



A Multiplex PCR Approach for Accurate Detection of Key Pathogens to Improve Disease Surveillance in Shrimp Aquaculture

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10.18805/IJAR.B-5531

ABSTRACT

Background: White spot syndrome virus (WSSV), Infectious hypodermal and haematopoietic necrosis virus (IHHNV) and *Enterocytozoon hepatopenaei* (EHP) are widely prevalent diseases affecting cultured and wild shrimp. These diseases lead to significant production and economic losses. There have been reports of co-infection of WSSV with IHHNV, WSSV with EHP and EHP with IHHNV. Since there are no proper management and treatment methods, early and timely detection of diseases is crucial. This study aimed to develop a multiplex PCR assay for timely and accurate diagnosis of WSSV, IHHNV and EHP, which is essential for minimizing production and economic losses in the shrimp aquaculture industry.

Methods: This study involves the screening and confirmation of penaeid shrimp samples for diseases viz, WSSV, IHHNV and EHP, designing of PCR primers, optimization of PCR parameters such as annealing temperature, primer concentration and validation of the assay.

Result: The multiplex PCR assay detected WSSV, IHHNV and EHP in a single PCR reaction. Analytical sensitivity was found to be 100 pg/μl of WSSV, 100 pg/μl of IHHNV and 10 pg/μl of EHP. Diagnostic sensitivity of the developed assay was 100%. Multiplex PCR assay developed in this study can be used for the screening of broodstock, post larvae, live food and environmental samples for early detection of WSSV, IHHNV and EHP.

Key words: Co-infection, Disease diagnosis, Multiplex PCR, Shrimp pathogens.

INTRODUCTION

In 2022, global shrimp production hit a new record of 9.4 million tonnes and farmed shrimp contributed about 63%. Shrimps and prawns accounted for 17% of aquatic animal products traded globally (FAO, 2024a; FAO, 2024b). The top five shrimp producing countries are Ecuador, China, India, Vietnam and Indonesia. They alone contribute about 74 per cent of the world's farmed shrimp supply (Jory, 2023). India's total shrimp aquaculture production was estimated at 11.6 lakh metric tonnes, including Pacific White leg shrimp (*Penaeus vannamei*), Black tiger shrimp (*Penaeus monodon*) and other shrimps in 2022-23 (MPEDA, 2023).

Shrimp farming predominantly employs intensive and semi-intensive techniques to meet the increasing global demand. The practice of high stocking density led to the emergence and spread of various diseases (Nunan *et al.*, 1998). Over the last decade, disease outbreaks have affected the global shrimp production. An estimated 60% of production losses are attributed to viral diseases, 20% to bacterial diseases and the remaining 20% to parasites, fungi and other causes (Flegel, 2012). The major diseases causing impacts are white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), yellow head virus (YHV), Taura syndrome virus (TSV), infectious myonecrosis virus (IMNV), necrotising hepatopancreatitis (NHP), acute hepatopancreatic necrosis disease (AHPND) and microsporidian parasite-*Enterocytozoon hepatopenaei* (EHP) (Tandel *et al.*, 2017; WOA, 2024).

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How to cite this article: Santhiya, V., Uma, A., Saravanan, S., Selvaraj, S. and Porselvan, S. (2026). A Multiplex PCR Approach for Accurate Detection of Key Pathogens to Improve Disease Surveillance in Shrimp Aquaculture. *Indian Journal of Animal Research*. 1-8. doi: 10.18805/IJAR.B-5531.

Submitted: 04-12-2024 **Accepted:** 05-01-2026 **Online:** 14-01-2026

WSSV causes white spot disease (WSD). WSSV in penaeid shrimp was initially documented in China and Taiwan in 1992. Subsequently, it has spread to many countries in Asian, African, American and Australian continents. WSSV causes 100% mortality within 3-10 days in infected shrimps resulting in severe economic losses in the shrimp farming industries of the world. Several reports suggest that global economic loss caused by WSD is approximately equal to 1/10th of shrimp produced annually (Stentiford *et al.*, 2012; Oakey *et al.*, 2019).

