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Original Research Article

INVESTIGATING THE PREVALENCE OF *PENAEUS MONODON* DENSOVIRUS INFECTION IN SELECTED SHRIMP FARMS IN CHILAW, SRI LANKA

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SUMMARY*: Penaeus monodon* Densovirus (*Pm*DNV) infects the hepatopancreas of penaeid shrimp and causes an asymptomatic disease. Heavy infections cause stunted growth, leading to a decline in farm production. While *Pm*DNV is widely distributed worldwide, there is no record of its prevalence in Sri Lanka. This study aimed to detect *Pm*DNV in *Penaeus monodon* in Chilaw region of Sri Lanka. Forty (n=40) shrimp samples, aged 94-105 days, were collected from four selected farms (A, B, C and D) in Chilaw. The hepatopancreas of shrimp was isolated, and separate sections were preserved in Davidson's fixative solution for histopathology and in 70% ethanol for DNA extraction. Fixed tissue samples were stained with Hematoxylin and Eosin (H&E), and infected hepatopancreas revealed hepatopancreatic cell necrosis and intranuclear inclusion bodies. DNA extraction was done according to the protocol of the Wizard Genomic DNA purification kit, and PCR was carried out using forward ($^{5'}$AATCTGCAGGGTACGGAAAAAAC$^{3'}$) and reverse ($^{5'}$TGTGGAACCATCTCAAATGCC $^{3'}$) primers. The study found that 60% (24/40) of the shrimp were infected with *Pm*DNV, and 27.5% of the samples were negative for the virus. From the studied samples, 12.5% of the samples did not show any bands in PCR. The severity of infection was assessed using band intensity, which revealed that 20% of samples were severely infected and that the majority (37.5%) were moderately infected. No significant difference was observed in the mean body weight and total length of infected and non-infected shrimps. Shrimp growth was not affected due to the moderate level of *Pm*DNV infection.

Keywords: *Penaeus monodon*, *Penaeus monodon* Densovirus, Histopathology, Polymerase Chain Reaction

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INTRODUCTION

Penaeus monodon Densovirus (*Pm*DNV), belonging to the family Parvoviridae family, subfamily Densovirinae (Catap *et al*., 2003), causes severe infections in penaeid shrimps, especially in *Penaeus monodon* species. This is also known as Hepatopancreatic parvo virus (HPV), and it has spread all over the world, including Thailand, China, Kuwait, Singapore and the Philippines (Catap *et al.,* 2003). This is a single-stranded DNA virus which infects both cultured and wild penaeid shrimps (Sukhumsirichart *et al*., 2006). Shrimp infected with *Pm*DNV often exhibit nonspecific gross symptoms of disease; however, there is unconfirmed evidence which shows that severe infections may result in stunted growth. (Sukhumsirichart *et al.*, 1999). Heavy viral infections could potentially cause significant economic losses to shrimp farmers due to mortality in the early phases of rearing. Infected shrimp species show nonspecific gross signs such as anorexia, atrophy of hepatopancreas, decreased preening activity, increased surface and gill fouling and sporadic opacity of tail musculature and stunting (Catap *et al*., 2003).

The incidence of the spread of viral diseases has increased due to the intensification of shrimp farming with the increased global demand for seafood. It is reported that *Pm*DNV leads to the retardation of growth of farmed shrimps, causing a dramatic decrease in farm profit (Pantoja and Lightner, 2000). In addition, diseased shrimp may be affected by secondary pathogens, which mask the actual effect of *Pm*DNV (Pantoja and Lightner, 2000), leading to difficulty in identifying *PmDNV*. Thus, appropriate diagnostic methods should be followed in the presence of suggestive clinical signs. *Pm*DNV detection was traditionally dependent upon histological evidence of large basophilic nuclear inclusions in H&E staining, but rapid, non-destructive molecular techniques have recently been developed (Phromjai *et al*., 2002). Polymerase Chain Reaction (PCR) is one of the molecular techniques that has been proven to be a powerful and sensitive diagnostic tool for shrimp viral infections and the detection of viral reservoirs in carrier species (Sukhumsirichart *et al*., 2006). Numerous studies have been carried out across the world to identify *Pm*DNV. This aspect still requires thorough investigation, as no experimental studies or published reports are currently available on the diagnosis of *Pm*DNV infection in penaeid shrimp species in Sri Lanka. Therefore, the main objective of this study was the detection of the prevalence of *Pm*DNV infection in *P. monodon* in Chilaw, a major shrimp farming area in Sri Lanka. Further, it was expected to correlate the growth of shrimps with *Pm*DNV infection.

