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Stock Health of Penaeid Shrimps in Sri Lanka: A Comprehensive Molecular-Based Study on Seven Diseases Found in the Asian Region

J.A. Athula[1*], C. N. Walpita[2], H.A.D. Ruwandeepika[2], T.N.S. Thilakasiri[3], E.G.R. Dayananda[3] and P.N. Chandraratne[3]

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ABSTRACT

Purpose: *Although shrimp aquaculture in Sri Lanka has mainly been plagued by recurrent outbreaks of White Spot Syndrome Virus (WSSV), recent symptomatic and analytical field data of shrimps were incoherent with typical WSSV infections. As transboundary infection of new diseases is not uncommon in world shrimp aquaculture, the possibility of new diseases established in the country surfaced. Hence, the present study was aimed at evaluating the stock health of shrimps in Sri Lanka for additional six diseases commonly available in the Asian region, namely Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Infectious Myonecrosis Virus (IMNV), Taura Syndrome Virus (TSV), Yellow Head Virus (YHV), Acute Hepatopancreatic Necrosis (AHPND) and Necrotizing Hepatopancreatitis Bacterium (NHPB).*

Research Method: *Sampling was performed using broodstocks, postlarvae, cultured Penaeus monodon, wild P. semisulcatus, P. indicus and P. monodon from different locations and broodstock collecting areas in the country. Other crustacean species were also collected as possible carrier species. The presence of pathogenic organisms was identified by Polymerase Chain Reaction using respective IQ 2000TM test kits and a total of 2060 PCR tests were conducted.*

Findings: *Of all the samples tested, no samples were positive for IMNV, TSV, YHV, AHPND and NHPB. However, confirmatory evidence was found in Sri Lanka for the presence of IHHNV with the prevalence of 31.3% in broodstock samples, 24.07% in hatchery-produced postlarvae and 18.03% in cultured shrimps. WSSV was also recorded with a prevalence of 10.34% in broodstock samples and 55.56% in cultured shrimp samples collected. None of the wild collected samples was positive for any of the tested diseases.*

Originality/value: *Reinstating the presence of WSSV in the Sri Lankan shrimp industry, provides confirmatory evidence of the presence of IHHNV in the country, highlighting the possibility of the emergence of new diseases in shrimp farming in Sri Lanka. Also, commonly suggested disease carriers seem to be not the major cause of disease spread during the sampling time. This study, therefore, provides much-needed information about the stock health of shrimps in Sri Lanka.*

Keywords: *IHHNV, P. monodon, Polymerase Chain Reaction, Stock health, WSSV*

INTRODUCTION

The development and expansion of a sustainable shrimp aquaculture industry are mainly affected by diseases caused by different causative agents. Most of these diseases cause major production losses and are a result of newly emerged pathogens and spread rapidly even across the national borders and these diseases are most

[1*] *Faculty of Graduate Studies, Sabaragamuwa University of Sri Lanka, PO Box 02, Belihuloya, 70140, Sri Lanka.*

athulaj@uwu.ac.lk

[2] *Department of Livestock Production, Faculty of Agricultural Sciences, Sabaragamuwa University of Sri Lanka, PO Box 02, Belihuloya, 70140, Sri Lanka.*

[3] *National Aquaculture Development Authority of Sri Lanka. No 49/1, New Parliament Rd, Pelawatte, Battaramulla, 10120, Sri Lanka.*

https://orcid.org/0000-0002-5663-1624

often caused by viruses (FAO, 2019).

Sri Lanka has experienced three major shrimp viral disease outbreaks and the first one was Monodon Baculo Virus (MBV) recorded in 1988. Although this disease is still reported, its severity level is considered as negligible as in many other countries, to a level where hosts hardly show typical symptoms and related economic losses (Fegan *et al.*, 1991). White Spot Syndrome Virus (WSSV) is the second reported shrimp viral disease, first started around May 1996 in Sri Lanka (Hettiarachchi and Epa, 1999) and greatly reduced the productivity, profitability and sustainability in the industry. Due to the severe impact of this disease on the shrimp aquaculture industry, the lowest annual farm production of 1570 Mt was recorded in 2005 (NAQDA, 2021). The third disease outbreak was reported in 1998 as a combination of WSSV and YHY disease (WB, NACA, WWF, FAO, 2001). The prevalence of WSSV not only affected the farmed shrimps but also wild stocks in lagoons and coastal areas. This was mainly due to contamination owing to the direct release of water and infected shrimps into the natural water bodies.

