Indian J. Fish., 70 (3): 98-106, 2023 doi:10.21077/ijf.2023.70.3.129795-13

Available online at: epubs.icar.org.in/index.php/IJF

A study on the natural host range of *Enterocytozoon hepatopenaei* in different species of shrimp and co-habiting aquatic fauna

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Abstract

Enterocytozoon hepatopenaei (EHP) is a serious pathogen impacting farmed shrimp production in Asian countries. Though many aquatic organisms were reported to be susceptible to EHP, the full range of susceptible hosts, which have the potential to transmit the pathogen to shrimp has not been identified. In this study, a range of farmed and wild invertebrates and other cohabiting fauna from different ecological niches located in the coastal and inland saline areas were subjected to PCR-based detection employing primers targeting three different genes coding for small subunit (ssu) rRNA, spore wall protein (SWP) and β-tubulin. The PCR analysis with ssu-rRNA primers showed positive amplification in *Penaeus vannamei, Penaeus monodon, Penaeus indicus*, marine shrimps, aquatic insect, mud crabs, freshwater crab and violet clam. PCR using SWP primers detected EHP in *P. vannamei, P. monodon,* aquatic insect and marine crabs, while the β-tubulin primers resulted in positive amplification only in mud crabs and *P. vannamei.* The PCR amplicons revealed 99-100% identity with the sequences of EHP. The present study forms the first attempt to screen a wide range of aquatic fauna for EHP employing three different PCR tests and the findings are significant as these organisms have the potential to be carriers of EHP and are likely to transmit the parasite to shrimp culture systems.

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Keywords:
Co-habiting aquafauna, *Enterocytozoon hepatopenaei,* EHP, Host range, Shrimp culture

Received : 04.11.2022
Accepted : 13.09.2023

Introduction

Shrimp aquaculture is a major contributor to the global aquaculture sector with a production of 6.5 million t (FAO, 2022). Currently, white leg shrimp (*Penaeus vannamei*) is the dominant farmed shrimp species, with a production of 5.8 million t, after the introduction of specific pathogen-free (SPF) stocks in 2009 against the backdrop of catastrophic mortalities caused by white spot syndrome virus (WSSV) in tiger shrimp *P. monodon*. For many years, infectious diseases have been the major constraints in shrimp farming and the major diseases encountered in Indian shrimp farming include the infection caused by WSSV, *Enterocytozoon hepatopenaei* (EHP), slow/retarded growth syndrome, white faeces/gut syndrome, running mortality syndrome, loose shell syndrome and white muscle disease (Patil *et al.,* 2021; Rajendran *et al.,* 2021).

In recent times, the most impactful production-limiting factor in Asian shrimp farming is the problem of slow/retardedgrowth (Rajendran *et al.,* 2021). Hepatopancreatic microsporidiosis (HPM), caused by the microsporidian *Enterocytozoon hepatopenaei,* is one of the pathogens associated with slow growth and has emerged as one of the most impactful pathogens of *P. vannamei* in all the shrimp growing countries in Asia, such as Thailand, Indonesia, Vietnam, India, Malaysia, China and Korea (Ha *et al.,* 2010; Rajendran *et al.*, 2016, Shen *et al.,* 2019; Kim *et al.*, 2021). In India, it has already been reported from all the major shrimp farming states (Biju *et al.,* 2016; Prathisha *et al.,* 2019; Behera *et al.*, 2019; Rathipriya *et al.,* 2019; Jithendran *et al.,* 2021) and the economic loss due to the infection was estimated to be USD 571.03 million (Patil *et al.,* 2021). It has been reported that EHP infection leads

to several non-specific clinical signs including retarded growth, which results in size variation, soft carapace, lethargy, poor feed consumption, empty gut, discolouration of hepatopancreas and occasionally floating white faecal matter (Rajendran *et al.*, 2016; Aranguren *et al.,* 2017; Janakiram *et al.,* 2018; Caro *et al.*, 2021). Further, infection with EHP is often followed by opportunistic infections, and it has been demonstrated that EHP is a risk factor for the emergence of acute hepatopancreatic necrosis disease (AHPND) and septic hepatopancreatic necrosis (SHPN) in shrimp (Aranguren *et al.,* 2017). Association of EHP with white gut/faeces syndrome in shrimp has also been reported (Caro *et al.,* 2021; Kumar *et al.*, 2022).

Due to the rapid spread of EHP worldwide and the consequent economic impact, researchers have made various attempts on different aspects of the pathogen and the disease. As of now, there are no effective therapeutants available for combating EHP infection and therefore, only sole reliance is on the adoption of good management practices and strict biosecurity measures that can prevent the disease. Chaijarasphong *et al.* (2020) indicated that the disease due to EHP is more threatening owing to the poor understanding of its reservoirs in diverse environment and transmission routes in the shrimp farming ecosystem. Further, it is imperative to know the presence of natural carriers and the potential risk associated with their presence to implement effective preventive measures (Kanchanaphum *et al.,* 1998). Previous studies demonstrated that live feed such as artemia (Karthikeyan and Sudhakaran, 2020), polychaetes (Desrina *et al.,* 2020; Krishnan *et al.,* 2021) and cohabiting fauna such as false mussel (Munkongwongsiri *et al.,* 2022) can serve as potential carriers of EHP. However, considering the wide distribution and high prevalence of EHP, it can be presumed that the pathogen might be present in a wide range of hosts. Thus, the present study explored the potential carrier hosts of EHP by screening some of the co-habiting fauna of shrimp farming systems as well as wild crustaceans using three reported EHP-specific PCR assays.

