve
29.12.16	Palghar	*P.vannamei* PL-15 (30)	HP, Gills	4.6g	Nested PCR +ve
30.12.16	Palghar	*P.vannamei* PL-15 (30)	HP, Gills	7g	Nested PCR +ve
4.05.17	Dhanu	*P.vannamei* Adult (30) 67 DOC	HP, Gills	10g	SS PCR –ve & Nested PCR +ve
07.06.17	Saphale	*P.vannamei* Adult(30) 60 DOC	HP, Gills	9g	SS PCR +ve
15.06.17	Saphale	*P.vannamei* Adult (30) 60 DOC	HP, Gills	8.7g	SS PCR +ve
20.06.17	Saphale	*P.vannamei* Adult (30) 68 DOC	HP, Gills	10.7g	Nested PCR +ve
28.06.17	Vasai	*P.vannamei* Adult (30) 68 DOC	HP, Gills	10.9g	Nested PCR +ve
19.09.17	Dhanu	*P.vannamei* Adult (30) 75 DOC	HP, Gills	13.2g	SS and Nested PCR +ve
19.09.17	Dhanu	*P.vannamei* Adult(30) 33 DOC	HP, Gills	4.4g	SS and Nested PCR +ve
28.09.17	Saphale	*P.vannamei* Adult (30) 43 DOC	HP, Gills	6.5g	Nested PCR +ve
03.06.17	Navasari	*P.vannamei* Adult (30) 70 DOC	HP, Gills	12g	Nested PCR +ve
03.06.17	Navasari	*P.vannamei* Adult(30) 67DOC	HP, Gills	10.3g	Nested PCR +ve
03.06.17	Navasari	*P.vannamei* PL- 40 (60)	HP, Gills	7g	Nested PCR +ve
03.06.17	Navasari	*P.vannamei* Adult (20) 66 DOC	HP, Gills	10.4g	Nested PCR +ve
12.08.17	Navasari	*P.vannamei* Adult (20) 70DOC	HP, Gills	12.6g	Nested PCR +ve
20.10.17	Navasari	*P.vannamei* Adult (30) 65 DOC	HP, Gills	9.4g	Nested PCR +ve
10.06.16	Khajod	*P.vannamei* Adult(30) 97 DOC	HP, Gills	16.8g	SS and Nested PCR +ve
10.06.16	Khajod	*P.vannamei* Adult(30) 90DOC	HP, Gills	15g	SS and Nested PCR +ve
10.06.16	Khajod	*P.vannamei* Adult(30) 44DOC	HP, Gills	5.3g	SS and Nested PCR +ve
10.06.16	Khajod	*P.vannamei* Adult (30) 70 DOC	HP, Gills	12.3 g	SS and Nested PCR +ve
16.06.16	Saras	*P.vannamei* Adult (15) 60 DOC	HP, Gills	9 g	SS and Nested PCR +ve

16.09.16	Saras	*P.vannamei* Adult (15) 73 DOC	HP, Gills	11.8 g	SS and Nested PCR +ve
16.06.16	Saras	*P.vannamei* Adult (15) 68 DOC	HP, Gills	11.6 g	SS and Nested PCR +ve
16.09.16	Saras	*P.vannamei* Adult (15) 105 DOC	HP, Gills	16.8 g	SS and Nested PCR +ve
11.10.16	Khajod	*P.vannamei* Adult(15) 67 DOC	HP, Gills	10 g	SS and Nested PCR +ve

4513 pond shrimp samples, 1408 samples were found to be EHP positive with 45% prevalence by PCR (Table 2). The first report for the slow growth in shrimps was reported by Anderson *et al*. (1989) at Malaysia in *Penaeus monodon*. And later, similar slow growth syndrome was reported by Hudson *et al.* (2009) in *Penaeus japonicus*. More evidence were obtained by knowing that retarded giant or black tiger shrimp *P. monodon* was reported with microsporidian infestation in hepatopancreas from Thailand in 2004. India EHP is also reported from shrimps showing slow growth (Sritunyalucksana *et al*., 2014) and very recently *P. vannamei* farm in India (Rajendran *et al.,* 2016; Raveendra *et al*., 2018). Rajendran *et al.,* 2016 reported that the prevalence of EHP was estimated to be 63.5% for 137 samples. Moreover, the EHP prevalence was observed as 16% for 50 samples by Raveendra *et al*. (2018), whereas, Prathisha *et al*. (2019) showed that 36.98% EHP prevalence in South Tamil Nadu. In the present study, the EHP prevalence was observed as 31.2% for 4513 samples.