METHODOLOGY

Sample Collection

Random samples were collected from four selected farms (A, B, C, and D) in Chilaw, North Western Province of Sri Lanka. Altogether, 40 shrimp were randomly collected, 10 from each grow-out pond. The total length and average weight of each sample were recorded at the sampling site.

Preparation of Hepatopancreas

Each shrimp was dissected, and the hepatopancreas was isolated on the same day of the sample collection. Part of the

hepatopancreas was stored in 70% ethanol, and the other part was stored in Davidson's fixative solution.

DNA Extraction and Quantification

These procedures were carried out in the Animal Science laboratory, Department of Animal Science, Faculty of Animal Science and Export Agriculture, Uva Wellassa University, Badulla, Sri Lanka.

DNA Extraction

DNA extraction from tissue samples was conducted using the Wizard Genomic (Promega A1120, USA) DNA purification kit by following the provided protocol at the Laboratory. Fresh tissue samples weighing 10-20 mg were utilised for each extraction, and their weights were measured using an analytical balance. Before extraction, the samples were washed with phosphate-buffered saline to eliminate excess ethanol. Subsequently, 600 µl of Nuclei Lysis Solution was added to a pre-chilled 1.5 ml centrifuge tube, and the fresh tissue sample was introduced to the solution. Homogenisation was performed for 10 seconds using a small homogeniser until all tissue particles dissolved. The lysate was then incubated at 65°C for 15–30 minutes, followed by cooling to room temperature for 5 minutes. To the room temperature sample, 200 µl of Protein Precipitation Solution was added and vortexed vigorously for 20 seconds. After chilling on ice for 5 minutes, centrifugation was carried out for 4 minutes at 13,000–16,000 × g, resulting in a visible white protein pellet at the bottom of the tube. The supernatant, containing the DNA, was carefully transferred to a clean 1.5 ml microcentrifuge tube with 600 µl of isopropanol at room temperature. The solution was gently mixed until the DNA formed visible thread-like strands, followed by centrifugation for 1 minute at 13,000–16,000 × g at room temperature. The DNA, visible as a small white pellet, was carefully decanted, and 600 µl of 70% ethanol kept at room temperature was added for DNA washing. After another centrifugation at 13,000–16,000 × g for 1 minute, the ethanol was aspirated, and the DNA pellet was air-dried on absorbent paper for 10–15 minutes. Subsequently, 50 µL of DNA Rehydration Solution was added to the tube to rehydrate the DNA. The sample was incubated at 65 °C for 1 hour, with gentle mixing by tapping the tube intermittently. Alternatively, DNA rehydration was acheived overnight at room temperature or at 4 °C**.** The extracted DNA was then stored at 2–8 °C until further use**.**

DNA Quantification

Extracted DNA samples were quantified in ng/µL, and the purity of the extracted DNA was analysed using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, USA).

Polymerase Chain Reaction and agarose gel electrophoresis

The DNA was amplified by specific primers for *Pm*DNV (F-$^{5'}$AATCTGCAGGGTACGGAAAAAAC$^{3'}$, R- $^{5'}$TGTGGAACCATCTCAAATGCC$^{3'}$) and Actin (F- $^{5'}$GAC TCG TAC GTC GGG CGACGA GG $^{3'}$, R - $^{5'}$AGC AGC GGTGGT CAT CAC CTG CTC $^{3'}$) which

act as an internal control. (Fernando *et al*, 2018).