IHHNV causes infectious hypodermal and haematopoietic necrosis. IHHNV is the smallest reported virus of the penaeid shrimps. IHHNV has been listed in the OIE since 1995 (Lightner *et al.*, 2012). This disease was reported for the first time in Hawaii causing 90% mortality in juveniles of *Penaeus stylirostris* imported from Ecuador and Costa Rica. However, *P. vannamei* did not show any mortality due to this infection (Lightner *et al.*, 1983). This disease caused huge mortalities in *P. stylirostris* and causes retardation in growth (Runt Deformity Syndrome) in *P. vannamei* (Aranguren Caro *et al.*, 2022).

EHP causes hepatopancreatic microsporidiosis (HPM) in shrimp. It is an intracellular parasite forming numerous spores in the tubule of epithelial cells of the shrimp hepatopancreas, causing growth retardation and size variation due to the impaired functions of hepatopancreatic cells (Babu *et al.*, 2024). EHP was first characterized based on 18S-rRNA from Thailand in 2009 (Tourtip *et al.*, 2009). HPM causes huge economic losses in shrimp farming (Thitamadee *et al.*, 2016; Jaroenlak *et al.*, 2018).

There have been reports of co-infection of WSSV, IHHNV with TSV in China (Tan *et al.*, 2009), EHP with IHHNV in China (Wang *et al.*, 2017), WSSV with EHP in India (Thamizhvanan *et al.* 2019) and IHHNV with WSSV in India (Saravanan *et al.*, 2021).

Since presumptive diagnostic methods are not reliable, more sensitive confirmatory diagnostic method like PCR is recommended (Pradeep *et al.*, 2012; Thitamadee *et al.*, 2016; WOA, 2019). PCR has transformed the diagnostic field of infectious diseases, but its application in diagnosis is constrained by limited volumes of samples, time and cost (Elrifro *et al.*, 2000). To address these limitations and strengthen the diagnostic efficiency of PCR, multiplex PCR assays were developed, enabling the simultaneous detection of several pathogens in a single reaction and thereby reducing both time and cost (Solanki and Devi, 2024; Sharma *et al.*, 2024). This study attempted to develop a multiplex PCR assay for simultaneous detection of WSSV, IHHNV and EHP in a single PCR assay.

MATERIALS AND METHODS

DNA extraction

Penaeid shrimp samples maintained in the repository of State Referral Laboratory for Aquatic Animal Health (SRLAAH), Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Madhavaram campus, Tamil Nadu, India was used in this study. This study was carried out for a period of 1 year at SRLAAH, TNJFU. Whole post larvae, gills, hepatopancreatic tissue and muscle from the infected and uninfected healthy shrimp samples were used for total DNA extraction and was done using a commercial DNA extraction kit (Qiagen, Germany) following the manufacturer's guidelines. The extracted DNA was then suspended in 30 µl of nuclease free water and used for the study either immediately or stored at -20°C for future use. The extracted genomic DNA was quantified using a nanophotometer (Thermo Fisher Scientific, USA) for PCR amplification.

Screening and confirmation of penaeid shrimp samples

The penaeid shrimp samples were screened for WSSV, IHHNV and EHP and confirmed using published PCR primers. The details of the published PCR primers are presented in Table 1.

Designing of primers

Sequence information of specific genes of WSSV, EHP and IHHNV collected from the NCBI GenBank database were used for designing of PCR primers for their specific diagnosis. 'PrimerBLAST' (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>) program was used for designing of primers. The details of the designed primers are presented in Table 2.

Standardization and validation of PCR assays

Optimization of primer annealing temperatures

The annealing temperature was optimized for the designed primers using a gradient PCR protocol in the temperature ranging from 58°C to 62°C. The annealing temperature that resulted in the sharpest and the most specific amplification, as confirmed by gel electrophoresis was selected and used for the assay.

Table 1: Details of the published PCR primers.