Under the government rehabilitation program of the shrimp farming industry initiated in 2004 in Sri Lanka, screening of brood shrimps for WSSV used for postlarvae (PL) production was initiated and it was implemented as a basic component of the Better Management Practices (BMP) in the shrimp hatchery operations and several BMP's including BMP's for shrimp farming, hatchery operations, supplying of feed and chemicals to the industry were introduced. Ever since many regulatory measures have been in place for controlling the spread and the economic significance of shrimp diseases in Sri Lanka, and the focal point was deservedly taken by WSSV. With all these efforts, 8180 MT of farmed shrimp production was reported in 2018 (NAQDA, 2021) operating 1987 ha of farms in North Western Province (NWP) and 121 ha of farms in Eastern Province (EP) (Department of Fisheries and Aquatic Resources, 2019). Although the production has increased, sporadic cases were reported in farming areas where unexpected mortalities and inconclusive symptoms were found in farmed shrimps. Some of these cases were not WSSV positive and symptoms were incoherent to WSSV, suggesting a possible new disease or disease complexes occurring in the country. It was therefore a timely necessity to evaluate the stock health of shrimps in the whole farming zones of the country for other diseases found in the region. World Organization for Animal Health (OIE) has listed 7 shrimp diseases of economic significance as found in the region, namely Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Infectious Myonecrosis Virus (IMNV), Taura Syndrome Virus (TSV), Yellow Head Virus (YHV), Acute Hepatopancreatic Necrosis (AHPND), White Spot Syndrome Virus (WSSV) and Necrotizing Hepatopancreatitis Bacterium (NHPB).

Therefore, the objective of the present study was to evaluate the stock health of *P. monodon* based on OIE listed seven diseases (IHHNV, IMNV, TSV, YHV/GAV, AHPND/EMS, WSSV and NHPB).

MATERIALS AND METHODS

Sample Collection

Samples were collected under nine different categories during the period of May to October 2017 from different areas of the country. These samples include *P. monodon* broodstock samples, PL samples (representing broodstock collecting areas), farmed shrimp samples and, as the common possible carrier species, wild-collected *P. monodon*, *P. merguiensis*, *P. indicus*, *Scylla serrata* (mud crab), *Portunus pelagicus* (blue swimming crab) and krill samples. Under the first three categories, samples were taken as individual samples and the rest of the samples were taken as pooled samples from the wild, including lagoons and canals which are fed with discharged effluent of shrimp farms. Purposive samples were collected targeting weak and moribund, with any clinical signs, or deformities

or directly from disease-recorded farms. Details of the sample collected areas are indicated in Table 01. Limited sampling locations during the experimental period were due to the unavailability of samples under rough sea seasons.

All the samples were collected as live or dead animals and transported to the PCR laboratory established under the National Aquaculture Development Authority at the Shrimp Farm Monitoring and Extension Unit, Bathulu Oya, Sri Lanka. These samples were stored under -20 ^{0}C. Brood shrimps separately stocked in the quarantine units were used to take samples and around 1 cm string of the faecal matter and pleopods were taken separately and stored using 70% alcohol until the DNA or RNA extraction.

DNA and RNA Extraction

DNA and RNA were extracted using different types of samples (Table 02) as prescribed in IQ 2000™ test kit manuals for seven diseases.

Reverse Transcription of RNA

RNA was extracted to detect the YHV/GAV, TSV and IMNV shrimp pathogens and the extracted RNA was subsequently converted into complementary DNA (cDNA) by using reverse transcription PCR (RT-PCR) as per the manufacturer's guidelines. RT-PCR reaction reagent mixture contained 7.0 µL of RT-PCR Pre Mix, 0.5 µL of IQzyme DNA Polymerase (2 U/µL) and 0.5 µL of RT Enzyme Mix. 2 µL of each RNA extract was mixed with 8 µL of RT-PCR reaction mixture separately and subjected to the RT-PCR reaction under the program of thermal cycle of 42 ^{o}C 30 min 94 ^{o}C 2 min; then 94 ^{o}C 20 sec; 62 ^{o}C 20 sec; 72 ^{o}C 30 sec, repeat 15 cycles, then add 72 ^{o}C 30 sec; 20 ^{o}C 30 sec at the end of the final cycle and followed by the nested PCR reaction (A200 series Peltier-based Thermal Cycler, China).

