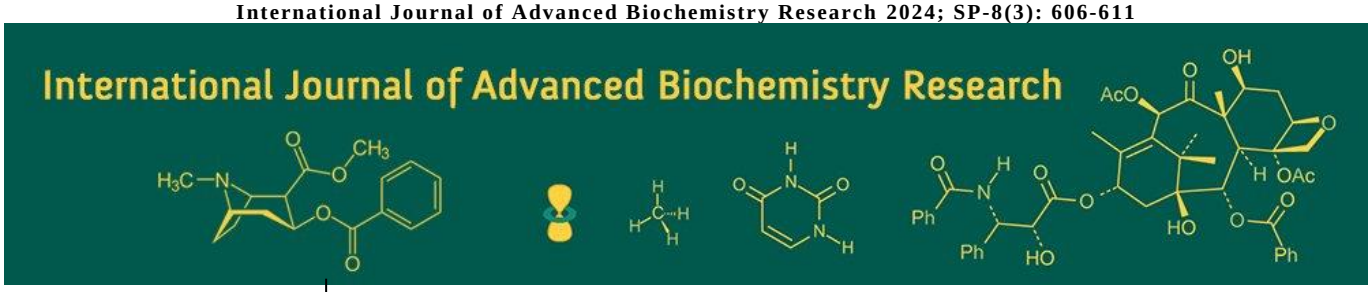

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Molecular detection of pathogens from cultured whiteleg shrimp farms in Karnataka, India

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Abstract
Litopenaeus vannamei, commonly known as the Whiteleg shrimp, emerges as one of the most economically lucrative species in India's commercial aquaculture sector. The primary objective of this study is to detect common pathogens affecting cultured shrimp in Karnataka using polymerase chain reaction (PCR) analysis. Over the period spanning from January 2020 to March 2022, a total of 278 samples were meticulously collected from shrimp farms in Karnataka. The screening process targeted both Office of Internationale des Epizootics (OIE) listed and non-OIE listed shrimp pathogens. The PCR analysis revealed the presence of white spot syndrome virus and *Enterocytozoon hepatopenaei* in the collected samples, with no detection of other pathogens. Out of the 278 samples, 63 (22.6%) were confirmed positive for EHP, while 14 (5%) showed infection by WSSV. Intriguingly, three samples were concurrently infected by both EHP and WSSV. Shrimp samples infected with WSSV exhibited distinctive white spots on the carapace, whereas EHP-infected shrimps displayed symptoms such as size variation and a white gut. Further histopathology analysis of gill tissue infected with WSSV revealed basophilic intra-nuclear inclusion bodies, while hepatopancreas tissue infected with EHP exhibited basophilic plasmodium. Notably, the study underscored a higher prevalence of EHP in shrimp farms across Karnataka. In light of these findings, the study strongly advocates for the implementation of stringent biosecurity measures and robust management practices in shrimp culture areas, emphasizing their critical role in sustaining the cultivation of *L. vannamei* in India.

Keywords: Whiteleg shrimp, OIE, EHP, WSSV, biosecurity

Introduction
Shrimp aquaculture represents a highly profitable and rapidly expanding segment within the aquaculture sector, boasting a global production of 4.45 million tons in 2018 (SOFIA, 2020). Shrimp production has a significant demand on the worldwide market, which helps developing countries grow economically. It also meets the increasing nutritional needs of the population and creates jobs. (Henchion *et al*., 2017) [6]. The whiteleg shrimp stands as one of the predominantly farmed shrimp species across various countries including China, India, Thailand, Japan, the Iran, Ecuador, Philippines, Taiwan and Vietnam and several other in Australia, South America and Southeast Asia. These nations have emerged as leaders in the field of shrimp aquaculture. (Ganjoor, 2015) [5]. The production of *L. vannamei* is pegged at 8, 15,745 metric tons in India with area under culture of 1.08 lakh hectares (MPEDA, 2021). Karnataka holds 7th position in India in shrimp culture with a production of 2185.84 metric tons under 970.39 hectares of culture area (MPEDA, 2021). In India during 2020-2021 590275 metric tons of frozen shrimp were exported valued at US$ 4426.19 million (MPEDA, 2021). Shrimp are being cultured extensively around the world due to increased public demand, and as a result, shrimp are being exposed to several infectious agent-like fungi, virus, bacteria, parasites, and these pathogens are responsible for high mortality and economic loss (Kennedy *et al*., 2016). According to Patil *et al*. (2021) [14] an estimated annual loss of 0.21 M ton shrimp of worth US$ 1.02 B was reported in India during 2018-2019 due to disease occurrence. The World Organization for Animal Health (Office International des Epizooties) has identified seven major diseases affecting shrimp aquaculture (OIE, 2021) *viz*., Infectious myonecrosis virus (IMNV), Acute hepatopancreatic necrosis disease (AHPND), Yellow Head Virus (YHV), Infectious hypodermal and haematopoietic necrosis virus (IHHNV), White spot syndrome virus (WSSV), Necrotising hepatopancreatitis