Pathogens	Primer code	Primers (5'-3')	Amplicon size	References
WSSV	WSSV- 982F	ATCATGGCTGCTTCACAGAC	982 bp	Kimura <i>et al.</i> , 1996
	WSSV- 982R	GGCTGGAGAGGACAAGACAT		
	WSSV- 570F	TCTTCATCAGATGCTACTGC	570 bp	
	WSSV- 570R	TAAGGCTATCCAGTATCACG		
IHHNV	IHHNV-309F	TCCAACACTTAGTCAAAACCAA	309 bp	Tang <i>et al.</i> , 2007
	IHHNV-309R	TGTCTGCTACGATGATTATCCA		
EHP	BEHP- 618F	CAGCTGGTTGAAAATGCAAA	618 bp	Han <i>et al.</i> , 2018
	BEHP- 618R	GTGCAAAAATGCCTTTCGTT		
	BEHP- 237F	GATATGCGCCTCTGTGTTCA	237 bp	
	BEHP- 237R	TGTTTGAATCCACTCGACA		

Optimization of primer concentrations for the designed PCR primers

The PCR amplification was optimized for the designed PCR primers (WSSV511-28, EHP337-BT and IHNV183-NS1) by varying the concentrations of primers viz., 30 picomoles, 20 picomoles, 15 picomoles, 10 picomoles and 5 picomoles.

Specificity of the designed PCR primers

The specificity of the designed PCR primers (WSSV511-28F and WSSV511-28R) for WSSV was confirmed with DNA from shrimp infected with WSSV, IHNV, EHP, MBV, HPV and WSSV uninfected healthy shrimp sample. The specificity of the designed PCR primers (IHNV183-NS1F and IHNV183-NS1R) for IHNV was confirmed with DNA from shrimp infected with IHNV, WSSV, EHP, MBV, HPV and IHNV uninfected healthy shrimp sample. Similarly, the specificity of the designed PCR primers (EHP337-BTF and EHP337-BTR) for EHP was confirmed with DNA from shrimp sample infected with EHP, WSSV, IHNV, MBV, HPV, *Agmasoma* spp. and EHP uninfected healthy shrimp sample.

Analytical sensitivity of designed primers and diagnostic sensitivity

The analytical sensitivity of the designed PCR primers was assessed with serially diluted DNA from WSSV, IHNV and EHP- infected shrimp samples at varying concentrations: 100 ng/μl, 10 ng/μl, 1 ng/μl, 100 pg/μl, 10 pg/μl and 1 pg/μl of total DNA. The diagnostic sensitivity of the designed primers was assessed using DNA extracted from samples that had been previously confirmed to be positive.

Multiplex PCR assay protocol for WSSV, IHNV and EHP

The PCR amplification was carried out in a thermal cycler (Biorad T100 Thermal cycler, USA) in a total volume of 50 μl reaction mixture containing 23 μl of 2X mastermix RED (Ampliqon, Denmark) (Tris-HCl pH 8.5, (NH₄)₂SO₄, 3 mM MgCl₂, 0.2% Tween 20®, 0.4 mM dNTPs, 0.2 units/μl AmpliqonTaq DNA polymerase), 1 μl (10 pmol) of WSSV511-28F and WSSV511-28R each, 1 μl (10 pmol) of IHNV183-NS1F and IHNV183-NS1R each, 1 μl (10 pmol) of EHP337-BTF and EHP337-BTR each, 1 μl (50 ng) of WSSV DNA, 1 μl (50 ng) of IHNV DNA, 1 μl (50 ng) of EHP DNA and 18 μl of nuclease free water. Since naturally occurring triple-positive (WSSV, IHNV and EHP) shrimp samples

were not available during the study period, validation of the multiplex PCR was performed using artificially mixed DNA templates. 17 double positive (WSSV and EHP) samples were used for validation of the developed assay. The diagnostic sensitivity of the multiplex PCR was assessed.

PCR analysis

All PCR experiments were carried out in 0.2 ml tubes in the thermal cycler with the following cycle parameters: initial denaturation at 94°C for 3 mins, followed by 35 cycles of 94°C for 30 s, annealing temp 59°C for 30 s and 72°C for 30 s and a final extension of 72°C for 7 min. The amplified PCR products were separated by agarose gel electrophoresis. Briefly, 2.0% agarose gel in 1X Tris-borate-EDTA buffer (TBE buffer) was used. About 8 μl of amplified PCR products were loaded along with a 100 bp molecular weight marker (2 μl) (GeneDirex, Taiwan) onto the gel. Gel electrophoresis was carried out at 120V for 30 mins. The resulting PCR bands were visualized and documented using a UV gel documentation system (Bio-Rad, Germany).