Table 01: Description of collected samples.

No	Sample category	The area of the sample collected
1	*P. monodon* broodstock	Handala and Beruwala
2	*P. monodon* postlarvae	Handala, Beruwala and Mullaitivu
3	*P. monodon* pond sample	NWP- Zone* 01 to Zone 05 EP- Batticaloa District
4	Wild *P. merguiensis*	Puttlam and Chilaw Lagoon and Mee Oya and Oddamavadi Lagoon area in Batticaloa
5	Wild *P. indicus*	Mannar, Puttlam, Kalpitiya and Chilaw Lagoon area
6	Wild *P. monodon*	Mannar, Puttlam, Kalpitiya, Chilaw and Batticaloa Lagoon area
7	Wild *Scylla serrata*	Zone 03 to 05 and Oddamavadi lagoon area in Batticaloa
8	Wild *Portunus pelagicus*	Zone 01, Zone 05 and 06, and Oddamavadi lagoon area in Batticaloa
9	Wild collected Krills	Zone 01 and 02 and Oddamavadi lagoon area in Batticaloa

**(Zone 01-Chilaw, Zone 02-Arachchikattuwa, Zone 03-Mundalama, Zone 04-Kalpitiya, Zone 05-Puttlam)*

Table 02: Type of samples taken for the DNA and RNA extraction.

Disease	Type of sample		
	postlarvae (PL)	Cultured/wild shrimps	Broodstock
IHHNV and WSSV	Abdomen of 25 PL	Pleopod (20mg)	Pleopod (20mg)
YHV/GAV, TSV and IMNV	5 PL (above PL-5)	Pleopod (20mg)	Pleopod (20mg)
AHPND/EMS and NHPB	10-30 PL (PL-6 to PL-8)	Hepatopancreas (10-25%)	Fecal sample (1 cm)

Table 03: Details of the samples tested for the study.

Sample category	Number of samples tested for each disease							
	TSV	YHV/ GAV	IMNV	IHHNV	NHPB	AHPND	WSSV	Total samples
P. monodon broodstock	145	145	145	131	138	138	145	987
P. monodon postlarvae	54	54	54	54	54	54	0	324
P. monodon pond sample	66	66	66	66	66	66	9	405
Wild *P. merguiensis*	13	13	13	13	13	13	0	78
Wild *P. indicus*	14	14	14	14	14	14	0	84
Wild *P. monodon*	5	5	5	5	5	5	0	30
Wild *Scylla serrata* (mud crabs)	8	8	8	8	8	8	8	56
Wild *Portunus pelagicus* (blue swimming crab)	10	10	10	10	10	10	10	70
Wild collected Krill	4	4	4	4	4	4	2	26
Total number of samples	319	319	319	305	312	312	174	2060

PCR Detection for WSSV, IHHNV, NHPB, YHV/GAV, TSV and IMNV

In the PCR reaction process of WSSV, IHHNV and NHPB, extracts of DNA were subjected to amplification protocol using the first PCR reaction reagent mixture and nested PCR reaction reagent mixture. First PCR reaction reagent mixture contained 7.5 µL of First PCR PreMix and 0.5 µL of IQzyme DNA Polymerase (2 U/ µL). 2 µL of each of the DNA extracts was mixed with 8 µL of first PCR reaction mixture separately and subjected to the first PCR reaction under the program of thermal cycle of 94 ^{0}C 2 min; then 94 ^{o}C 20 sec; 62 ^{o}C 20 sec; 72 ^{o}C 30 sec, repeat 15 cycles, then add 72 ^{o}C 30 sec; 20 ^{o}C 30 sec at the end of the final cycle. After completion of the first PCR reaction 15 µL of the Nested PCR reaction mixture was added and subjected to the Nested PCR reaction under the program of thermal cycle of 94 ^{o}C 20 sec; 62 ^{o}C 20 sec; 72 ^{o}C 30 sec, repeat 30 cycles, then add 72 ^{o}C 30 sec; 20 ^{o}C 30 sec at the end of the final cycle. The same nested PCR reaction was followed in the PCR reaction process of YHV/GAV, TSV and IMNV diseases using the product of RT-PCR reaction.