Materials and methods

Sample collection and sampling area

Samples of live *Penaeus monodon* (length: 16.0-17.5 cm; weight: 35.0-40.0 g) and *Penaeus indicus* (length: 9.0-10.0 cm; weight: 4.0-5.0 g) were collected from shrimp farms located in South 24 Parganas District, West Bengal, India. Samples of *Penaeus vannamei* (length: 6.5-15.1 cm; weight: 1.6-25.0 g) were collected from farms located in Maharashtra and inland saline farms of Punjab and Rajasthan. Samples of mud crab *Scylla serrata* (length: 11.5-15.5 cm; weight: 46.0-87.0 g) were collected from Mumbai, Maharashtra, and *Scylla olivacea* (length: 8.0-12.0 cm; weight: 29.0-40.0 g) were collected from shrimp farms located in South 24 Parganas District, West Bengal, India. Samples of wild freshwater crab *Sartoriana spinigera* (length: 13.0-15.0 cm; weight: 52.0-69.0 g) were collected from the Keleghai River in the West Medinipur District, West Bengal. An aquatic insect *Dytiscus* sp. was collected from an inland saline shrimp farm located in Rohtak District, Haryana. For snail, violet clam and green mussels, DNA extracted from samples collected previously from different locations in the Palghar District, Maharashtra were used. The details of the samples and sampling locations are depicted in Fig. 1 and 2 as well as Table 1. All the samples were either transported live; in ice or in 70% ethanol to the laboratory and then stored at -80^{0}C or at room temperature according to the samples until further used.

DNA extraction

DNA was extracted from the hepatopancreas of crustaceans and internal organs of insect samples using DNAzol™ reagent (Invitrogen, USA) as per the manufacturer's protocol. Briefly, 100 mg tissue was homogenised in a 1.5 ml microcentrifuge tube in 1 ml of DNAzol™ reagent. In the case of green mussel, clam and snail, previously extracted DNA samples available in the laboratory were used. The concentrations and purity of the isolated DNA were

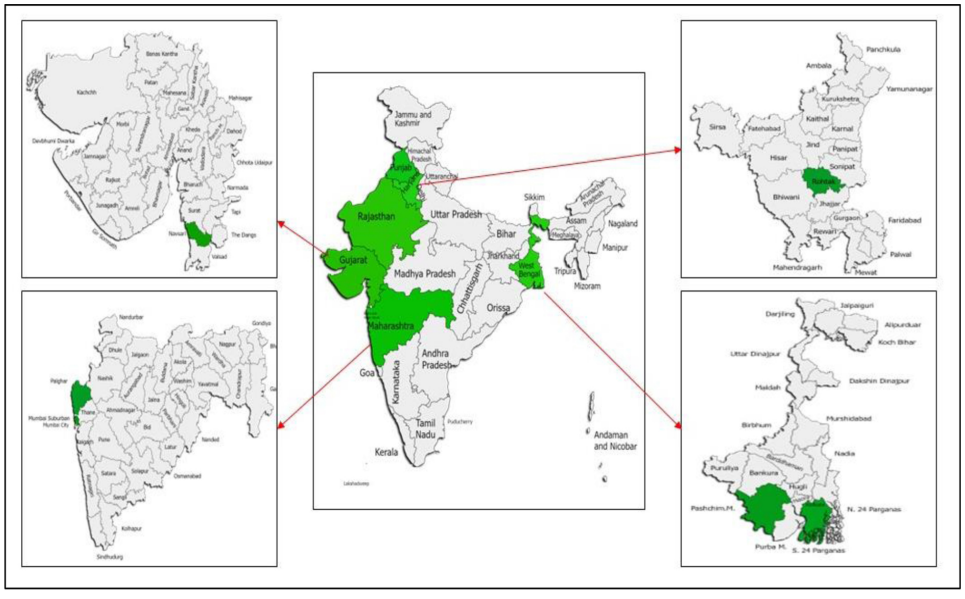
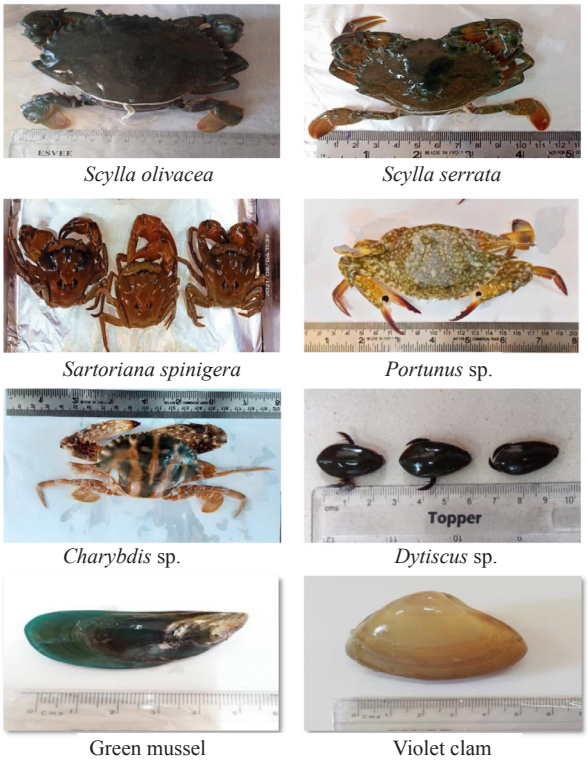