RESULTS AND DISCUSSION

The standardized annealing temperature of designed primers targeting WSSV, IHNV and EHP was 59°C (Fig 1A, 1B, 1C). The best PCR amplification was observed at using 10 pmol of PCR primers (Fig 2A, 2B, 2C). The designed PCR primers (WSSV511-28F and WSSV511-28R) for WSSV, (IHNV183-NS1F and IHNV183-NS1R) for IHNV and (EHP337-BTF and EHP337-BTR) for EHP specifically detected WSSV, IHNV and EHP respectively and no cross reaction was observed when tested with other DNA (Fig 3A, 3B, 3C). The analytical sensitivity of designed PCR primers for WSSV, IHNV and EHP based on DNA concentration was 100pg, 100pg and 10pg of template DNA respectively (Fig 4A, 4B, 4C). The diagnostic sensitivities of the primers designed for WSSV, IHNV and EHP were 100% (75/75), 100% (38/38) and 100% (95/95) respectively when tested with the previously confirmed samples (Table 3). Optimization of multiplex PCR assay using various primer concentrations is represented in Fig 5. Analytical sensitivity of the developed multiplex PCR assay was 100pg of template DNA is represented in Fig 6. The multiplex PCR assay for WSSV, IHNV and EHP was optimized using various concentrations of the designed

Table 2: Details of the designed PCR primers.

Pathogens	Primer code	Accession numbers of reference sequences used	Target gene	Annealing temperature	Amplicon size
WSSV	WSSV511-28F WSSV511-28R	KT963933	VP 28	59°C	511 bp
IHNV	IHNV183-NS1F IHNV183-NS1R	KJ830753	NS 1	59°C	183 bp
EHP	EHP337-BTF EHP337-BTR	OQ622243	β-tubulin	59°C	337 bp

primers. The most effective amplification of DNA from WSSV-infected, IHHNV-infected and EHP-infected penaeid shrimp samples was observed at concentrations of 10 picomoles

(WSSV511-28), 10 picomoles (IHHNV183-NS1) and 10 picomoles (EHP337-BT) by PCR (Fig 7). The diagnostic sensitivity of the developed multiplex PCR assay was 100%

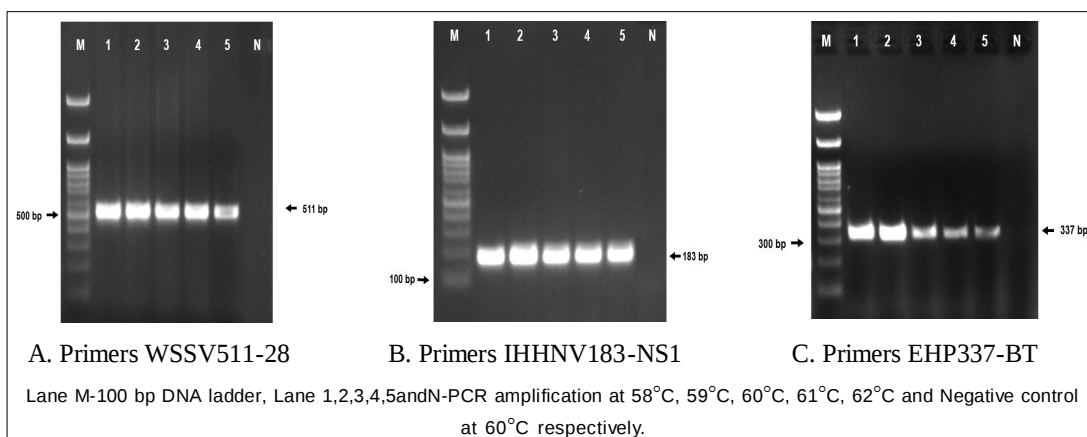


Fig 1: Optimization of annealing temperatures of designed primers targeting WSSV, IHHNV and EHP.

