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Histological Changes in the Hepatopancreas and Stomach of *Litopenaeus vannamei* Experimentally Induced with White Spot Syndrome Virus Infection

May Flor S. Muegue [a*], Philip Ian P. Padilla [b], Ma. Ramela Angela C. Bermeo-Capunong [b], Christopher Marlowe A. Caipang [b], Rommel J. Gestuveo [b], Mary Jane A. Amar [c] and Jane S. Geduspan [b]

[a] Institute of Fish Processing Technology, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miagao, Iloilo, Philippines.
[b] Division of Biological Sciences, College of Arts and Sciences, University of the Philippines Visayas, Miagao, Iloilo, Philippines.
[c] Institute of Aquaculture, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miagao, Iloilo, Philippines.

Authors' contributions

This work was carried out in collaboration among all authors. Author MFSM designed the study, wrote the methodology, performed the experiments, and prepared the first draft of the manuscript. Author PIPP designed the study, revised the manuscript for significant intellectual content of the manuscript. Author MRACBC performed the experiments and helped revise the manuscript. Author CMAC did conceptualized the study, provided inputs for the improvement of the manuscript. Author RJG assisted in the conduct of the experiments, data interpretation, and manuscript writing. Author MJAA designed the methodology, provided inputs for the improvement of the manuscript. Author JSG designed the study and revised the manuscript for significant intellectual content. All authors read and approved the final manuscript.

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**Corresponding author: Email: msmuegue@up.edu.ph;*

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ABSTRACT

Litopenaeus vannamei is an important aquaculture species in the Philippines and in the world. Its production continues to increase due to its fast growth and lower protein requirement, making it a favorable species to culture. However, sustainability of the this industry is hampered by the occurrence of viral diseases. White spot syndrome virus (WSSV) is considered the most devastating shrimp viral pathogen which can cause up to 100% mortality few days upon the onset of the infection. To understand the rapid mortality of the infected shrimps, this study examined histological changes in the stomach and hepatopancreas of *Litopenaeus vannamei* induced with WSSV infection. Results showed that cells in the stomach of infected *L. vannamei* undergo cellular abnormalities including hypertrophied nucleus, chromatin margination and basophilic inclusion bodies. The hepatopancreas was also observed to present basophilic inclusion bodies and chromatin margination. Disruption of the tubular epithelium in the hepatopancreas were further observed. These cellular damages that result to tissue and organ destruction as the infection progresses lead to the loss of the functions of these organs which probably contributed to the rapid mortality of shrimp infected with WSSV.

Keywords: White spot syndrome virus; Litopennaeus vannamei; hypertrophied nucleus; chromatin margination; basophilic inclusion bodies.

1. INTRODUCTION

Litopenaeus vannamei locally known as Pacific white shrimp is an important aquaculture commodity in the Philippines. Since the lifting of the ban on importation of *L. vannamei* in the country in 2007, production of this species continues to increase to supply the increasing local demand for shrimp. World production of *L. vannamei* in 2008 reached to 3.2 million metric tons and continues to increase, accounting to nearly 90% of global cultured shrimp [1]. *Litopenaeus vannamei* is a preferred cultured species over the local *Penaeus monodon* (Black tiger prawn) because of its ability to adapt to a broader range of salinity and temperature, higher resistance to environmental stress factors, faster growth, and lower nutritional requirements which renders this species cheaper to produce [2]. However, sustainability of *L. vannamei* aquaculture industry is hampered by viral diseases which caused significant economic losses.

"There are more than 20 viruses that infect both wild and cultured penaeid shrimps" [3]. "Among these, the white spot syndrome virus (WSSV) is considered as one of the most widespread and devastating virus that has affected the shrimp culture industry. This virus does not only infect shrimps but other crustaceans as well including crabs and crayfish that are found in both freshwater and marine environment" [4]. "The virus affects all life stages of the shrimps and mortality can reach up to 100% in a short period of time" [5-7]. "WSSV has been detected in the postlarvae of both wild and hatchery-reared shrimps" [8,9]. "Thus, the disease caused by this viral pathogen is not only a significant threat to the shrimp culture industry but to the marine ecology as well" [10].