The PCR amplification was performed in a 25 µL of total reaction mixture containing 1x PCR buffer, 10 mM dNTP mixture (Promega, US), 50 mM $MgCl_2$, 10 µM each primer, 200 ng template DNA, 1 U of Taq DNA polymerase (Thermo Scientific, US) and RNAse-free water (Fernando *et al.*, 2018). The PCR programme consisted of initial denaturation at 94 °C for 2 min. followed by 30 cycles of denaturation at 94 °C for 10 s, annealing of primers at 55 °C for 30 s and primer extension at 72 °C for 6 minutes (Fernando *et al*., 2018). Amplified DNA was run in 2% agarose gel at 120V for 30 minutes. Ethidium bromide-stained gel was observed under UV for the presence of bands (Figure 1). The band intensity was expressed relative to the staining of the band for the actin gene as an internal control, and densitometric analyses were performed using the Image J Software Package.

Histopathology

Histopathology procedures were carried out at the Histopathology Laboratory, Department of Pathobiology, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka. After fixation in Davidson fixative solution (1:10 w/v), hepatopancreas samples were sectioned into 2 mm slices, placed in cassettes, and processed through dehydration, clearing, and wax infiltration. Wax-embedded samples were sectioned at 4-5µm thick sections using a rotary microtome (Cat No. MR2258). Sectioned slides were stained with H&E, followed by mounting with DPX mounting solution. Air-dried, mounted tissue sections were then examined using a light microscope under high power and oil immersion. Microscopic images of the tissue were captured for further analysis.

Data Analysis

Total length and weight of the infected and non-infected shrimps were analysed to find the correlation between the growth of the shrimps and the infection. All the collected data were analysed using Microsoft® Excel and Minitab statistical software version 17 (LLC, Pennsylvania, USA).

RESULTS

Among the 40 samples tested, 24 (60%) were confirmed to be positive for *Penaeus monodon* Densovirus (*Pm*DNV), while 11 samples (27.5%) were tested negative for the infection. Notably, no bands were observed in 5 samples (12.5%). The severity of the *Pm*DNV infection was assessed by measuring the band intensity for both the *Pm*DNV virus and actin protein using Image J software. The ratio of the band intensity of the two bands was used to grade the severity level. A ratio greater than 1 was categorised as a severe infection, while a ratio ranging from 0.1 to 0.9 was considered a moderate infection. Ratios less than 0.1 were indicative of a negative infection status (Fernando *et al.*, 2018). According to the findings, only 20% of the total samples exhibited severe infection, while 37.5% showed a moderate infection with *Pm*DNV.