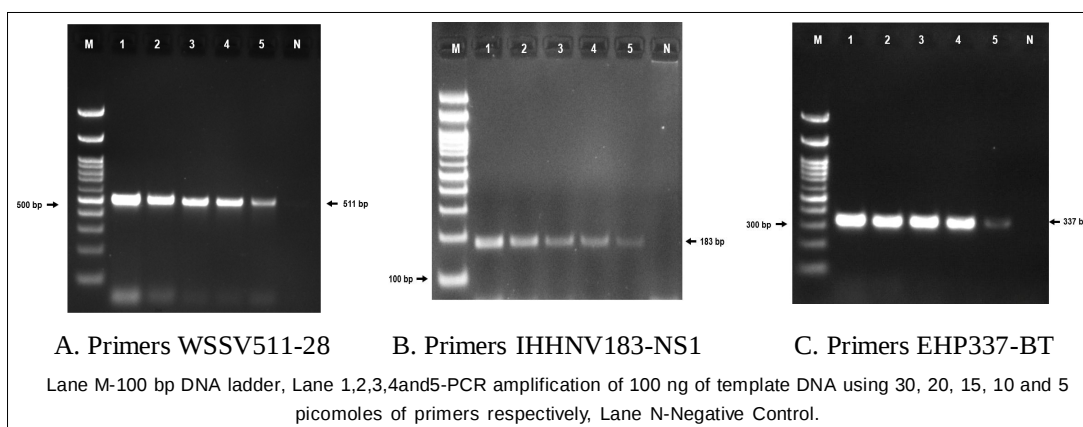


Fig 2: Optimization of primer concentrations of designed PCR primers of WSSV, IHHNV and EHP.