Fig. 1. Map showing the different sampling locations

Table 1. Details of host species, location of collection and number of samples collected for the study

Host	Place of collection	No. of samples
Shrimp		
Penaeus monodon	West Bengal	10
P. monodon	Gujarat	07
Penaeus indicus	West Bengal	04
Penaeus vannamei	Maharashtra	40
P. vannamei	Punjab	08
P. vannamei	Rajasthan	05
Marine shrimp	Maharashtra	07
Crabs		
*Scylla serrata**	Maharashtra	10
*S. olivacea**	West Bengal	07
Portunus sp. and *Charybdis* sp.**	Maharashtra	10
Sartoriana spinigera#	West Bengal	03
Aquatic insect		
Dytiscus sp.	Haryana	10
Mollusc		
Green mussel	Maharashtra	08
Violet clam	Maharashtra	05
Snail	Maharashtra	10

*Mud crabs; **Marine crabs; #Freshwater crabs



Scylla olivacea | *Scylla serrata*
Sartoriana spinigera | *Portunus* sp.
Charybdis sp. | *Dytiscus* sp.
Green mussel | Violet clam

Fig. 2. Different host species (crabs, insect and molluscans) used in the study

measured using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The isolated DNA was stored at -20°C until further use.

Polymerase chain reaction and sequencing

For PCR amplification, three different published nested PCR protocols targeting different genes of EHP were used. Primarily, all the samples were screened using the ssu-rRNA gene of microsporidians (Tangprasittipap *et al.,* 2013). Subsequently, all the samples were subjected to PCR assay targeting SWP (Jaroenlak *et al.,* 2016) and positive samples were further screened with β-tubulin-PCR (Han *et al.,* 2018). The details of primers used are provided in Table 2. The PCR amplification was carried out in a 25 µl reaction mixture and PCR amplification was carried out in a Veriti® Thermal Cycler (Applied Biosystems, USA).

The PCR amplified products were analysed on 1.5% agarose gel stained with ethidium bromide (0.5 µg ml^{-1}) by mixing an aliquot of PCR product (6 µl) with 2 µl of 6X DNA loading dye (Thermo Scientific, USA) and loaded in the wells along with DNA ladder. Electrophoresis was carried out at 80 V for 40 min. The gel was then visualised in a Syngene InGenius3 Gel Documentation System (Syngene, UK) and the images were captured for further analysis.

The PCR products of all three genes (ssu-rRNA, SWP and β-tubulin) from selected samples were sequenced (Eurofins Genomics India Pvt. Ltd. Bengaluru, India). The sequences were verified and further subjected to BLAST (Basic Local Alignment Search Tool) analysis using National Centre for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov) to find out the homology with already reported sequences in the GenBank. Multiple sequence alignments were performed using the Clustal X.2.0 program (http://www.ebi.ac.uk/tools/clustalw2).

Results

Polymerase chain reaction

The PCR screening with ssu-rRNA-specific PCR showed positive amplification yielding 176 bp amplicon in *P. monodon, P. indicus, P vannamei,* marine shrimp, mud crab (*S. serrata* and *S. olivacea*), freshwater crab (*Sartoriana spinigera*), aquatic insect, marine crab

Table 2. Details of primers used in the study

Primer name	Sequence	Product size (bp)
18S (small sub-unit) rRNA gene		
First step		
ENF779	CAGCAGGCGCGAAAATTGTCCA	
ENR779	AAGAGATATTGTATTGCGCTTGCTG	779
Nested step		
ENF176	CAACGCGGGAAAACTTACCA	
ENR176	ACCTGTTATTGCCTTCTCCCTCC	176
SWP (spore wall protein) gene		
First step		
SWP_1F	TTGCAGAGTGTTGTTAAGGGTTT	
SWP_1R	CACGATGTGTCTTTGCAATTTTC	514
Nested step		
SWP_2F	TTGGCGGCACAATTCTCAAACA	
SWP_2R	GCTGTTTGTCTCCAACTGTATTTGA	148
β-tubulin gene		
First step		
EHP-618F	CAGCTGGTTGAAAATGCAAA	
EHP-618R	GTGCAAAAATGCCTTTCGTT	618
Nested Step		
EHP-237F	GATATGCGCCTCTGTGTTCA	
EHP-237R	TGTTTGGAATCCACTCGACA	237