bacterium (NHPB), and Taura syndrome virus (TSV). These diseases cause significant economic losses globally, particularly in Thailand, China and India (Sahul Hameed *et al*., 2017) [18]. Additionally, emerging disease such as infection with Decapod iridescent virus (DIV-1) or Shrimp hemocyte iridescent virus (SHIV) are also threats to shrimp farming (Qiu *et al*., 2017) [15]. In Indian shrimp culture recent studies have reported the microsporidian disease caused by *Enterocytozoon hepatopenaei* (EHP) (CIBA, 2016; Rajendran *et al*., 2016) [3, 17], Hepatopancreatic Parvovirus (HPV), Monodon-type Baculovirus (MBV) (Tandel *et al*., 2017) [23], Infectious Myonecrosis Virus (IMNV) (Sahul Hameed *et al*., 2017) [18] and Vibriosis (Raja *et al*., 2017) [16]. Due to the rapid expansion of shrimp farming, several existing and emerging pathogens are causing a significant impact on shrimp aquaculture. Hence, to overcome this issue, disease surveillance plays a major role in regular monitoring, screening for disease outbreaks in shrimp aquaculture sector.

In this study, dead and moribund *L. vannamei* shrimp specimens were gathered from shrimp farms located in Karnataka, India. These samples were analyzed to identify the causative agent responsible for their condition.

Material and methods

Collection of samples

Post-larvae and juveniles of *L. vannamei* were obtained from shrimp farms situated in Udupai, Uttara Kannada and Dakshina Kannada districts of Karnataka, India (Fig. 1). The study spanned from January 2020 to March 2022, during which a total of 278 samples were collected. Shrimps exhibiting moribund conditions, mortality, or displaying clinical symptoms such as white spots on the carapace, size variation, lethargy, empty gut, and red discoloration were selected. These specimens were carefully transported to the laboratory on ice and subsequently fixed in 70% ethanol, while RNA samples were preserved using RNA fixative.

Nucleic acids isolation

L. vannamei collected from shrimp farm were dissected out aseptically. DNA and RNA were extracted from gill, hepatopancreas and pleopods. DNA was extracted as per Otta *et al*. (2003) [13]. Extracted DNA was used to detect the presence of WSSV, EHP, AHPND, NHP, MBV, HPV, IHHNV and DIV1. RNA was extracted by using 1 ml of RNA X-press (HiMedia, India). After a 5-minute incubation period with vigorous mixing, 100 µl of chloroform was introduced and allowed to incubate for 10 minutes at room temperature, followed by a 15-minute centrifugation at 12,000 g at 40 °C. The supernatant was carefully transferred to a new tube and mixed with 750 µl of 100% isopropanol before undergoing centrifugation at 12,000 g for 15 minutes at 40 °C to induce precipitation. The resulting RNA pellet was washed by adding 70% (v/v) ethanol, air-dried, and subsequently suspended in 50 µl of RNase-free water. The concentration of RNA was determined by measuring the absorbance at 260 nm. The extracted RNA was utilized for the detection of IMNV, YHV, and TSV.