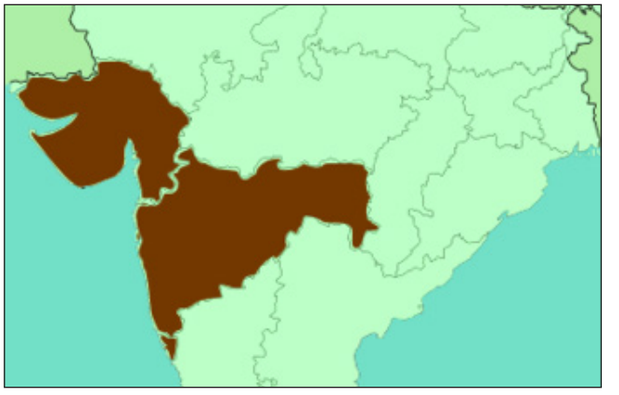

Fig. 1. Study area (Maharashtra & Gujarat)

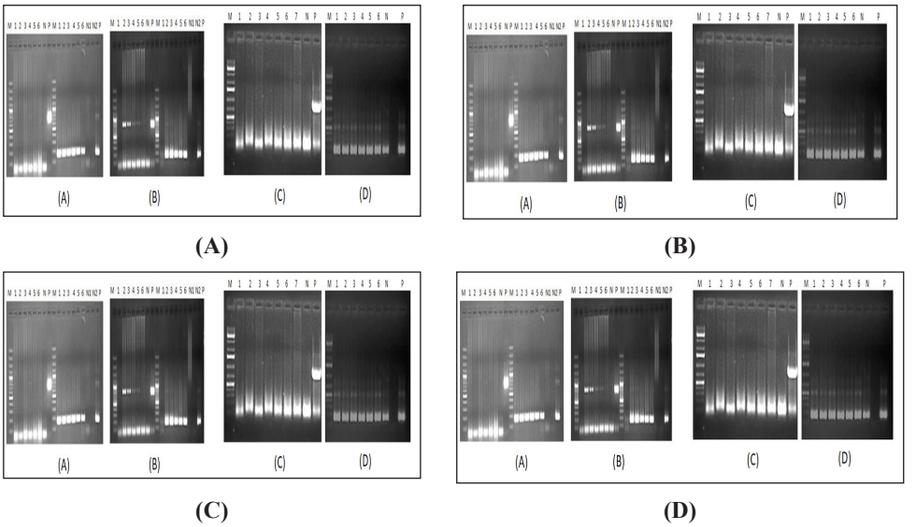

Fig 2. (A) Sample- showing Negative for single step PCR and Positive in Nested PCR of SSU primer. **(B)** Sample- showing positive for single step PCR and Positive in Nested PCR of SSU primer. **(C) & (D)** Sample- showing Negative for single step PCR and Positive in Nested PCR of SWP primer

Correlation of EHP with DOC and region

The prevalence of EHP based on DOC was correlated to indicate the EHP infection irrespective of their size and weight and found that, the DOC of the infected samples started from a minimum of 18 to a maximum of 150. The maximum and minimum range of infection was found at DOC of 61- 80 and 21 - 40 of shrimp samples (Fig. 3) whereas, Prathisha *et al*. (2019) reported maximum range of infection with respect to DOC was between 31 to 60 DOC. This results conclude that the grow out phase of shrimps were greatly affected which is supported by Prathisha *et al.* (2019). The region wise prevalence of EHP in Maharashtra and Gujarat was estimated and found that the highest positive samples were obtained from Thane which is clearly showed in the Fig. 4, which is followed by Surat, Navsari and Raigad.