and clam. Interestingly, all these samples were found to be positive only in the second step of PCR. Among these hosts, all four samples of *P. indicus* screened were found to be PCR-positive. The other host species which showed a high prevalence (80.0%) in terms of positive PCR amplification were *P. monodon, S. serrata* and violet clam. A relatively high prevalence of EHP was noticed in freshwater crab (66.6%) and aquatic insect (60.0%). Of the 20 samples of *P. vannamei* tested with ssu-rRNA primers, a prevalence of 40% could be noticed. A lower prevalence was noticed in *S. olivacea* (28.5%), marine shrimp (14.3%) and marine crab (10.0%). No PCR amplification could be noticed in the samples of green mussel and snail (Fig. 3-4; 6-8; Table 3).

Following ssu-rRNA-PCR, all the samples were subjected to SWP-specific PCR. In this, many samples of *P. vananmei* showed discernible amplification in the first-step PCR, yielding a 514 bp amplicon. However, *P. monodon,* aquatic insect, *S. serrata* and marine crab along with some of the samples of *P. vannamei* showed positive amplification in the second-step PCR yielding 148 bp amplicon. Interestingly, aquatic insect showed the maximum prevalence of EHP (70%) followed by *P. vannamei* (50%) collected from Punjab. The prevalence of EHP in *P. monodon* and *S. serrata* and marine crabs was found to be 20%. *P. indicus,* marine shrimp, *S. olivacea,* violet clam, green mussel and snail were found to be negative in the PCR. Among the host species tested, green mussel and snail were found to be negative in both the PCR tests (Figs. 3, 5, 7 and 8; Table 3).

Host species that have shown positive PCR amplification in SWP-specific PCR were further subjected to PCR analysis using primers targeting the β-tubulin gene of EHP. Similar to SWP-specific PCR, many samples of *P. vannamei* showed positive amplification in the first-step PCR yielding a 618 bp amplicon. In second-step PCR, *P. vannamei* showed positive amplification yielding a 237 bp amplicon with a prevalence of 47.8%. Although *S. serrata* showed a high prevalence (70%) of EHP in β-tubulin-PCR, this requires further confirmation. Samples of *P. monodon* and marine crab which showed positive amplification in the SWP-specific primers did not show any amplification with the β-tubulin-specific primers (Fig. 5 and 8; Table 3).

Sequencing of EHP genes

Sequence analysis of the 176 nt sequence amplified from the aquatic insect *Dytiscus* sp. using ssu-rRNA primers showed 100% identity to the ssu-rRNA gene of microsporidian reported

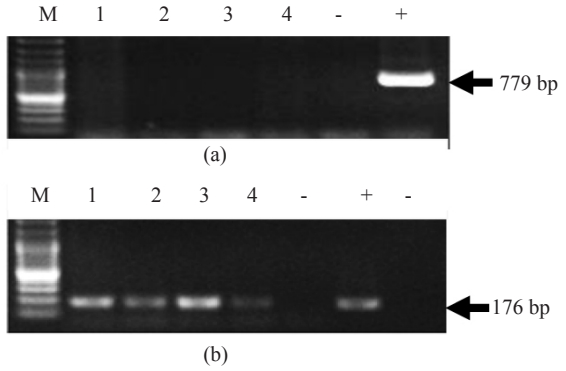


Fig. 4. Agarose gel electrophoresis of the PCR products amplified from the hepatopancreatic DNA of *P. indicus* using ssu-rRNA primers. A and B: First-step and second-step PCR. Lane M: 100 bp plus molecular weight marker; Lane 1-4: Sample DNA; - : Negative control; + : Positive control

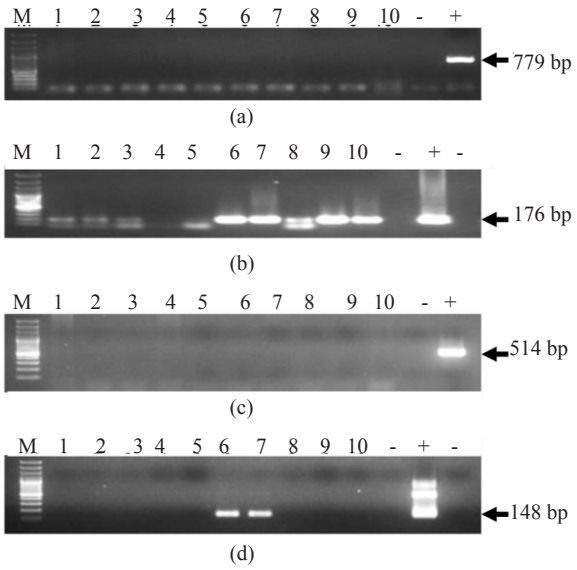


Fig. 3. Agarose gel electrophoresis of the PCR products amplified from the hepatopancreatic DNA of *P. monodon* using two different sets of primers. A and B: First-step and second-step PCR using primers targeting ssu-rRNA gene of microsporidians; C and D: First-step and second-step PCR using primers targeting spore wall protein gene of EHP. Lane M: 100 bp plus molecular weight marker; Lane 1-10: Sample DNA; - : Negative control; + : Positive control