PCR and RT-PCR analysis

The extracted DNA templates were utilized for detecting the presence of WSSV, EHP, AHPND, HPV, MBV, IHHNV, NHP, DIV1, while RNA templates were employed for detecting IMNV, YHV, and TSV. Detailed information regarding PCR primer sequences, annealing temperatures, and the sizes of the amplified products for each pathogen is provided in Table 1. PCR amplification was conducted in 30 µl reaction mixtures containing 1X PCR buffer, 10 pmol of each primer, 50 µmol of each dATP, dCTP, dGTP, and dTTP, 0.9 units of Taq DNA polymerase (HiMedia, Mumbai), 2 µl of nucleic acid, and adjusted to a final volume of 30 µl using Millipore water. The PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara, Japan) was employed for synthesizing cDNA. For each RNA sample, a total of 20 µl of reaction mixture was prepared, comprising 4 µl of 5X PrimeScript Buffer, 1 µl of Random 6 mers (50 µM), 1 µl of Oligo dT Primer (50 µM), 1 µl PrimeScript Reverse Transcriptase (200 U/µl), and 13 µl of normalized RNA. The reaction mixture was incubated at 37 °C for 15 minutes, followed by enzyme deactivation at 85 °C for 5 seconds. After cDNA synthesis, PCR was performed as described earlier using 2 µl of cDNA as a template. The amplified PCR products were subjected to electrophoresis in 1.5% agarose gels stained with ethidium bromide and visualized under ultraviolet transillumination.

Histopathology

Hepatopancreas and gill tissues were obtained from both infected and healthy shrimp specimens and preserved in Davidson's fixative (Humason, 1972) [7] for a duration of 48 hours. Subsequently, tissue sections were prepared following standard procedures and stained with haematoxylin and eosin. These stained sections were then examined under a light microscope to observe any histopathological alterations.

Result

Sample collection and clinical symptoms

A total of 278 samples of L. vannamei were gathered from shrimp farms across various districts of Karnataka, including Udupi (n=71), Dakshina Kannada (n=49) and Uttara Kannada (n=158). and Samples exhibiting clinical indications such as white spots on the carapace, size discrepancies, and pale hepatopancreas were procured and transferred to the laboratory (Fig. 2). Shrimp mortality ranged from 10% to 50%.

PCR and RT-PCR analysis

Out of 278 samples, all samples were diagnosed for WSSV, EHP, AHPND, HPV, MBV, IHHNV, NHP, DIV1 and 64 RNA samples were screened for IMNV, TSV and YHV. Among this 14 samples were positive for WSSV and 63 samples were positive for EHP. A prominent band of 486 bp confirms the presence of WSSV (Figure 3C) and a prominent band of 176 bp confirms the presence of EHP (Figure 3D). Both WSSV and EHP exhibited higher prevalence rates in the Uttara Kannada district, followed by Udupi and Dakshina Kannada, as indicated in Table 2. Among this one positive sample from Uttara Kannada and two sample from Dakshina Kannada were positive for both EHP and WSSV which indicates shrimp have Co- infection.

Histopathology

Histopathology analysis of WSSV infected shrimp shows severe basophilic intra-nuclear inclusion bodies in gill tissue (Figure 4A). EHP infected shrimp hepatopancreas shows presence of plasmodium which appeared as basophilic structures confined within a vacuole in the cytoplasm of hepatopancreatic epithelial cells (Figure 4C). Tissues from healthy shrimps are shown in Figure 4B&D.

Table 1: List of PCR primer and cycling conditions for the detection of shrimp pathogens