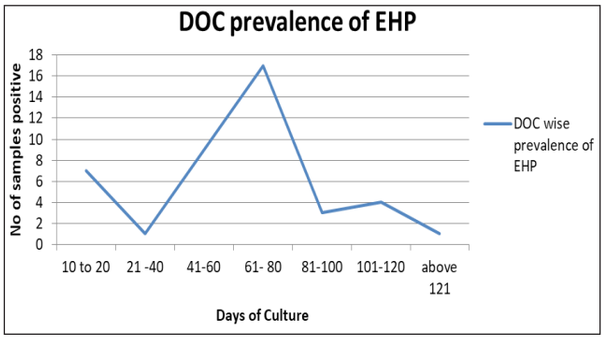

Fig. 3: Showing DOC prevalence of EHP in Maharashtra and Gujarat

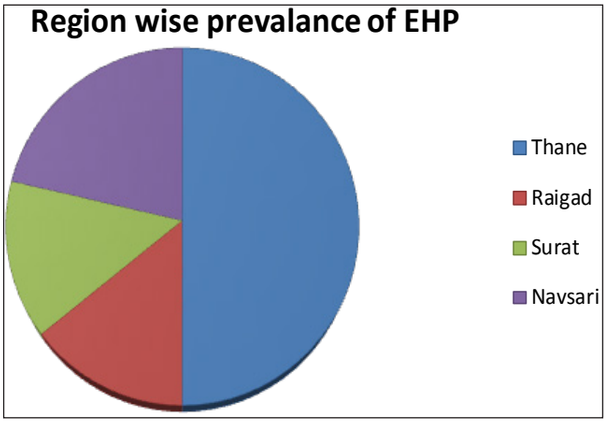

Fig. 4: Showing Region wise prevalence of EHP in Maharashtra and Gujarat

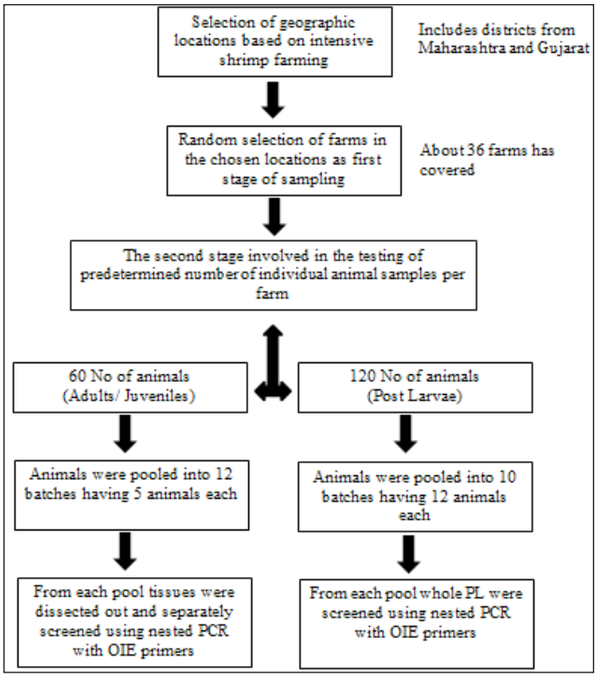

Fig. 5: Flow diagram showing the sampling frame and screening process

Molecular confirmation

The molecular diagnostic PCR was shown to be the reliable tool for the diagnosing the EHP in shrimp samples. The study was earlier conducted with SSU primers of EHP and later the reported SWP (Spore Wall Protein) primers were used as there has been several reports on cross reactivity of 18s rRNA primers of EHP with other closely related microsporidia. The results were confirmed with the SWP primers and tabulated below (Table 2).

CONCLUSION

The current study determined the prevalence of EHP in one third of the shrimp farming ponds in Maharashtra and Gujarat (Thane 21%, Raigad 9%, Surat 6%, Navsari 6%). The parasite was identified as severe outbreaks from Indian shrimp farms since 2015. PCR could be used as a diagnostic tool for studying epidemiological distribution of parasites in shrimp farming using archived shrimp tissue samples. It can also help monitor shrimp farms for microsporidian infection and to study parasite life cycle

and transmission dynamics in shrimp ponds. The authors insisted the shrimp farmers undertaking *P. vanammei* farming operations should take necessary precautionary measures to avoid this devastating parasite causing retarded growth and associated production and economic loss

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CONFLICT OF INTEREST

On behalf of all authors, the corresponding author declares that there is no conflict of interest.

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