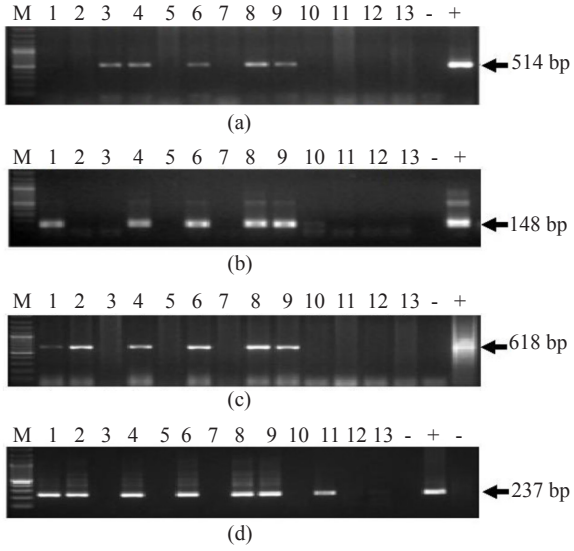


Fig. 5. Agarose gel electrophoresis of the PCR products amplified from the hepatopancreatic DNA of *P. vannamei* collected from inland saline aquaculture farms using two different sets of primers. A and B: First-step and second-step PCR using primers targeting spore wall protein gene of microsporidians; C and D: First-step and second-step PCR using primers targeting the β-tubulin gene of EHP; Lane M: 100 bp plus molecular weight marker; Lane 1-8: Sample DNA from Punjab; Lane 9-13: Sample DNA from Rajasthan; - : Negative control; + : Positive control

Table 3. Prevalence of *E. hepatopenaei* in shrimp and other co-habiting aquatic fauna screened by different PCR protocols

Host	Location	SSU-PCR			SWP-PCR			β-tubulin-PCR		
		Total No.	No. Positive	Prevalence (%)	Total No.	No. Positive	Prevalence (%)	Total No.	No. Positive	Prevalence (%)
P. monodon	West Bengal	10	08	80	10	02	20	10	00	0
P. monodon	Gujarat	07	00	0	07	00	0	ND	ND	ND
P. indicus	West Bengal	04	04	100	04	00	0	ND	ND	ND
P. vannamei	Maharashtra	20	07	35	40	08	20	10	04	40
P. vannamei	Punjab	ND	ND	ND	08	04	50	08	05	62.5
P. vannamei	Rajasthan	ND	ND	ND	05	01	20	05	02	40
S. spinigera (Freshwater crab)	West Bengal	03	02	66.6	03	00	0	ND	ND	ND
Marine shrimp	Maharashtra	07	01	14.3	07	00	0	ND	ND	ND
Dytiscus sp. (Aquatic insect)	Haryana	10	06	60	10	07	70	10	00	0
S. serrata	Maharashtra	10	08	80	10	02	20	10	07	70
S. olivacea	West Bengal	07	02	28.5	07	00	0	ND	ND	ND
Marine crabs*	Maharashtra	10	01	10	10	02	20	10	0	0
Green mussel	Maharashtra	08	00	0	08	00	0	ND	ND	ND
Violet clam	Maharashtra	05	04	80	05	00	0	ND	ND	ND
Snail	Maharashtra	10	00	0	10	00	0	ND	ND	ND

**Portunus* sp. and *Charybdis* sp.

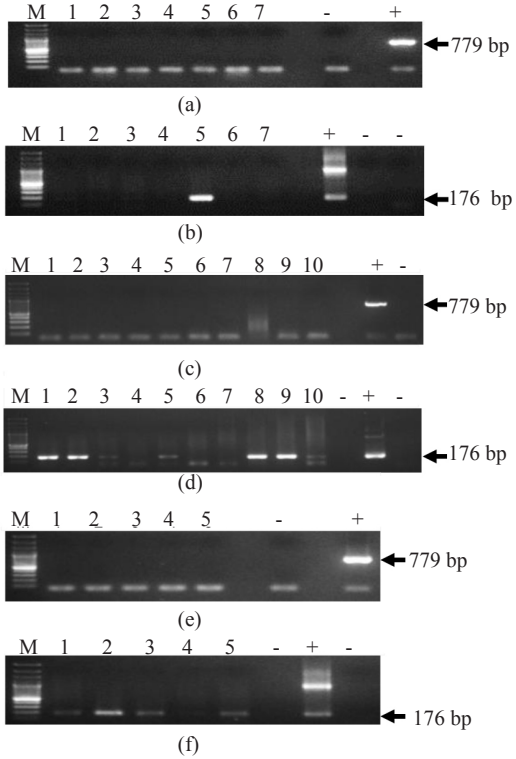


Fig. 6. Agarose gel electrophoresis of the PCR products amplified using primers targeting ssu rRNA gene (nested PCR) from the hepatopancreatic DNA of, A and B: Wild marine shrimp; C and D: Crabs, *S. olivacea and S. spinigera;* E and F: Violet clam; Lane M: 100 bp plus molecular weight marker; Lane 1-10: Sample DNA; - : Negative control; + : Positive control