Pathogen	Primer Name	Sequence (5′-3′)	Annealing Temperature (°C)	Product size (bp)	Reference
WSSV	IK1	TGGCATGACAACGGCAGGAG	55 °C	486	Hossain *et al*., 2001a [28]
	IK2	GGCTTCTGAGATGAGGACGG			
	146F1	ACTACTAACTTCAGCCTATCTAG	55 °C	1447	Lo *et al*. 1996a, b [29]
	146R1	TAATGCGGGTGTAATGTTCTTACGA			
	146F2	GTAACTGCCCCTTCCATCTCCA	55 °C	941	
	146R2	TACGGCAGCTGCTGCACCTTGT			
HPV	H441F	GCATTACAAGAGCCAAGCAG	55 °C	441	Phromjai *et al*., 2002 [30]
	H441R	ACACTCAGCCTCTACCTTGT			
MBV	MBV 1.4F	CGATTCCATATCGGCCGAATA	65 °C	533	Belcher and Young (1998) [31]
	MBV 1.4R	TTGGCATGCACTCCCTGAGAT			
IHHNV	IHHNV309F	TCCAATCGCGTCTGCGATACT	55 °C	309	Tang *et al*., 2007 [32]
	IHHNV309R	TGTCTGCTACGATGATTATCCA			
TSV	9992F	AAGTAGACAGCCGCGCTT	60 °C	231	Nunan *et al*., 1998 [33]
	9195R	TCAATGAGAGCTTGGTCC			
IMNV	4587F	CGACGCTGCTAACCATACAA	60 °C	328	Poulos and Lightner, 2006 [34]
	4914R	ACTCGGCTGTTCGATCAAGT			
YHV	10F	CCGCTAATTTCAAAAACTACG	58 °C	135	Mohr *et al*., 2015 [35]
	144R	AAGGTGTTATGTCGAGGAAGT			
EHP	ENF779	CAGCAGGCGCGAAAATTGTCCA	58 °C	779	Tangprasittipap *et al*., 2013 [36]
	ENR779	AAGAGATATTGTATTGCGCTTGCTG			
	ENF176F	CAACGCGGGAAAACTTACCA	64 °C	176	
	ENF176R	ACCTGTTATTGCCTTCTCCCTCC			
AHPND	AP4-F1	ATGAGTAACAATATAAAACATGAAAC	55 °C	1269	Sritunyalucksana *et al*., 2015 [37]
	AP4-R1	ACGATTTCGACGTTCCCCAA			
	AP4-F2	TTGAGAATACGGGACGTGGG	55 °C	230	
	AP4-R2	GTTAGTCATGTGAGCACCTTC			
NHP	NHPF2	CGT-TGG-AGG-TTC-GTC-CTT-CAGT	60 °C	379	Aranguren *et al*., 2010 [38]
	NHPR2	GCC-ATG-AGG-ACC-TGA-CAT-CAT-C			
DIV1	SHIV-F1	GGG CGG GAG ATG GTG TTA GAT	59 °C	457	Qiu *et al*., 2017 [15]
	SHIV-R1	TCG TTT CGG TAC GAA GAT GTA			
	SHIV-F2	CGG GAA ACG ATT CGT ATT GGG	59 °C	129	
	SHIV-R2	TTG CTT GAT CGG CAT CCT TGA			