PCR Detection for AHPND/EMS

The presence of AHPND/EMS was checked by PCR as described by the manufacturer (IQ 2000^{TM} test kit manuals). Briefly, the PCR reaction mixture contained 12.5 µL of PreMix regent and 0.5 µL of IQzyme DNA Polymerase (2 U/ µL). 2 µL of each DNA extract was mixed with 13 µL of PCR reaction mixture separately and subjected to the PCR reaction under the program of thermal cycle of 94 ^{o}C 20 sec; 62 ^{o}C 20 sec; 72 ^{o}C 30 sec, repeat 15 cycles, then add 72 ^{o}C 30 sec; 20 ^{o}C 30 sec at the end of the final cycle.

Subsequently, the PCR products were resolved in agarose gel (2%), and stained with ethidium bromide (5 ng mL^{-1}). The presence of WSSV, IHHNV, NHPB, YHV/GAV, TSV, IMNV and AHPND/EMS were visualized in a gel documentation system (GenoSens 1660 gel documentation and analysis system, Clinex, China).

Totally 2060 PCR tests were done to investigate the presence of pathogens in the shrimps and other crustacean samples (Table 03). Reporting of error results in some of the PCR tests was the reason for the variation in the number of samples tested for each disease.

RESULTS AND DISCUSSION

With the aim of evaluating the stock health of *P. monodon* for seven OIE listed diseases, broodstock shrimps, postlarvae, pond-reared juveniles, wild-caught shrimp species and other crustaceans considered as possible host species were PCR tested using IQ 2000™ test kits. As White Spot Disease (WSD) has been widely recorded in the country for a long period, a limited number of samples were tested for WSSV in the present study. Among the 145 tested broodstock samples that were taken into the hatcheries for breeding purposes, 15 samples were positive, representing 10.34% of infection for WSSV. Of the 9 farmed shrimp samples purposively collected from zone 2 where the highest farm density in NWP is reported (Munasinghe *et al.*, 2010), 5 samples were positive for WSSV, representing 55.55% of infection. (Table 04). Similar results were explained by Lakmal *et al* in 2012 indicating 20.56% and 23.26% infection rates of WSSV in brood shrimps taken into the hatcheries in NWP respectively in 2010 and 2011 indicating the seasonal variation of the rate of infection. Gayathri *et al* in 2016 reported the prevailing infection levels of WSSV in the farmed *P. monodon* as 58.05% in 2011 and 59.06% in 2012 from the farm samples collected from the NWP in the country. The present study revealed the recording of a high infection rate of WSSV even after more than 25 years of the first infection of WSD in Sri Lanka. Under a similar study, 35% and 63% of WSSV infection rates were recorded respectively from the 84 broodstock samples and 350 farmed shrimp samples collected from 18 different culture ponds on the East coast of India (Vaseeharan *et al.*, 2003) and WSSV is still the most serious viral threat to shrimp farmers in Asia for both *P. vannamei* and *P. monodon* (Flegel, 2012; Lo *et al.*, 2012; Patil *et al.*, 2021).

From all the tested wild samples of possible carrier species {8 mud crab samples (*Scylla serrata*), 10 Blue swimming crab samples (*Portunus pelagicus*) and 2 Krill samples} none were positive for the White Spot Disease. On the contrary, studies from elsewhere reported WSSV-infected wild species as carriers. One of the studies conducted by Vaseeharan *et al* in 2003 reported 22.72% infection of WSSV (n=22) in wild-collected *Scylla serrata* on the East coast of India. In 1998 Supamattaya *et al* indicated the high infection possibilities and mortality under the artificial contamination of WSSV in *Scylla serrata, Portunus pelagicus* and Krills having the ability to carry infections persisting for a significant period in shrimp farming environments. Hence, negative results of WSSV in all the tested carrier species in our study are not in agreement with the known facts, yet, due mainly to a limited number of samples tested (Table 04).