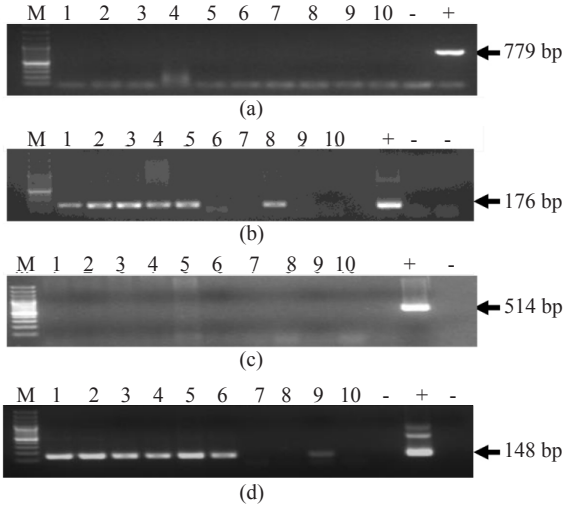


Fig. 7. Agarose gel electrophoresis of the PCR products amplified from the DNA of aquatic insect *Dytiscus* sp. using two different sets of primers. A and B: First-step and second-step PCR using primers targeting ssu-rRNA gene of microsporidians; C and D: First-step and second-step PCR using primers targeting spore wall protein gene of EHP; Lane M: 100 bp plus molecular weight marker; Lane 1-10: Sample DNA; -. : Negative control; +. : Positive control

from shrimp such as *P. vannamei* and *P. monodon* from India, Vietnam, South Korea, China and Thailand (GenBank Accession Nos. MH260592, MF134829, KX981865, KF362129). Further, the sequence showed 100% similarity with the ssu-rRNA gene sequence reported from polychaete from India (GenBank Accession No. MH259888). Analysis of the 148 nt sequence obtained from *P. vannamei, P. monodon* and the aquatic insect *Dytiscus* sp. using SWP-specific primers were sequenced and a comparison of the sequences showed 100% identity among the sequences. BLAST analysis of the identified sequences revealed 100% identity with SWP gene sequence of EHP reported from India, Thailand, Latin America and Malaysia. Further, sequence analysis of first-step PCR product (494 nt) obtained from *P. vannamei* showed 99% identity with the β-tubulin gene of EHP available in the GenBank. Multiple alignment of the nucleotide sequence of EHP β-tubulin from *P. vannamei* is shown in Fig. 9.

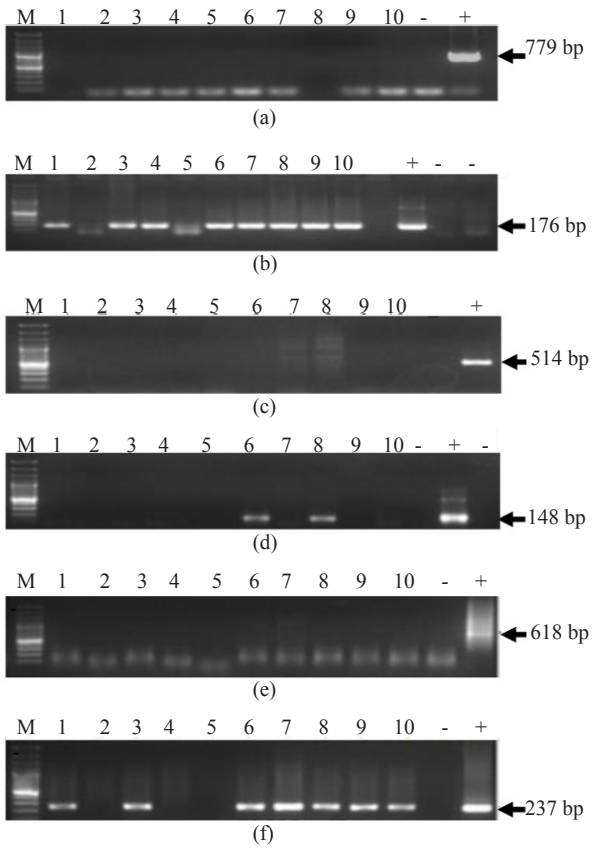


Fig. 8. Agarose gel electrophoresis of the PCR products amplified from the DNA extracted from *S. serrata* using three different sets of primers. A and B: First-step and second-step PCR using primers targeting ssu- rRNA gene of microsporidians; C and D: First-step and second-step PCR using primers targeting spore wall protein gene of EHP; E and F: First-step and second-step PCR using primers targeting the β-tubulin gene of EHP; Lane M: 100 bp plus molecular weight marker; Lane 1-10: Sample DNA; - : Negative control; + : Positive control

The nucleotide sequences of ssu-rRNA gene and SWP gene of EHP from *Dytiscus* sp. and β-tubulin gene from *P. vannamei* have been submitted to GenBank under the accession numbers OM802323, OP480798 and OP490297, respectively.