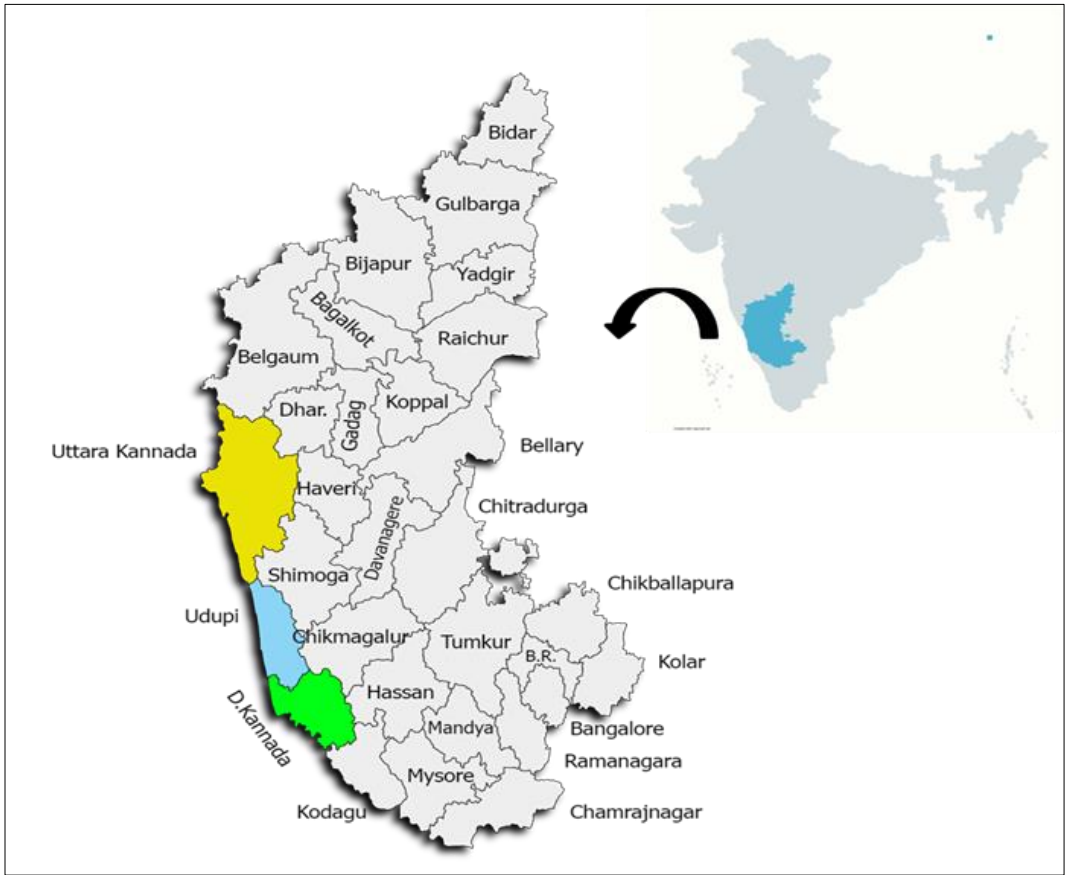

Fig 1: A map illustrating the locations where samples were collected in Karnataka, India

Table 2: Disease surveillance carried out in shrimp farms located in different districts of Karnataka

District	Sample collected	DNA samples screened	RNA samples screened	Pathogen detected	Incidence of Co-infection
Uttara Kannada	158	126	32	WSSV (6) and EHP (31)	WSSV with EHP (1)
Udupi	71	51	20	WSSV (4) and EHP (20)	Not reported
Dakshina Kannada	49	37	12	WSSV (4) and EHP (12)	WSSV with EHP (2)

Fig 2: Clinical symptoms noted in the collected shrimp samples include: A) Presence of white spots on the carapace, B) Variation in size, C) Paleness of the hepatopancreas, and D) Significant mortality rates

Discussion

Shrimp culture have significant contribution in economy in India and the migration of species from one area to another heightens the risk of the emergence of new pathogens in aquatic animals (Tendencia *et al.*, 2018) [25]. Diseases are recognized as substantial concerns and challenges for the aquaculture sector on a global scale, including in India (Stentiford *et al.*, 2017) [22]. WSSV is major shrimp pathogen that has been causing widespread damage to the shrimp culture industry since the early 1990s (Lightner, 2005 and Flegel, 2006) [9, 4]. *Enterocytozoon hepatopenaei* an emerging microsporidian parasite have a severe impact on shrimp industry worldwide and now it became concern in India. In early stages there are no clinical signs of disease but after 35 to 40 days it can be recognized by observing growth variation in the shrimp (Thitamadee *et al.*, 2016) [27]. Studies have reported incidence of WSSV has been reported from different parts of India (Balakrishnan *et al*., 2011; Thamizhvanan *et al*., 2019; Otta *et al*., 2014) [2, 26, 12]. Similarly, EHP has been reported from all over India (Rajendran *et al*., 2016; Santhoshkumar *et al*., 2017; Thamizhvanan *et al*., 2019) [17, 18, 26]. Co-infection of WSSV and EHP have been reported from different parts of India (Babu *et al*., 2021; Thamizhvanan *et al*., 2019) [1, 26]. To tackle these issues, we conducted a 26-month disease surveillance in shrimp farms situated in the different district of Karnataka, India. We reported 5% prevalence of WSSV, 23.02% prevalence of EHP and 0.7% prevalence of co-infection of WSSV and EHP from shrimp farms of Karnataka. The PCR findings were corroborated through histopathological examination. Histopathology analysis of WSSV infected shrimp shows severe basophilic intra-nuclear inclusion bodies in gill tissue (Figure 4A) which are typical characteristics of WSSV infection. This is similar to the previous reports (Saravanan *et al*., 2021; Babu *et al*., 2021) [20, 1]. EHP infected shrimp hepatopancreas shows presence of plasmodium which appeared as basophilic structures confined within a vacuole in the cytoplasm of hepatopancreatic epithelial cells (Figure 4C). which are typical characteristics of EHP infection. This is similar to the previous reports (Tang *et al*., 2015; Rajendran *et al*., 2016; Santhoshkumar *et al*., 2017, Babu *et al*., 2021) [24, 17, 18, 1].