Table 04: Presence of White Spot Syndrome Virus in *P. monodon* broodstock samples, farmed *P. monodon* samples and some possible carrier species.

Sample category	Number of samples tested for WSSV	Number of positive samples
P. monodon broodstock	145	15
P. monodon pond sample	9	5
Wild *Scylla serrata* (mud crabs)	8	0
Wild *Portunus pelagicus* (blue swimming crab)	10	0
Wild collected Krill	2	0

In the present study, a considerably high prevalence of IHHNV was recorded from the *P. monodon* broodstock, PL and farmed shrimp samples showing amplification of approximately 333 bp and 630 bp (Figure 01). As indicated in the manual of the IQ 2000™ for the detection of IHHNV (Figure 02), out of the 131 broodstock samples tested, 41 samples were positive for IHHNV (Table 05) indicating 31.3% of total infection and out of 54 PL samples, 13 samples were positive (24.07% of total infection). All the tested broodstock samples represented the broodstock collecting areas of Handala and Beruwala and all the PL samples tested were from Mullathive, Handala and Beruwala areas of the country. Out of 66 farmed shrimp samples collected from five shrimp farming zones in NWP and EP, infection of IHHNV was only recorded from zone 2 in NWP with 11 samples of infection indicating a 44% infection rate of zone 2 and 18.03% infection rate for the NWP (Table 05).

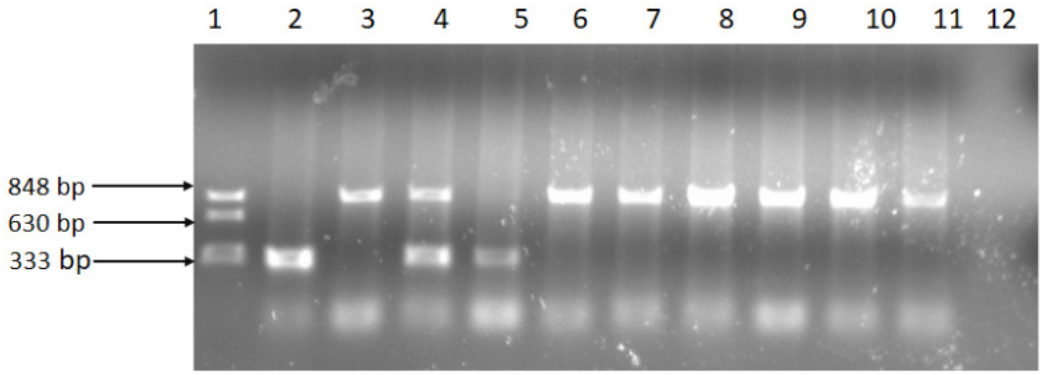


Figure 01: **PCR detection of Infectious Hypodermal and Hematopoietic Necrosis Virus DNA in *P. monodon* samples. Lane 01: Molecular weight markers 848bp, 630bp, 333bp, Lane 02 and 05: sample of light IHHNV infection, Lane 04: sample of very light IHHNV infection, Lane 03 and 06 to 11: IHHNV negative samples, Lane 12: negative control.**