Discussion

EHP is an emerging pathogen that mainly affects farmed *P. vannamei* in several South-east Asian countries and causes growth retardation and subsequent economic losses. Currently, there are no effective remedial measures against EHP infections; therefore, adoption of good management practices and strict biosecurity measures is the only solution to prevent the disease. The main route of entry of any pathogen is soil, water, feed, or any exogenous source that may be a potential host. Recently, it has been reported that management of EHP is more challenging owing to the limited knowledge on its natural reservoirs and transmission routes in the shrimp production system (Chaijarasphong *et al.,* 2020). Earlier reports demonstrated that live feed such as artemia and polychaetes served as potential carriers of EHP (Kummari *et al.,* 2018; Desrina *et al.,* 2020; Krishnan *et al.,* 2021) and as a precautionary measure, such feed should be used only after the stringent screening for EHP.

To contribute more insights into the host range of EHP, the present study examined a range of potential hosts for the presence of the parasite using established PCR protocols. The study attempted to look into the potential carrier status of farmed and wild crustaceans, aquatic insect and molluscs, apart from screening some of the farmed shrimp to assess the prevalence of EHP infection. In the present study, *P. monodon* samples collected from the polyculture system of West Bengal were subjected to EHP-specific PCR. Though both SSU-PCR and SWP-PCR gave positive results, *P. monodon* samples did not show positive amplification with β-tubulin-PCR. Earlier, all the research works on EHP in *P. monodon* were based on SSU-PCR (Chayaburakul *et al.,* 2004; Tourtip *et al.,* 2009; Ha *et al.,* 2010; Biju *et al.,* 2016; Marimuthu *et al.,* 2021). However, the present study provides the first SWP-PCR based evidence of EHP infection in *P. monodon*.

P. vannamei samples were collected from different brackishwater farms of Maharashtra and inland saline shrimp farms of Punjab and Rajasthan and, as expected, PCR screening for EHP revealed positive amplification in many samples. Prevalence of EHP in *P. vannamei* farms from various shrimp-growing states has already been reported by many researchers from India (Biju *et al.,* 2016; Rajendran *et al.,* 2016; Giridharan and Uma, 2017; Santhoshkumar *et al.,* 2017; Kummari *et al.,* 2018; Jithendran *et al.,* 2021; Sajiri *et al.,* 2021) and other countries (Tang *et al.,* 2017; Shen *et al.,* 2019; Kim *et al.,* 2021). Along with these, the present observation indicates that EHP has become a widely prevalent pathogen in the shrimp farming environment in India.

It has been reported that crabs can be a potential host of EHP (Kummari *et al.,* 2018). The researchers detected the presence of EHP using SSU-PCR in crab co-habiting the shrimp ponds; however, the report did not mention the species of the crab host. The potential of mud crabs (*Scylla serrata, S. olivacea* and *S. paramamosain*) as a carrier of shrimp pathogens, especially WSSV, has been established by many researchers (Rajendran *et al.,* 1999; Somboonna *et al.,* 2010). Wild crab species such as *Uca* sp., *Scylla* sp. and *Sesarma* sp., which are common dwellers of aquatic systems adjoining shrimp culture ponds were also proved as carriers of TSV and IHHNV (Kiatpathomchai *et al.,* 2008; Das *et al.,* 2016; Saravanan *et al.,* 2021). The present study confirmed that mud crab (*S. serrata*) could act as carrier of EHP.

Many aquatic fauna are reported to act as mechanical vectors for the transmission of potential shrimp pathogens. In an earlier study, the water boatman *Trichocorixa reticulata* has been reported as a carrier of the TSV (Lightner, 1996). Similarly, many aquatic insects such as *Ephydridae* sp., *Gerries, Notonecta, Nepa, Ranatra* and Dragon nymph fly have been described as the vector of WSSV (Lo *et al.,* 1996). In another study, aquatic insects such as *Belostoma* sp., *Aesohna* sp., *Cybister* sp. and *Notonecta* sp. were reported as the potential hosts of *Macrobrachium rosenbergii* nodavirus (MrNV) and extra small virus (XSV), causing white tail disease (Sudhakaran *et al.,* 2008). In the present study, an aquatic insect *Dytiscus* sp. was found to be a carrier of EHP. This finding needs more attention as these insects, which are natural inhabitants of the culture system, may pose a significant threat to the transmission of EHP to nearby ponds. Therefore, the existing