WSSV is a circular double stranded DNA virus with size of about 300KB classified under the *Nimaviridae* family [11-13]. It is an enveloped and rod-shaped virus with a tail-like projection [6]. "The gross signs of WSSV infection includes reduced feeding, broken sizes, reddish discoloration, gathering around the ponds, and loose cuticle with white spots (which represent abnormal deposits of calcium salts by the cuticular epidermis) of 0.5 - 2.0 mm in diameter" [14]. WSSV infects different tissues and organs in shrimp. The virus has been reported to infect the heart, gills, and stomach of *P. japonicus*, hepatopancreas of *P. semisulcatus*, and gills and connective tissues of *Aristeus antennatus* [15]. "In *Penaeus monodon*, WSSV infection was observed in the gills, lymphoid organs, hematopoietic tissue, foregut (including the stomach), cuticular epidermis, connective tissue, striated muscle, heart, haemocytes, antenna

gland, nervous tissue and the ovary" [14]. "Separate reports further show the presence of the virus in the hepatopancreas, gills, lymphoid organ, connective tissue, hematopoietic, cuticular epidermis, digestive epithelium and striated muscles of *L. vannamei* co-infected with infectious hypodermal and hematopoietic necrosis virus (IHHNV)" [16,17]. Histologically, infected cells have intranuclear basophilic inclusions and hypertrophied nuclei with chromatin margination [2].

The stomach and hepatopancreas are the two biggest and morphologically distinct organs found in the cephalothorax of shrimp. Both organs are part of the digestive system and therefore play important roles in the survival of the shrimp. The stomach functions as storage and site of partial digestion of ingested food while hepatopancreas or the digestive gland of shrimp functions in food absorption, synthesis and secretion of digestive enzymes and also as storage of lipids, glycogen and minerals especially during the intermoult stages of the shrimp [18]. The hepatopancreas on the other hand functions as both the liver and the pancreas, making it very important to the survival of shrimp. The stomach and hepatopancreas of shrimps may be the target organs of WSSV, along with other organs and tissues [16,17,19] however, limited studies have been conducted to determine the degree of histological changes and damage caused by the virus in these important organs of *L. vannamei*.

This work aims to describe the morphology and histology of *Litopenaeus vannamei* stomach and hepatopancreas, and to observe histological changes in these organs as a consequence of WSSV infection.

2. MATERIALS AND METHODS

2.1 Collection of Experimental Animals

A total of 40 healthy juveniles of *L. vannamei* (3–5 g), were collected from a rearing tank in Freshwater Aquaculture Section Hatchery, University of the Philippines Visayas, Miagao, Iloilo, Philippines. These shrimps were equally divided in four (4) improvised tanks (5-L plastic container supplied with aeration and water inflow and outflow) and acclimatized for 2 days (salinity 28-30 ppt; ambient temperature 25-28^{o}C). The shrimp were fed with commercial feed (35% protein) on the second day of acclimatization. Each group were randomly screened to confirm absence of white spot syndrome virus (WSSV) infection by Polymerase Chain Reaction using the published primers of [20].

2.2 Preparation of the Viral Inoculum

The viral inoculum used in this study were obtained from the WSSV infected shrimp tissue stored in the ultralow freezer (-80^{o}C) at the University of the Philippines Visayas-National Institute of Molecular Biology and Biotechnology (UPV-NIMBB) Laboratory. To revive the virus and amplify its quantity, WSSV-free *L. vannamei* were fed with the infected tissue.

Representative samples of moribund shrimp were sampled for confirmation of WSSV infection by PCR, while other moribund shrimps were collected and stored at -80^{o}C until use.

2.3 Experimental Infection

Four aquariums were prepared and filled with filtered and UV sterilized seawater with salinity between 28-30ppt, temperature of 25–28^{o} C and pH of 8.0–8.2. Healthy juveniles of *L. vannamei* shrimp (3–5 g) were randomly allocated to the aquariums at a density of 10 shrimp per 20-L aquariums. Continuous aeration was employed in all tanks with water exchange of 50% daily as described by [21]. Food, molts and fecal material is siphoned out every 12 hours. The replicated treatment groups were kept in infected tanks 1, 2 and 3, while the fourth tank held the negative control. Inoculum of WSSV was administered orally by feeding the stock with WSSV infected shrimp tissue at 15% of the average body weight (ABW) of shrimp samples. Control group were given the same amount of WSSV-free shrimp tissue. The stocks were monitored and sampling was performed 2 days post-challenge. Moribund shrimps were collected and immediately preserved in Bouin's solution for histological examination.

2.4 Morphology and Gross Pathology

Healthy shrimp was used to study the general morphology of *L. vannamei*. The carapace was removed to examine the cephalothorax region. The cuticular epithelium was excised to further study the morphology, location and orientation of the stomach and hepatopancreas with respect with the other organs found in the cephalothorax.