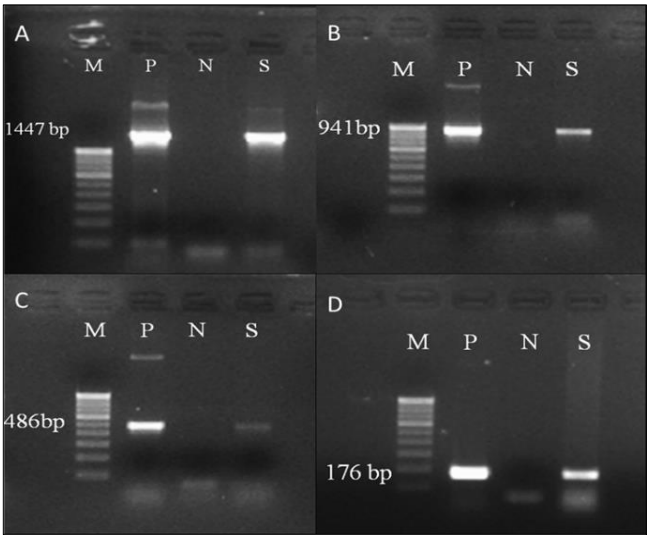

Fig 3: The agarose gel electrophoresis image validates pathogen infection in shrimp tissue samples as follows A – The PCR detection of WSSV carried out using specific primer sets 146F1 and 146R1.; B – The PCR detection of WSSV carried out using specific primer sets 146F2 and 146R2; C – The PCR detection of WSSV carried out using specific primer sets IK1 and IK2; D – The PCR detection of EHP carried out using specific primer sets ENF176F and ENF176R. M – 100 bp ladder; P – Positive control; N – Negative control; S – Sample

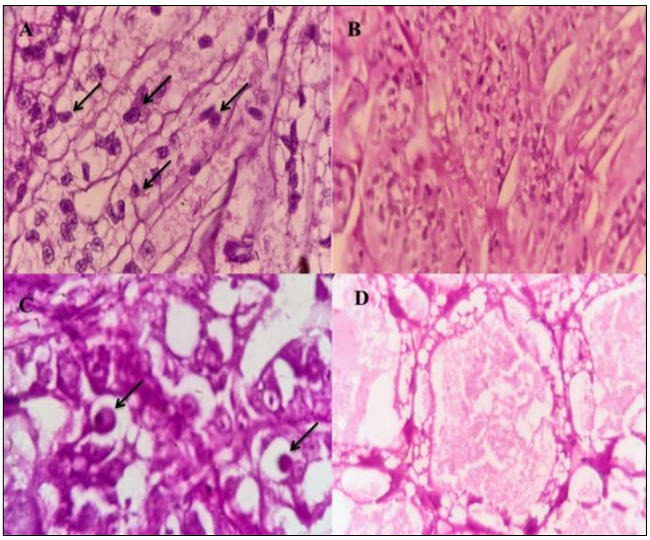

Fig 4: Histopathological examination of shrimp tissues (magnification 40X): A) Gill tissue from WSSV-infected shrimp displays basophilic intra-nuclear inclusion bodies. B) Gill tissue from a healthy shrimp appears normal. C) Hepatopancreas tissue from EHP-infected shrimp exhibits basophilic plasmodium. D) Hepatopancreas tissue from a healthy shrimp appears normal

Conclusion

In India, the utilization of Specific Pathogen-Free (SPF) brooders is widespread among hatcheries for post-larvae production; however, despite this practice, cultured shrimps are frequently afflicted by various pathogens. This study emphasizes the frequent presence of EHP and WSSV infections in *L. vannamei*, either occurring individually or as co-infections. Particularly noteworthy is the higher incidence of EHP compared to WSSV across Karnataka. Consequently, we strongly advice for the implementation of good management practices by farmers to mitigate infection occurrences.