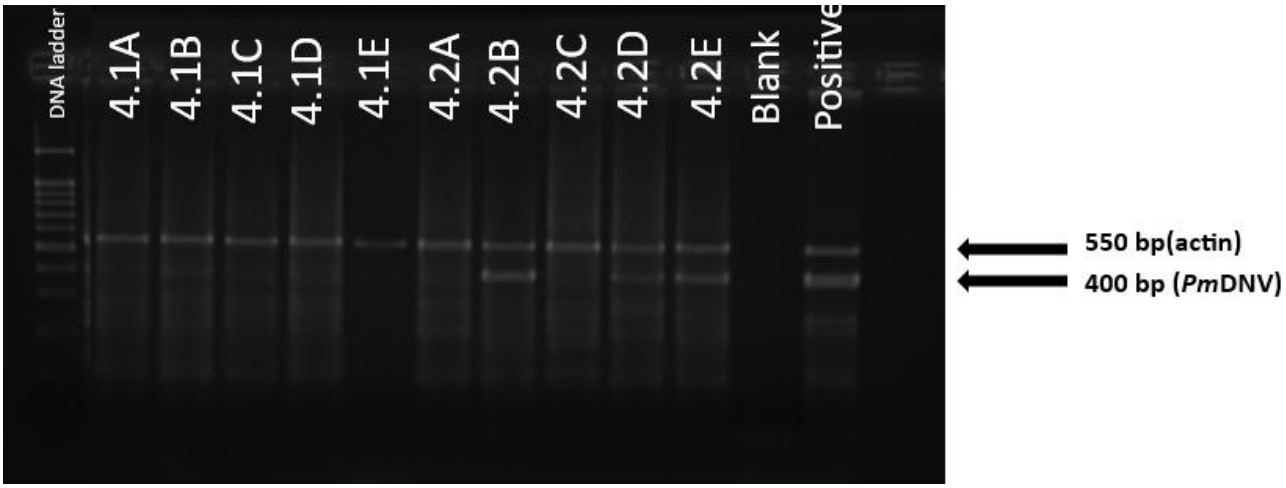

Figure 1. Results obtained from the DNA sample of *Penaeus monodon* (farm D) used for electrophoresis gel for the detection of *Penaeus monodo*n Densovirus (*Pm*DNV)-specific PCR amplicons using the primer set of Fernando *et al*., (2018). Actin was used as the internal control. It is present in both infected and non-infected samples. The second band (400bp) is specific for shrimp *Pm*DNV. Lane 4.2B, 4.2D, and 4.2E show the presence of both bands at varying intensities, indicating *Pm*DNV. The level of band intensity could be a reflection of the state of viral load. Lane 4.1A, 4.1B, 4.1C, 4.1D, 4.1E, 4.2A, 4.2C show the band only for Actin, indicating the absence of *Pm*DNV.

Samples for PCR testing were collected from four shrimp farms in Chilaw, North Western Province of Sri Lanka. The sampled populations differed between farms, with average ages ranging from 95 to 130 days. In addition, different commercial feed brands, including EGOFEED, Lion Shrimp Feed, and Mega Feed, were used, with varying feeding regimes across farms. The average body weight of shrimp also varied between farms (20–30 g), reflecting differences in culture period and feeding practices.

Table 1. Prevalence of *Penaeus monodon* Densovirus (*Pm*DNV) in four selected farms in Chlaw, Sri Lanka

Farm	Positive	Negative	No bands
Farm A	80%	20%	0
Farm B	70%	30%	0
Farm C	40%	10%	50%
Farm D	50%	50%	0

Given these variations, it was necessary to assess the prevalence and severity of *Pm*DNV infection separately for each farm. Ten samples were collected from each of the four farms (A, B, C, and D), and the prevalence (Table 1) and severity (Table 2) of infection are presented as percentages based on these ten samples per farm.

Table 2. Severity of *Penaeus monodon* Densovirus (*Pm*DNV) in four selected farms in Chlaw, Sri Lanka

Farm	Severely	Moderately	No infection	No Bands
Farm A	30%	50%	20%	0
Farm B	30%	40%	30%	0
Farm C	10%	30%	10%	50%
Farm D	10%	30%	60%	0

The most popular pathological method to detect the presence of *Pm*DNV is staining the histological sections of hepatopancreas using H&E and identification of intranuclear inclusion body that develops in E- and F-cells, which is a recognisable cytopathic result of *Pm*DNV infection (Pantoja and Lightner, 2000). The E cells were described as embryonic cells, with cuboid to prismatic form and a big, basophilic nucleus that takes up most of the cytoplasmic volume. These cells have microvilli in the apical region and extend into the tubule lumen. We noticed that this cell is primarily present in the distal part of the hepatopancreatic tubule and is lacking in the proximal and middle parts. Fibril cells, or F cells, possessed a large, somewhat elongated nucleus. The cytoplasm also had prominent basophilia, and the cells were prismatic or triangular in shape. This cell type also possesses microvilli and can be found in the tubule lumen. Similar features have been identified in epithelial tissues of the hepatopancreas of shrimp samples collected for the present study. All the PCR-positive samples displayed typical single *Pm*DNV inclusions on the epithelial tissue sample, whereas the PCR-negative samples did not. H&E staining showed basophilic intranuclear inclusions in epithelial cells of the tubules in hepatopancreatic tissues in shrimp samples (Figure 3).