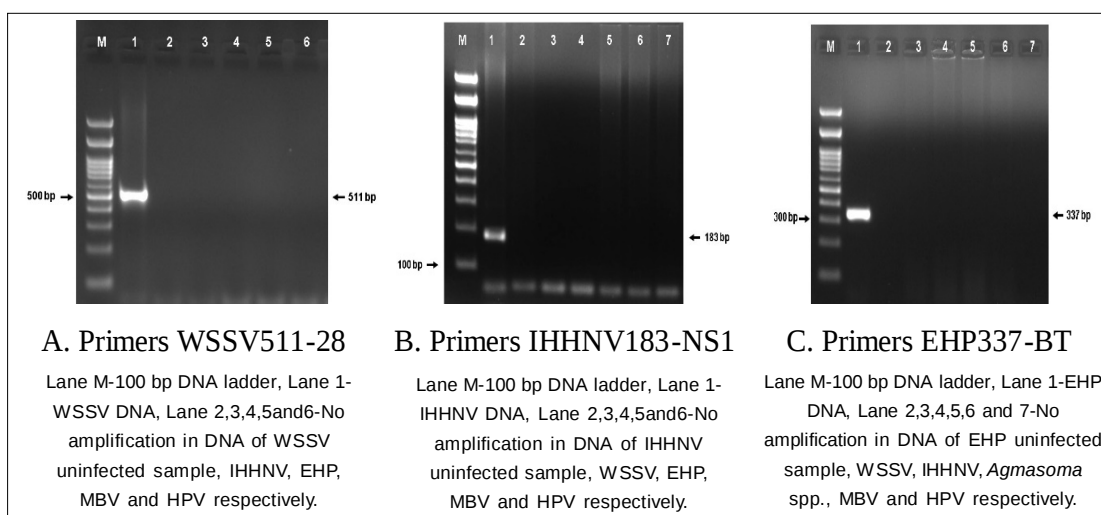
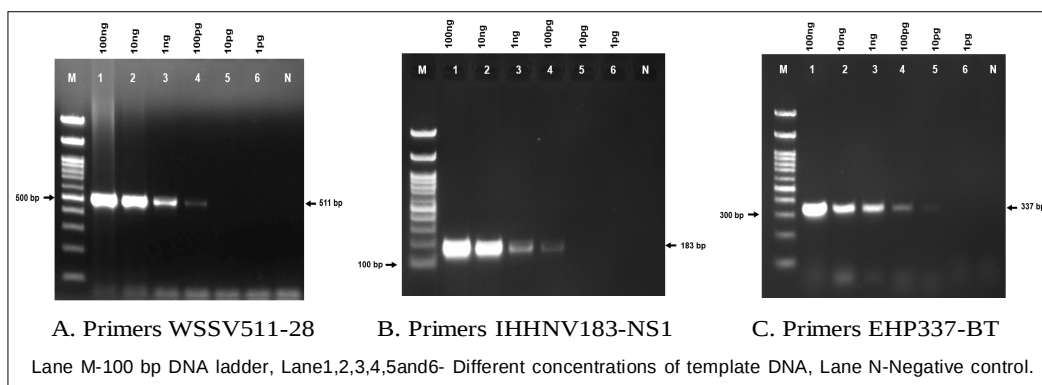
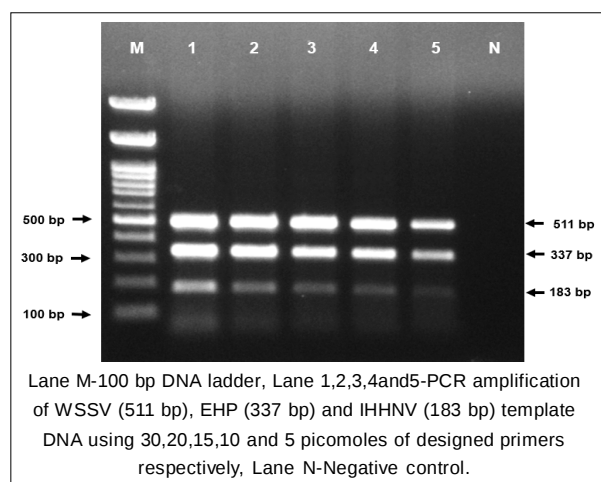
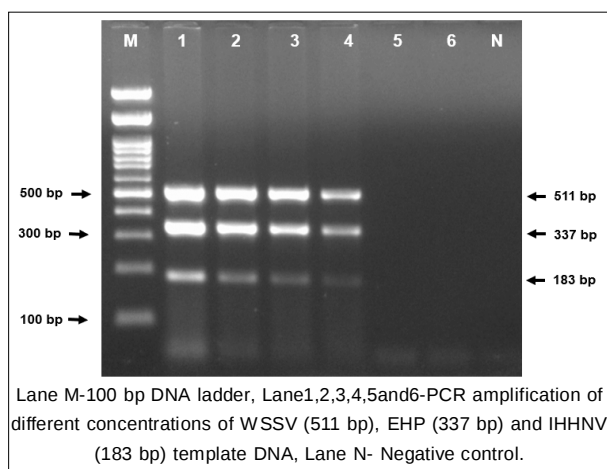


Fig 3: Specificity of designed PCR primers for detecting WSSV, IHHNV and EHP.

Table 3: Diagnostic sensitivity of designed primers.

Pathogens	Samples confirmed (published primers)	Samples diagnosed (designed primers)	Diagnostic sensitivity	95% Confidence interval (CI)	Significance level
WSSV	75	75	100 %	95.20%-100%	$\alpha = 0.05$
IHHNV	38	38	100 %	90.75%-100%	$\alpha = 0.05$
EHP	95	95	100 %	96.19%-100%	$\alpha = 0.05$

**Fig 4:** Analytical sensitivity of designed PCR primers for WSSV, EHP and IHHNV.**Fig 5:** Optimization of primer concentrations of multiplex PCR assay.**Fig 6:** Analytical sensitivity of the developed multiplex PCR assay.

(38/38), as all previously confirmed positive samples were correctly detected for their respective target pathogens.