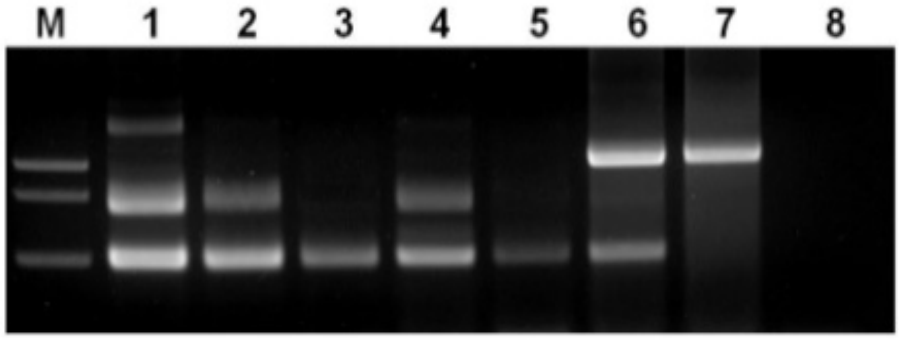


Figure 02: **Reference gel picture as given in the manual of IQ 2000™ for the detection of Infectious Hypodermal and Hematopoietic Necrosis Virus DNA in *P. monodon* samples. Lane M: Molecular weight markers, 848bp, 630bp, 333bp, lane 01: IHHNV P (+) standard, 2000 copies/reaction, lane 02: IHHNV P (+) standard, 200 copies/reaction, lane 03: IHHNV P (+) standard, 20 copies/reaction, lane 04: sample of moderate IHHNV infection lane 05: sample of light IHHNV infection, lane 06: sample of very light IHHNV infection, lane 07: IHHNV negative sample, lane 08: negative control (Yeast tRNA or ddH_2O)**

Table 05: Status of IHHNV in *P, monodon* and other wild crustaceans.

Sample category	Subzone /Area	Total number of samples	Number of samples positive for IHHNV	% Infection
P. monodon samples collected from the farming industry				
Broodstock	Hendala and Beruwala	131	41	31.3
PL samples	Broodstock collected from Mullaitivu Beruwala and Hendala	54	13	24.07
Farmed shrimp samples (NWP)	Zone 01	15	0	0
	Zone 02	25	11	44
	Zone 03	6	0	0
	Zone 04	6	0	0
	Zone 05	9	0	0
Total		66	11	18.03
Farmed shrimps (EP)	Batticaloa	05	0	0
Crustacean samples collected from wild				
P. merguiensis	NWP	13	0	0
P. indicus		14	0	0
P. monodon		5	0	0
Scylla serrata		8	0	0
Portunus pelagicus		10	0	0
Krills		4	0	0
Total		54	0	0

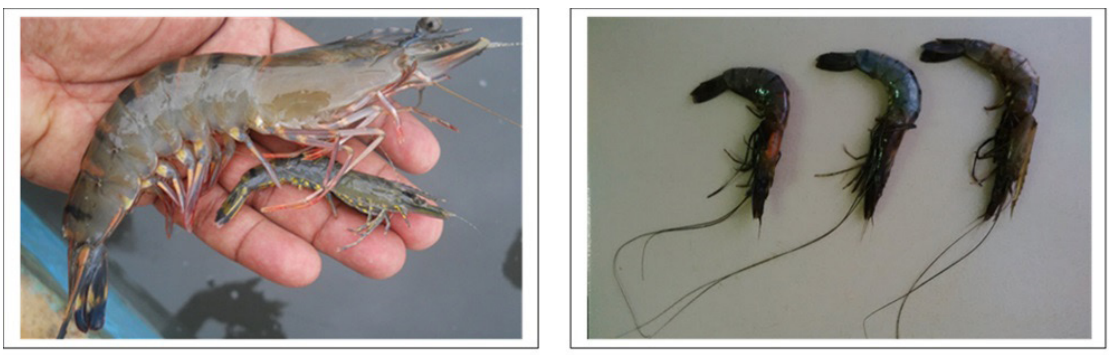
The first incidence of IHHNV in Sri Lanka was reported in 2011 (Wijegoonawardane and Chanthirika, 2011) indicating 44%, 25% and 31% rates of IHHNV infection in the postlarvae, sub-adults and wild brooder shrimps, respectively. However, their detection methodology was not properly explained, leaving uncertainties about the methodology followed. Our findings were based on the IQ 2000™ IHHNV detection kits validated and recommended by the OIE, and this further confirms the presence of IHHNV in Sri Lanka.

It was reported that (Motte *et al.,* 2003) all the life stages of the *P. monodon* are susceptible to infection of IHHNV, lowering the hatching success of the eggs and survival and culture performances of early larval stages. As our findings brood shrimps, PLs' and farmed shrimps were also reported their susceptibility to the IHHNV. Some common clinical signs viz: size variation/ growth retardation (Lightner *et al.,* 1992) (Figure 03a) and blue appearance of moribund shrimps (Figure 03b), low hatching success of the eggs and late-stage transferring in hatchery production cycles (personal information) were observed in the field providing symptomatically evidence of the presence of IHHNV and it was confirmed by the present molecular study. Therefore, even a probable threat of IHHNV may have been present even from the past that might indicate atypical clinical signs incoherent with common WSD