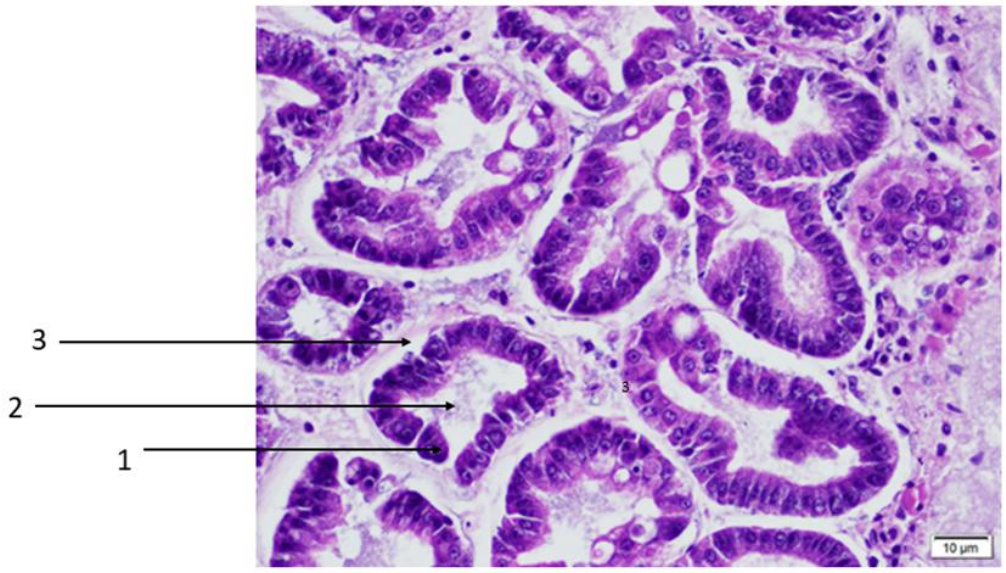

Figure 2. Cross-section of the *P. monodon* hepatopancreas- H& E
1-Distal blind end, 2-HP tubule lumen, 3- Basement membrane, scale bar- 10µm.

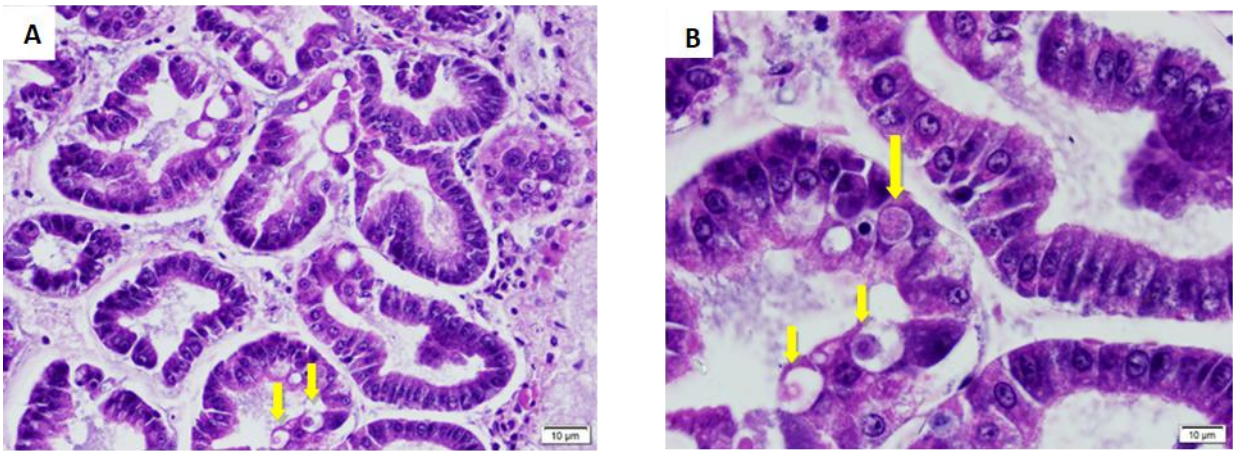

Figure 3. An image of an infected hepatopancreas tissue sample. Image (A) at low magnification, and image (B) at a higher magnification. Short arrows in panels A and B depict the advanced stage of the Densovirus infection, with large inclusions, and the long arrow in panel B depicts the initial stage of the Densovirus infection. Scale bar in both panels = 10 µm.

Statistical analysis revealed that there is no significant ($p > 0.05$) difference between the mean length and mean weight of the infected and non-infected shrimps collected from all four farms (Table **3**).