2.5 Histology

The stomach and hepatopancreas from normal and experimentally-infected shrimp were excised

aseptically and immediately fixed with Bouin's solution (1:10 tissue to fixative ratio) for 48 hours. The preserved tissues were processed by a routine histological method [22]; dehydrated in alcohol series and embedded in paraffin wax. They were cut into 5µm sections by a rotary microtome (Reichert-Jung Biocut 2030). The thin sections of the hepatepancreas and stomach tissues were stained with hematoxylin and eosin (H&E). Photomicrographs of the specimen were taken using a Sony Camera (14.5 MP) fitted to an Olympus light microscope (4X to 100X).

2.6 Polymerase Chain Reaction

WSSV infection in shrimp samples was confirmed by Polymerase Chain Reaction (PCR) technique. Stomach and hepatopancreas tissues (50 to 100 mg) of infected and control shrimps were used for DNA extraction following previously described procedures [23]. PCR was carried out according to [20] using their published primers with sequences; WSSV F-5' GTACGGCAATACTGGAGGAGGT 3' and WSSV R-3'GGAGATGTGTA AGATGGACAAG 5'. The expected size of the amplification product is 232-bp. PCR products were electrophoresed in 0.8% agarose gel, stained with gel red, and visualized using Bio-Rad Molecular Imager Gel Documentation System with Image Lab Software.

3. RESULTS AND DISCUSSION

The WSSV infected shrimp died in less than 2 days upon oral infection and this supports the claim of previous researches that WSSV causes rapid mass mortality few days upon the onset of infection [6,17]. Dead *L. vannamei* were collected and processed histologically to study the effects of WSSV infection in the hepatopancreas and stomach shrimp. Samples from the control group were also randomly selected to serve as control or WSSV-free samples.

3.1 Morphology and Histology of *L. vannamei* Stomach and Hepatopancreas

Shrimp body is divided into 2 parts, the head and body section. The head is fused with the chest and is called the cephalothorax. The body and the abdomen consists of six segments, each segment has a pair of swimming feet which are also segmented (Fig. 1a and b).

The cardiac stomach is dorsal in the cephalothorax and looks like an oval sac. It leads into the pyloric stomach, which is situated in a ventro-posterior position in relation to the cardiac stomach. The pyloric stomach is reduced in size, elliptically shaped and comprises two chambers: an upper chamber leading into the midgut, and a lower chamber or gland filter. The hepatopancreas occupies much of the cephalothoracic cavity. The hepatopancreatic lobes are dorsally located and surround completely the midgut. The hepatopancreas is reddish, large compact organ, occupying greater part of the cephalothorax cavity, posterior to the cardiac portion of the stomach. Fig. 1 (c and d) shows the hepatopancreas and stomach of healthy *L.vannamei*.

Histologically, the cardiac stomach is lined by a simple cylindrical epithelium, whose height varies in the different zones. Nuclei are situated at different heights in the cells usually at the medial and basal zones. Next to the epithelium is a thin layer of dense connective tissue and surrounded by circular and longitudinal striated fibres. A serrated cuticle lays on the epithelium. The stomach wall forms many small folds projecting into the lumen. The size and number of folds vary according to distension degree. In areas where folds are bigger, the epithelium appears stratified and connective tissue is well developed with big haemolymphatic lagoons (Fig. 2, a to c). The cardiac stomach is separated from the pyloric one by the cardiopyloric valve (Fig. 2 d), which is a fold of the ventroposterior wall of the cardiac stomach extending dorsally and leaving a narrow canal towards the pyloric stomach.

The pyloric stomach (Fig. 3a) is small compared with the cardiac stomach. The wall histology is similar to that of the cardiac stomach, but the striated muscle in this region is more developed (Fig. 3b). The lumen of the two stomach regions are easily distinguishable based on their cross-section (Fig. 2a and Fig. 3a). Cardiac stomach has 2 chambers in which the lower one has more folds than the upper one. The pyloric stomach has three chambers with deeper folds in the transition that reduce the lumen (Fig. 3a). In the lower chamber, the cuticle forms the double structure of the filter (Fig. 3c). The filter consists of an outer row of elongated setae and an inner row comprising dorsally curved setae where each seta overlaps the next. The inner row of filtration setae form longitudinal channels of 16-18µm wide. Fig. 3c also show that the epithelium in the filter zone is underlined by thin connective tissue.