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References

1. Babu B, Sathiyaraj G, Mandal A, Kandan S, Biju N, Palanisamy S, *et al*. Surveillance of disease incidence in shrimp farms located in the east coastal region of India and *in vitro* antibacterial efficacy of probiotics against Vibrio parahaemolyticus. J Invertebr. Pathol. 2021;179:107536.
2. Balakrishnan G, Peyail S, Kumaran R, Theivasigamani A, Anil KS, Jitesh SB, *et al*. First report on White Spot Syndrome Virus (WSSV) infection in white leg shrimp *Litopenaeus vannamei* (Crustacea, Penaeidae) under semi intensive culture condition in India. Aquaculture, Aquarium, Conservation & Legislation. 2011;4(3):301-305.
3. CIBA. Managing *Enterocytozoon hepatopenaei* (EHP), microsporidial infections in vannamei shrimp farming: An Advisory. E-publication No.29; c2016 Jan.
4. Flegel TW. Detection of major penaeid shrimp viruses in Asia, a historical perspective with emphasis on Thailand. Aquaculture. 2006;258(1-4):1-33.
5. Ganjoor M. A short review on infectious viruses in cultural shrimps (Penaeidae family). J Fish Sci. 2015;9(3):1.
6. Henchion M, Hayes M, Mullen AM, Fenelon M, Tiwari B. Future protein supply and demand: strategies and factors influencing a sustainable equilibrium. Foods. 2017;6(7):53.
7. Humason GL. Animal tissue techniques. Animal tissue techniques; c1962.
8. Kennedy DA, Kurath G, Brito IL, Purcell MK, Read AF, Winton JR, *et al*. Potential drivers of virulence evolution in aquaculture. Evol. Appl. 2016;9(2):344-354.
9. Lightner DV. Biosecurity in shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance. J World Aquac Soc. 2005;36(3):229-248.
10. MPEDA, (2020-21). Available from: https://mpeda.gov.in/?page_id=651
11. OIE. OIE-listed diseases 2021. Paris, France: Office International des Epizooties, World Organisation for Animal Health.
12. Otta SK, Arulraj R, Ezhil Praveena P, Manivel R, Panigrahi A, Bhuvaneswari T, *et al*. Association of dual viral infection with mortality of Pacific white shrimp (*Litopenaeus vannamei*) in culture ponds in India. VirusDisease. 2014;25:63-8.
13. Otta SK, Karunasagar I, Karunasagar I. Detection of monodon baculovirus and white spot syndrome virus in apparently healthy *Penaeus monodon* postlarvae from India by polymerase chain reaction. Aquaculture. 2003;220(1-4):59-67.
14. Patil PK, Geetha R, Ravisankar T, Avunje S, Solanki HG, Abraham TJ, *et al*. Economic loss due to diseases in Indian shrimp farming with special reference to *Enterocytozoon hepatopenaei* (EHP) and white spot syndrome virus (WSSV). Aquaculture. 2021;533:736231.
15. Qiu L, Chen MM, Wan XY, Li C, Zhang QL, Wang RY, *et al*. Characterization of a new member of Iridoviridae, Shrimp hemocyte iridescent virus (SHIV), found in white leg shrimp (*Litopenaeus vannamei*). Sci Rep. 2017;7(1):11834.
16. Raja RA, Sridhar R, Balachandran C, Palanisammi A, Ramesh S, Nagarajan K, *et al*. Pathogenicity profile of Vibrio parahaemolyticus in farmed Pacific white shrimp, *Penaeus vannamei*. Fish Shellfish Immunol. 2017;67:368-381.
17. Rajendran KV, Shivam S, Praveena PE, Rajan JJ, Kumar TS, Avunje S, *et al*. Emergence of *Enterocytozoon hepatopenaei* (EHP) in farmed Penaeus (Litopenaeus) vannamei in India. Aquaculture. 2016;454:272-80.
18. Sahul Hameed AS, Abdul Majeed S, Vimal S, Madan N, Rajkumar T, Santhoshkumar S, *et al*. Studies on the occurrence of infectious myonecrosis virus in pond-reared *Litopenaeus vannamei* (Boone, 1931) in India. J Fish Dis. 2017;40(12):1823-1830.
19. Santhoshkumar S, Sivakumar S, Vimal S, Abdul Majeed S, Taju G, Haribabu P, *et al*. Biochemical changes and tissue distribution of *Enterocytozoon hepatopenaei* (EHP) in naturally and experimentally EHP-infected whiteleg shrimp, *Litopenaeus vannamei* (Boone, 1931), in India. J Fish Dis. 2017;40(4):529-539.
20. Saravanan K, Praveenraj J, Kiruba-Sankar R, Devi V, Biswas U, Kumar TS, *et al*. Co-Infection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) and white spot syndrome virus (WSSV) in the wild crustaceans of Andaman and Nicobar Archipelago, India. Viruses. 2021;13(7):1378.
21. Sofia; c2020. Available from: http://www.fao.org/state-of-fisheries-aquaculture
22. Stentiford GD, Sritunyalucksana K, Flegel TW, Williams BA, Withyachumnarnkul B, Itsathitphaisarn O, *et al*. New paradigms to help solve the global aquaculture disease crisis. PLoS Pathog. 2017;13(2):1006160.
23. Tandel GM, John KR, George MR, Jeyaseelan MP. Current status of viral diseases in Indian shrimp aquaculture. Acta Virol. 2017;61(2):131-137.
24. Tang KF, Pantoja CR, Redman RM, Han JE, Tran LH, Lightner DV, *et al*. Development of in situ hybridization and PCR assays for the detection of *Enterocytozoon hepatopenaei* (EHP), a microsporidian