Table 3. Mean values of length and weight of infected and non-infected samples collected from all four farms. ($p > 0.05$)

Farm	Parameter	Infected	Non infected	p value
Farm A	Total weight	13.46± 4.30	13.56± 6.93	0.995
	Total length	12.49 ±1.62	11.95 ±2.33	0.81
Farm B	Total weight	17.47 ±3.26	15.40 ±1.85	0.251
	Total length	13.36 ±1.21	12.7 ±0.436	0.25
Farm C	Total weight	12.80 ±2.21	3.98 ±7.95	0.122
	Total length	12.22± 1.02	3.17 ±6.35	0.067
Farm D	Total weight	13.32± 2.04	12.82 ±3.17	0.777
	Total length	12.24 ±1.38	12.66 ± 2.00	0.711

DISCUSSION

*Pm*DNV originated in Asia, and all the initial reports of *Pm*DNV-infected shrimp were from Asia or the Indo-Pacific region, so it was considered to be of Indo-Pacific origin, but it eventually spread to wild shrimp in the Americas through the importation of live Asian shrimp for aquaculture (Safeena *et al.*, 2012). *Pm*DNV was first reported in cultured populations of 4 different penaeid shrimp species from 4 separate culture facilities in China (*Penaeus chinensis*), Singapore (*P. merguiensis*), the Philippines (*P. monodon*) and Kuwait (*P. semisulcatus*) (Lightner and Redman, 1985). After the first report in 1984 involving wild *Penaeus merguiensis* from Singapore, *Pm*DNV was reported in 1985 from *F. chinensis* in Korea (Jeeva *et al*., 2012). The presence of *Pm*DNV in hatchery-reared, early post larvae and mass mortalities in shrimp larvae has been reported in India, especially in *P.monodon* post larvae (Safeena *et al*., 2012). These findings suggest that *Pm*DNV is distributed all over Asia. In this study, PCR has been used to detect *Pm*DNV and this technique is expected to be much more sensitive compared to direct microscopic observation. According to the PCR results, 60% of the total collected samples were infected with *Pm*DNV and 27.5% were not infected with *Pm*DNV. This study demonstrates that there is a gradation of infection in farmed shrimps in Sri Lanka.

Shrimp affected by *Pm*DNV show non-specific gross signs, including atrophy of the hepatopancreas, anorexia, poor growth rate, reduced preening activities and a consequent increased tendency for surface and gill fouling and sporadic opacity of tail musculature. (Manjanaik *et al.*, 2005). *Pm*DNV infects epithelial cells of the hepatopancreas and midgut and causes disease in several species of cultured and wild shrimps worldwide and in freshwater prawns. Heavy infection may result in stunted growth, reduced production, and cause death, which leads to the production retardation (Jeeva *et al*., 2012).

Our methodology was effective in comparing the severity of the infection among the positive samples for the *Pm*DNV virus. During the first month following stocking, the virus was fatal to shrimp larvae. More recent findings have demonstrated a substantial statistical association between *Pm*DNV infection and small size. The majority of shrimp infected with *Pm*DNV generally develop very slowly, stopping at about 6 cm in length (Flegel, 2006). In our study, samples for analysis were collected from four different farms in the Chilaw, North Western Province of Sri Lanka. Therefore, it is necessary to determine the correlation with the PmDNV infection of samples collected from different farms separately. According to the statistical results, there were no infected samples from all four farms (Table 3). The lack of significant differences in growth ($p>0.05$) in mean length and mean weight between infected and non-parameters between infected and non-infected shrimp contradicts some existing literature suggesting that viral infections, including *Pm*DNV, may have negative impacts on the

growth of shrimp. Several studies have reported stunted growth and reduced production in shrimp infected with various viruses, emphasising the economic consequences for the aquaculture industry (Flegel *et al*., 1999; Pantoja and Lightner, 2000).

According to PCR results, 37.5% of the total samples were moderately infected with the virus. Therefore, infection does not significantly affect shrimp growth, and the infection is at a manageable level in Penaeus monodon.

Histopathology was also used to confirm the preliminary data obtained from the PCR analysis. According to the results gained from histopathological examination, we have identified different degrees of acidophilic and basophilic colours in each inclusion, indicating that the infection was at various stages (Flagel 2006). Here, we have identified different types of cells in the epithelial tissues of the tubule of the hepatopancreas of shrimps. They are E cells, F cells, B cells and R cells. (Silva *et al*., 2018). *Pm*DNV has been observed in only infected cells that go through the S phase of cell division. It has been recognised that the E-typed cells of the hepatopancreas are particularly sensitive to the virus since they are the only type of cells that can undergo mitotic division (Kerdmusik *et al.*, 2018). Characteristic F cells, which are likewise incapable of mitosis, were found to have *Pm*DNV intranuclear inclusions that showed a positive response to the *Pm*DNV. These results imply that an E cell can still differentiate into an F cell following *Pm*DNV infection because F cells arise through differentiation from E cells.