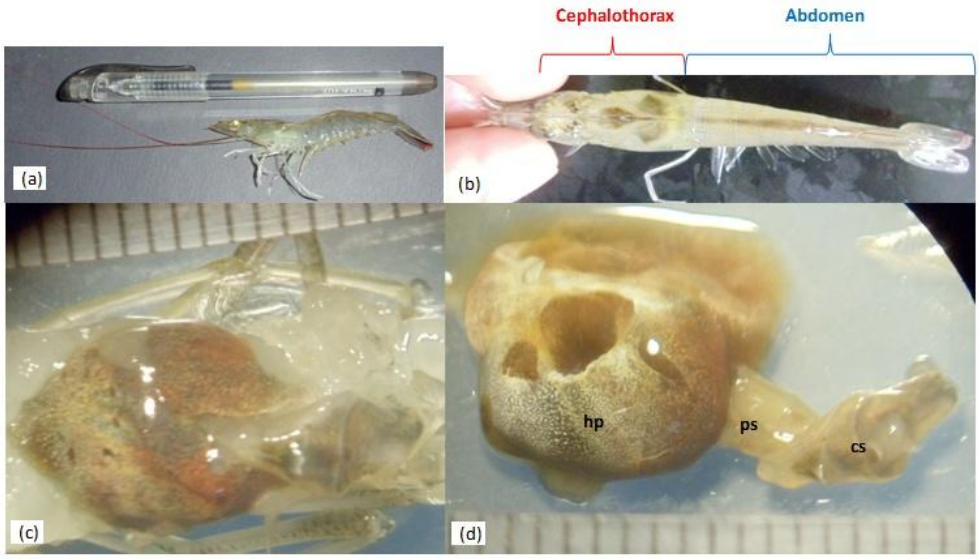

Fig. 1. Macroscopic examination of *L. vannamei.* (a) relative length of shrimp used in the study, (b) two main parts of shrimp body, the cephalothorax and the abdomen, (c) close-up image of the hepatopancreas and stomach of shrimp, (d) hepatopancreas and stomach excised from the cephalothorax,hepatopancreas (hp), pyloric stomach (ps) and cardiac stomach (cs)

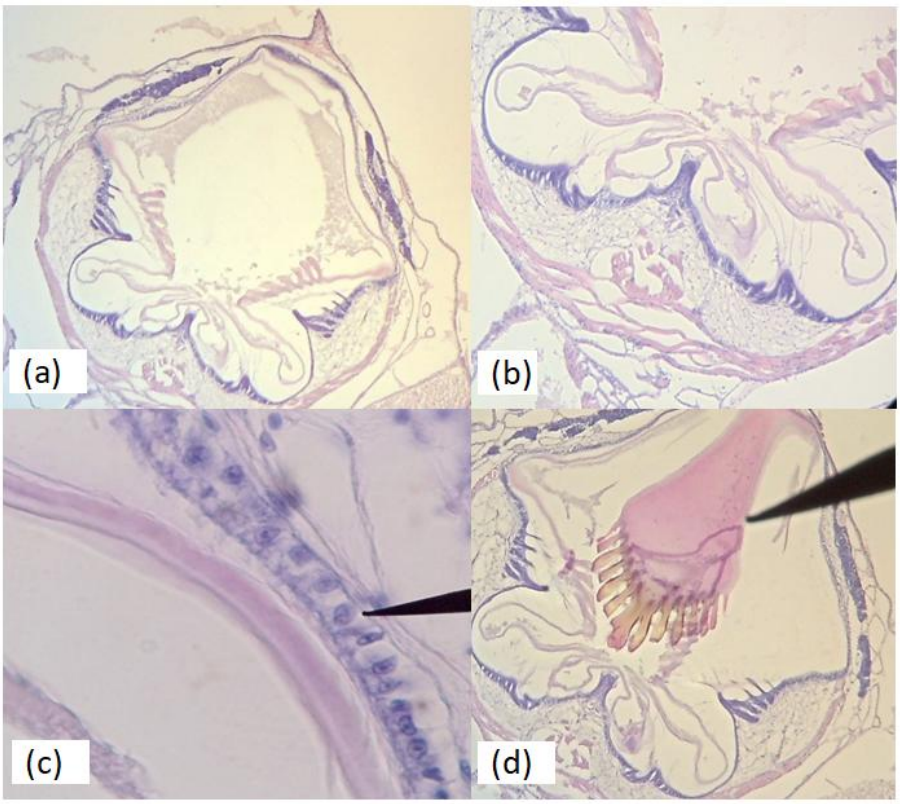

Fig. 2. Histology of the cardiac stomach of *L. vannamei.* (a). Cross-section of the cardiac stomach at low magnification (200X); (b). High magnification of the cardiac stomach showing the folds (1000X). (C). Epithelium of the cardiac stomach (1000X). (D). Pyloric valve that seperates the cardiac stomach from the pyloric stomach. H&E stain