Conventional PCR based on 18S-rRNA for WSSV detection by Lo *et al.* (1996), has produced false positives (Claydon *et al.*, 2004). Recent findings suggest the emergence of a new WSSV strain in India due to the deletion of several ORF regions. VP28 gene was detected in both old and new strain of WSSV (Sivakumar *et al.*, 2018). VP28, a key envelope protein involved in viral infection of shrimp cells, is highly conserved across different geographical isolates (Mendoza-Cano and Sánchez-Paz, 2013). Hence, primers detecting WSSV were designed based on VP28 in this study. Diagnostic assays based on the SSU rRNA gene, often led to false positive results due to the high sequence similarity (68%–90%) with other aquatic microsporidians. Diagnostic assays based on the SSU rRNA gene are typically preferred for screening tissue samples such as post-larvae and hepatopancreas, whereas assays targeting the spore wall protein (SWP) coding gene are more suitable for environmental samples like soil and faeces (Jaroenlak *et al.*, 2016). A nested PCR based on β -tubulin for detection of EHP was developed, which is a more conserved gene in EHP isolates. Thus, making β -tubulin as a more reliable target for PCR assay development (Han *et al.*, 2018). β -tubulin gene sequences show sufficient variability at species level, the diagnostic assays based on this gene did not show any cross reaction with other parasites of shrimp. It is a single copy gene in the genome of EHP (Piamsomboon *et al.*, 2019). In this study, primers for detecting EHP were designed based on β -tubulin, as it can be used for screening of shrimp, live foods and environmental samples. Due to the conserved nature of non-structural protein-1 of IHHNV, the genes encoding NS-1 was chosen for primer designing. Annealing

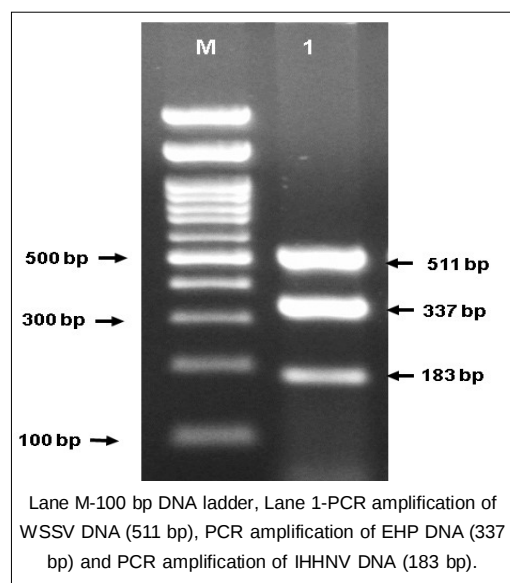


Fig 7: Multiplex PCR assay developed for detection of WSSV, EHP and IHHNV.

temperature at 59°C resulted in clear bands during gel electrophoresis. No cross reaction was observed in other published reports and these results correlated with the present study (Natividad *et al.*, 2006; Yang *et al.*, 2006; Khawsak *et al.*, 2008). The analytical sensitivity of multiplex PCR assay when compared to that of uniplex PCR assay is usually reduced 10 to 100 times in most cases (Yang *et al.*, 2006). In this study, the analytical sensitivity for WSSV and IHHNV in uniplex PCR assays were similar to multiplex PCR assays. But the analytical sensitivity for EHP in multiplex PCR assay is reduced 10-fold compared to that of uniplex PCR assay. Multiplex PCR assay developed for simultaneous detection of WSSV and EHP could detect WSSV and EHP at concentration of 100pg (Tamizhvanan *et al.*, 2019). The optimized PCR protocol specifically detected WSSV, IHHNV and EHP, thereby making it a convenient tool for simultaneous screening at reduced time and handling.

CONCLUSION

The developed multiplex PCR protocol offers a more rapid, cost-effective and high specificity in screening of shrimp samples since WSSV, IHHNV and EHP can be detected in a single PCR reaction tube. Using the developed assays, detection of WSSV, IHHNV and EHP starting from sample preparation can be completed within 4-5 h. These developed duplex and multiplex PCR assays are effective in rapid detection of WSSV, IHHNV and EHP that cause mortalities and growth retardation in penaeid shrimps. It can be used for screening of post larvae in hatcheries for infection before stocking them in grow-out ponds, broodstock, live foods and environmental samples; thus, preventing huge economic losses due to these pathogens.

ACKNOWLEDGEMENT

The present study was supported by Tamil Nadu Fisheries University, Nagapattinam, Tamil Nadu, India for extending the research facilities and funding's.

Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal Care and Handling Techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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