During the advanced stage of the infection, nearly all of the epithelial cells of the distal tubules of the hepatopancreas are infected with the large, single, *Pm*DNV inclusion bodies. The inclusion bodies cause the lateral displacement of the nucleoli and margination of the chromatin. Inclusion bodies are seen in certain advanced stages of infection in the lumen of the infected hepatopancreas. The infected hepatopancreatic tissues do not show a remarkable inflammatory response or necrosis (Catap and Traviña, 2005). Different stages of inclusions have been shown by different levels of acidophilic and basophilic colours (Kerdmusik *et al*., 2018). Eosinophilic inclusion bodies can be observed in the early stage of the infection, and they turn to basophilic and dark inclusions when the infection progresses to an advanced stage. Initially the generative E cells of the distal tubules of the hepatopancreas become infected, and thereafter the infection spread to all cells of the hepatopancreas and anterior midgut. This will lead to establishing the viral infection faster (Catap and Traviña, 2005).

H&E staining showed basophilic intranuclear inclusions in epithelial cells of the tubules in hepatopancreatic tissues samples. Even though Kerdmusik *et al.*. (2018) observed that more than 10 inclusion bodies in the infected tissue samples depicting infection at a severe level, we only observed two to three inclusion bodies in the infected cells (Figure 3). Therefore, we can conclude that the Densovirus infection

was at an early stage in the four shrimp farms in Chilaw. Furthermore, we did not observe any inclusion bodies in the lumen of the hepatopancreas (Figure 2), and there were no remarkable necrosis or inflammatory responses associated with advanced infection. These observations also confirm an early stage Densovirus infection in the Chilaw shrimp farms.

*Pm*DNV can spread both vertically and horizontally (Catap and Traviña, 2005). The feeding experiment demonstrating the horizontal transmission of *Pm*DNV from infected *Artemia* to healthy *P. monodon* post-larvae increases the likelihood that this vector could also introduce *Pm*DNV into rearing systems (Safeena *et al*., 2012). Oral ingestion of *Pm*DNV-contaminated material (the entire body of infected *P. monodon* post-larvae) resulted in horizontal transmission of *Pm*DNV among *P. monodon* post-larvae (Catap and Traviña, 2005). PmDNV can also be transmitted vertically from parental brood stock to F1 progeny.

One of the major challenges faced by shrimp farmers in Sri Lanka is the impact of viral diseases, specifically White Spot Syndrome Virus (WSSV) and Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) (Athula *et al*., 2024). These pathogens have led to an increase in mortality rates among cultured shrimp, resulting in substantial economic losses for the industry in Sri Lanka (Athula *et al*., 2024). In Sri Lanka, *Pm*DNV is still present at a moderate level, and measures must be taken to control the virus before it develops into a severe condition. Aquatic biosecurity should include preventive measures, including the establishment of disease surveillance systems and the development of molecular techniques for early carrier detection. Implementation of Better Management Practices (BMP) is crucial to identify disease risk factors, empirical observations and successful industry practices, and provincial, national and international regulatory requirements. These practices will help to support farmers to increase efficiency and productivity by reducing the risk of shrimp health problems, reduce impact by viral and pathogenic infections and improving food safety and the quality of shrimp farm product (Walker and Mohan, 2009). Additionally, as part of health management in aquaculture systems, extensive practical research is needed in areas including vaccines, RNAi, probiotics, immunostimulants, and other molecular techniques for enhanced diagnostics.

CONCLUSION

Based on the histopathological and molecular findings, *Penaeus monodon* Densovirus was detected in all four shrimp farms selected in Chilaw, in the North Western Province of Sri Lanka. However, there was no significant difference in the mean length and body weight between infected and non-infected shrimp. This suggests that the level of infection in the Chilaw region is currently moderate and does not appear to have a measurable impact on growth parameters**.**

This study provides the first confirmatory evidence of the presence of *Pm*DNV in Sri Lanka**.** In addition, the findings highlight the importance of employing multiple

diagnostic approaches**,** such as PCR and histopathological examination, to improve the accuracy and reliability of disease detection in shrimp populations.

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