clinical signs, leading to a suspicion of possible new diseases or red coloration of the farmed shrimps (Figure 04) and sudden death of the brood shrimps during the field observations may be a result of the IHHNV infection individually or as a combined effect of IHHNV and WSSV. Although the IHHNV has been recorded as an economically less affected disease to the *P. monodon* farming industry (Chayaburakul *et al.*, 2004; Withyachumnarnkul *et al.*, 2006) *Litopenaeus vannamei* is identified as the most susceptible species to the IHHNV resulting high economic losses owing to the Runt Deformity Syndrome (Chai *et al.*, 2016)China, samples were collected during two cultivation seasons and subjected to diagnostic PCR. The results of this study showed that 167 out of 200 shrimp were positive for IHHNV, indicating a high viral prevalence (83.5 %. *L. vannamei* was introduced in 2018 into Sri Lanka as a new species and has presently been expanding as a dominant farming shrimp species having an apparent threat of IHHNV in the farming industry.

Although different host and carrier species of IHHNV disease including crabs, penaeid and non-penaeid shrimps were recorded elsewhere (Yu *et al*., 2021), out of 54 samples represented with shrimps, Blue swimming crabs and krills sampled in the present study were negative for the IHHNV (Table 05). Though the limited number of samples tested here was not sufficient to eliminate the risk of carrier species established in the wild, it is at least a relief to have comparatively low risk from wild carrier species to the farmed shrimps.

Out of seven diseases investigated in the present study total number of 1581 samples (representing 300 plus samples for each disease) were tested for other five diseases of TSV, YHV, IMNV, AHPND and NHPB and yet, none of the samples were positive (Table 03). FAO (2017) reported the presence of YHV in Sri Lanka in 1998 as a dual infection of YHV and WSSV indicating a high occurrence of YHV during the early phase and a low occurrence during the later phase of the disease outbreak justifying a low occurrence may be due to the rapid development of tolerance in pond cultured *P. monodon* to the YHV. There was a reported high prevalence of YHV from the 150 samples collected from the hatcheries in NWP as 78% and 22% infection rates under the wet mount observations and histopathology studied respectively. The same study revealed that 56.6% of the average prevalence of YHV was comparatively tested for the years 2010, 2011 and 2012 using IQ2000 commercial kits (Gayathri *et al*, 2017). On the contrary, molecular detection using the same IQ2000 test kits was conducted by the NAQDA for a total of 110 random samples including 50 broodstock samples, 18 PL samples and 42 farmed *P. monodon* samples in 2009, and all the samples were tested negative for YHV (Unpublished data). YHV is a critical disease to *P. monodon* causing severe mortalities up to 100% within 3– 5 days of the first appearance of clinical signs (Chantanachookin *et al.*, 1993; Flegel *et al*., 2012).



(a) (b)

Figure 03. a & b: Observed clinical signs of cultured *P. monodon*: (a) size variation (b) Shrimps appeared in blue color.

Figure 04: Shrimps appeared in red.

However, we have not observed any symptomatic evidence of YHV during our study period in 2017 and also during the disease screening program (including YHV) implemented by NAQDA in 2009 (Unpublished data). Further, no mass mortalities were recorded in shrimp farms showing YHV clinical signs during the recent past. Our present molecular detection study conducted using the IQ200 test kits for 319 samples including 145 broodstock samples, 54 postlarvae samples, 66 farm samples 5 wild collected *P. monodon* samples and 49 samples of possible carrier species also confirmed the absence of YHV in the country. Considering the absence of YHV in the present study and also in other recent studies, it is likely that YHV is no longer a commercially significant disease in the country, at least at present.

CONCLUSION

Molecular-based evidence of the present study reveals that, out of the seven shrimp diseases found in the Asian region, only WSD and IHHNV are reported in Sri Lanka. While reiterating the heavy presence of WSD in *Penaeus monodon* stocks in Sri Lanka long after its first appearance, this provides the first confirmatory evidence of IHHNV in the country. Formerly reported YHV in *P. monodon* seems to be no longer present and none of the carrier species tested are positive for any diseases tested. The presence of IHHNV in *P. monodon* may pose considerable economic losses in newly introduced *Litopenaeus vannamei* shrimps, highlighting the need for better-controlling mechanisms in place to avoid such losses.

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