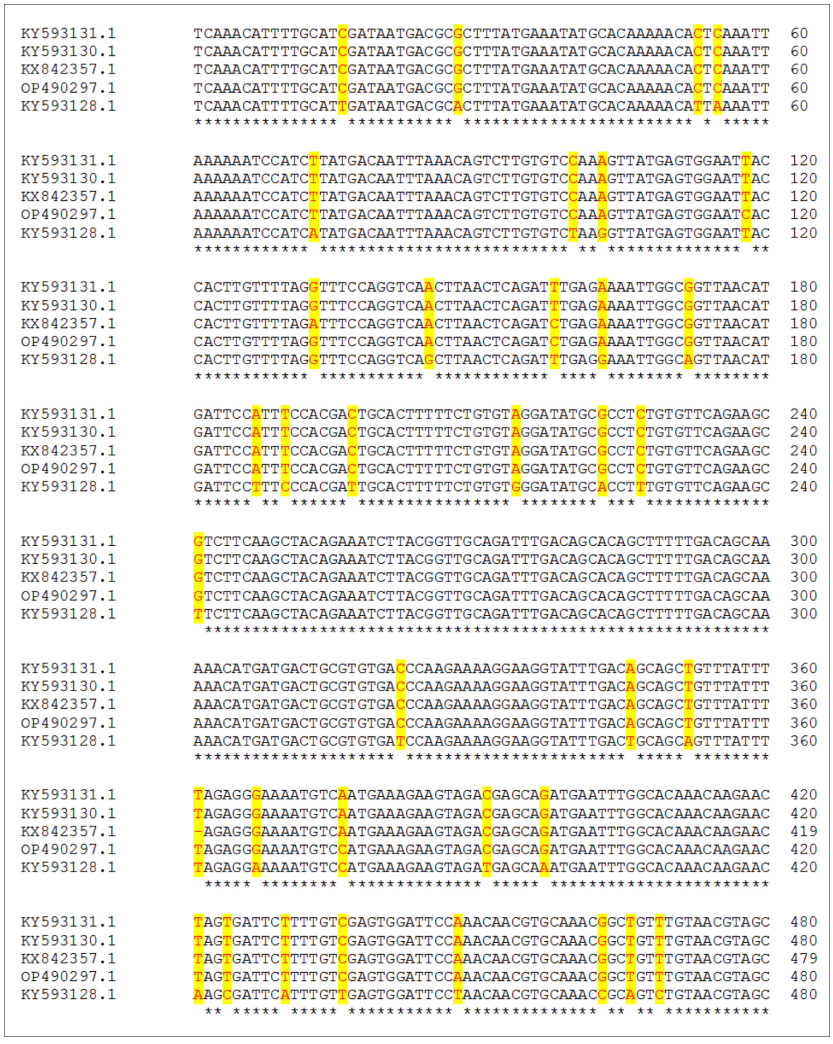


Fig. 9. Multiple alignments of the nucleotide sequences of β-tubulin from EHP amplified from *P. vannamei* with other EHP sequences reported in the GenBank

biosecurity measures to prevent the entry of potential carrier hosts may be improvised to avoid aquatic insects in shrimp farms. A recent study by Munkongwongsiri *et al*. (2022) revealed EHP-positive samples of false mussels *Mytilopsis leucophaeata* from shrimp culture ponds. In the present study, though other co-habiting aquatic fauna including clams, snail and green mussel were screened, only a few clam samples were tested EHP-positive in SSU-PCR. Nevertheless, further studies are needed to know the role of these aquatic fauna in carrying EHP, by increasing the sample size and including farms from many locations.

Most of the previous studies used either SSU-PCR or SWP-PCR for the screening of EHP from different aquatic organisms. The present study forms the first one which utilised all three PCR tests (SSU-nested PCR, SWP-PCR and β-tubulin-PCR) available for the detection of EHP in various potential hosts. SSU-PCR was used as a preliminary screening test to compare how many of the SSU-PCR positive samples will be tested positive for EHP using EHP-specific primers. It can be seen from the data that the number of samples tested positive in SSU-PCR was higher than that tested positive in SWP-PCR, except for the aquatic insect in which 7/10 samples were found positive in SWP-PCR and only 6/10 were positive in SSU-PCR. Similarly, higher prevalence of EHP was noticed in all organisms using β-tubulin-PCR compared to SWP-PCR, except for *P. monodon* in which all the samples were negative in β-tubulin-PCR, whereas 2/10 samples were positive with SWP-PCR (Table 3).

The present study indicated that EHP can be transmitted from infected ponds to naïve culture systems by the aquatic insect *Dytiscus* sp., which are natural inhabitants of shrimp ponds. Mud crabs, *S. serrata* and *S. olivacea* are also likely to carry EHP as potential hosts and thus, can cause infection in co-habiting shrimp. *P. monodon* and *P. vannamei* which were already reported to be hosts for the pathogen were again confirmed by different PCR methods. *P. indicus* was also found to be potential host, but the present data is not conclusive because of the smaller sample size. The report of EHP in aquatic insect and *P. vannamei* from inland saline-affected states warrants the necessity of implementing proper biosecurity measures in these states for the control of EHP. Extensive targeted surveillance covering a large sample size and wide geographical locations is needed to have more insights into the natural host range of EHP.

Acknowledgements

The authors are thankful to Director and Vice-Chancellor, ICAR-CIFE, Mumbai, for providing necessary facilities to conduct the research work. Part of the research work has been supported by National Agriculture Higher Education Project (NAHEP).

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