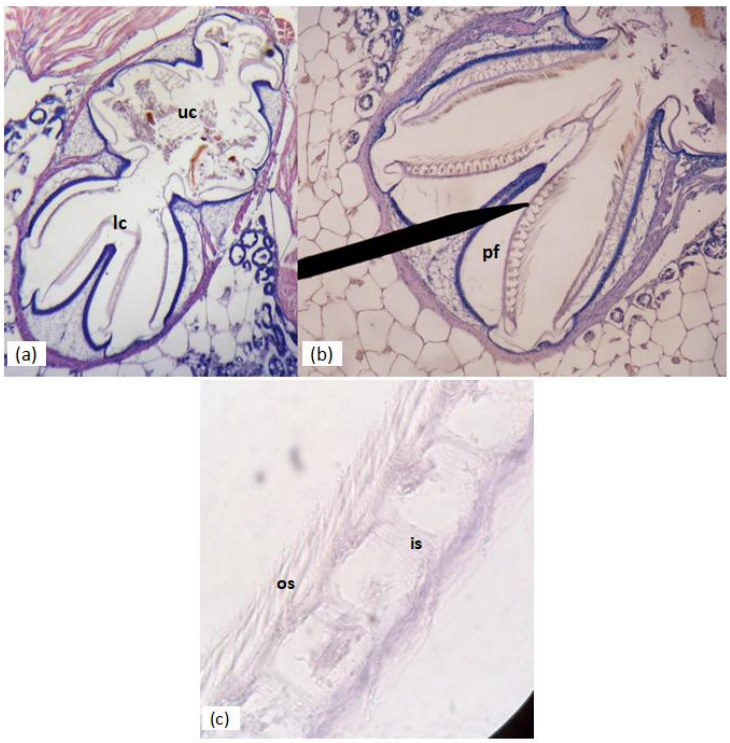

Fig. 3. Cross-section of the pyloric stomach. (a) whole image of pyloric stomach cross-section, (b) the two chambers are upper chamber (uc) and lower chamber (lc), 200X; C. The epithelium and pyloric filter (pf) at the lower chamber of the stomach (400x). (c) double structure of the pyloric filter consists of an outer row of elongated setae (os) and an inner row of dorsally curved setae (is) (1000X). H&E stain

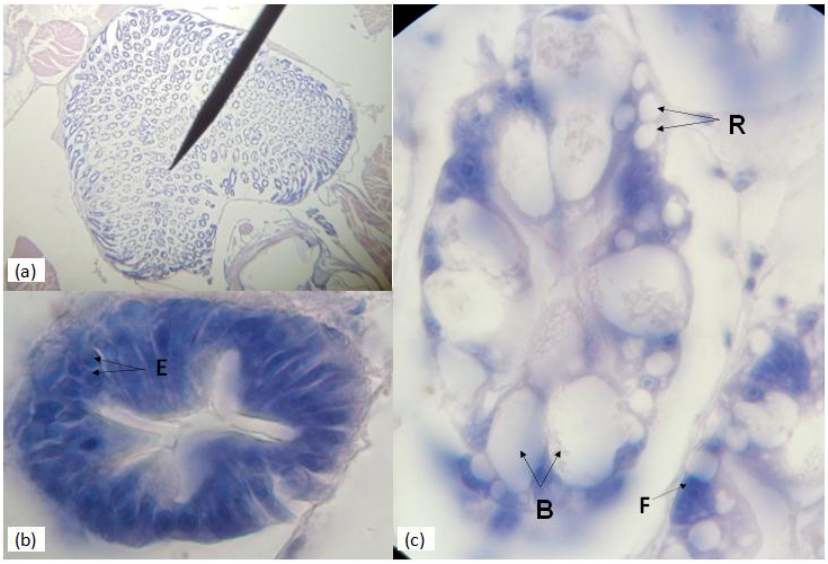

Fig. 4. (a) shows the cross-section of hepatopancreas at low magnification; (b) and (c). High magnification of the hepatopancreas showing the different cell-types (E, F, B and R cells) at 1000X. H&E stain

The general morphology of *L. vannamei* hepatopancreas is reddish brown, large compact organ which occupies the greater part of the cephalothoracic cavity, posterior to the cardiac portion of the stomach. The hepatopancreas has two separate lobes which are composed of

compact arrays of tubules. Fig. 4 (a to c) shows that each tubule under the light microscope is consists of four cell types which can be classified as E, F, B, and R cells [24]. The E-cells 'Embryonic cells' are undifferentiated and localized at the blind end of the tubule. The F and R-cells occupy mid distal region while the B cells occupy the mid region and are mostly interspersed with R-cells. The proximal portion of the tubule is lined predominantly by R-cells with few F-cells in between.