parasite infecting penaeid shrimp. J Invertebr Pathol. 2015;130:37-41.
25. Tendencia EA, Leobert D, de la Pena JM, de la Cruz (Eds). AEPRS: Aquatic emergency preparedness and response systems for effective management of transboundary disease outbreaks in Southeast Asia: proceedings of ASEAN Regional Technical Consultation, Centara Grand Central, Ladprao, Bangkok, Thailand; c2018.
26. Thamizhvanan S, Sivakumar S, Santhosh Kumar S, Vinoth Kumar D, Suryakodi S, Balaji K, *et al*. Multiple infections caused by white spot syndrome virus and *Enterocytozoon hepatopenaei* in pond-reared Penaeus vannamei in India and multiplex PCR for their simultaneous detection. J Fish Dis. 2019;42(3):447-454.
27. Thitamadee S, Prachumwat A, Srisala J, Jaroenlak P, Salachan PV, Sritunyalucksana K, *et al*. Review of current disease threats for cultivated penaeid shrimp in Asia. Aquaculture. 2016;452:69-87.
28. Werstuck GH, Lentz SR, Dayal S, Hossain GS, Sood SK, Shi YY, *et al*. Homocysteine-induced endoplasmic reticulum stress causes dysregulation of the cholesterol and triglyceride biosynthetic pathways. The Journal of clinical investigation. 2001 May 15;107(10):1263-1273.
29. Lo CF, Ho CH, Peng SE, Chen CH, Hsu HC, Chiu YL, *et al*. Infection of white spot syndrome associated virus (WSBV) in cultured and wild-caught shrimps, crabs and other arthropods. Dis Aquat Org. 1996a;2:215-225.
30. Phromjai J, Boonsaeng V, Withyachumnarnkul B, Flegel TW. Detection of hepatopancreatic parvovirus in Thai shrimp Penaeus monodon by in situ hybridization, dot blot hybridization and PCR amplification. Diseases of aquatic organisms. 2002 Oct 4;51(3):227-232.
31. Belcher CR, Young PR. Colourimetric PCR-based detection of monodon baculovirus in whole Penaeus monodon postlarvae. Journal of virological methods. 1998 Sep 1;74(1):21-29.
32. Tang YY, Ma Y, Wang J, Fan Y, Feng S, Lu Q, *et al*. Short-term meditation training improves attention and self-regulation. Proceedings of the national Academy of Sciences. 2007 Oct 23;104(43):17152-17156.
33. Nunan D. Teaching grammar in context; c1998. p.101-109.
34. Poulos BT, Tang KF, Pantoja CR, Bonami JR, Lightner DV. Purification and characterization of infectious myonecrosis virus of penaeid shrimp. Journal of General Virology. 2006 Apr;87(4):987-996.
35. Mohr SH, Wang J, Ellem G, Ward J, Giurco D. Projection of world fossil fuels by country. Fuel. 2015 Feb 1;141:120-135.
36. Tangprasittipap A, Srisala J, Chouwdee S, Somboon M, Chuchird N, Limsuwan C, *et al*. The microsporidian *Enterocytozoon hepatopenaei* is not the cause of white feces syndrome in whiteleg shrimp Penaeus (Litopenaeus) vannamei. BMC veterinary research. 2013 Dec;9:1-0.
37. Sirikharin R, Taengchaiyaphum S, Sanguanrut P, Chi TD, Mavichak R, Sritunyalucksana K. Characterization and PCR detection of binary, Pir-like toxins from Vibrio parahaemolyticus isolates that cause acute hepatopancreatic necrosis disease (AHPND) in shrimp. PloS one. 2015 May 27;10(5):e0126987.
38. Eichhorn SJ, Dufresne A, Aranguren M, Marcovich NE, Capadona JR, Rowan SJ, *et al*. Current international research into cellulose nanofibres and nanocomposites. Journal of materials science. 2010 Jan;45:1-33.