3.2 Histopathology of the Stomach and Hepatopancreas of WSSV infected *L. vannamei*

Examination of the WSSV infected *L. vannamei* compared with the healthy shrimp show that healthy shrimp has white spot-free and intact carapace. The carapace is difficult to remove or detach from the cephalothorax. Healthy shrimp also has a carapace with dark coloration representing the cuticular epithelium that is difficult to separate from the shell. WSSV-infected shrimp on the other hand has easily detached carapace with opaque spots either the spots are individual or clumpy. These spots are typical of WSSV infection. Moribund shrimp was reddish compared to the healthy one with likewise reddish and easily raptured hepatopancreas. These observations coincides with previous studies showing enlargement and increased fragility of the hepatopancreas of WSSV-infected shrimps [1,17,25].

Histological observations revealed that cellular changes upon viral infection were similar to both organs under study. Healthy shrimp shows normal epithelial cells of the stomach both in the cardiac and pyloric region show distinct nucleus and other organelles as basophilic with unstained or lucent to lightly stained cytoplasm. The muscle layer in this organ is intact (Fig. 5a). Induced WSSV infection in shrimp resulted to several abnormalities to the cells of the stomach. Hypertrophied nucleus, chromatin margination and basophilic inclusion bodies were observed in stained tissue (Fig. 5b). These abnormalities are very distinct and were easily distinguished from the normal cells. These results conform with earlier studies with WSSV infected cultured *L. vannamei* [2] and other penaeid shrimps [17]. In another report [15], the stomach of WSSV-infected *Penaeus japonicus* show intranuclear inclusion bodies specifically in the cuticular and sub-cuticular connective tissue.

Histological observation of the hepatopancreas showed that the organ is consist of tubules. The tubular epithelium vary in appearance and sizes of lumen, depending on the cell types that compose the epithelium. Healthy shrimp sample has intact epithelium with normal E-, B-, R- and F-cells (Fig. 6a). Induced WSSV infection in shrimp showed notable tissue disruptions in the hepatopancreas and cellular abnormalities including basophilic inclusion bodies, chromatin margination, and disrupted epithelium (Fig. 6b). The four cell types lining the hepatopancreatic tubules are difficult to distinguish in WSSV-infected samples. This results conform with the observations in hepatopancreas of other shrimp species infected with the virus during culture [14,15]. The observed disruption in the heptopancreatic epithelium of WSSV- infected shrimp suggest dysfunction of the organ [14]. Histopathological examination of hepatopancreatic tubules showed that the hepatopancreas exhibit vacuolization of the entire tissue. The observed increase in fragility of hepatopancreas of WSSV-infected *L. vannamei* is in agreement with the previous reports showing enlargement and increase fragility with vacuolization of hepatopancreatic tissue attributed to the increased hemolymph from this organ to promote systems of immunity cells [1,25]. The same observation was reported in *F. chinensis*, *M. japonicus, F. indicus, F. merguiensis* and *P. monodon* [17]. It has also been reported that lysis of some parts of hepatopancreas was observed in the later stages of infection without detecting any virus possibly due to the already poor health status of the shrimp and autolysis of the infected tissue [26] Hepatopancrease of WSSV-infected *Penaeus semisulcatus* was reported to show hepatopancreatic degeneration and necrosis and basophilic hypertrophied nuclei [15].

Other cellular abnormalities caused by WSSV infection was reported by several researchers to be present in different tissues, mostly of ectodermal and mesodermal of origin, including the gills, cuticular epithelium, legs, lymphoid organ, muscle and stomach [1,2,14,16,17]. Common histological findings were basophilic intranuclear inclusion bodies, marginated chromatin and hypertrophied nuclei of cells. In the early stage of viral infection, the inclusion bodies are termed as Cowdry-type A inclusions and as the infection progresses, the nuclei undergo further degeneration and finally develop into prominent basophilic type of inclusions [17].

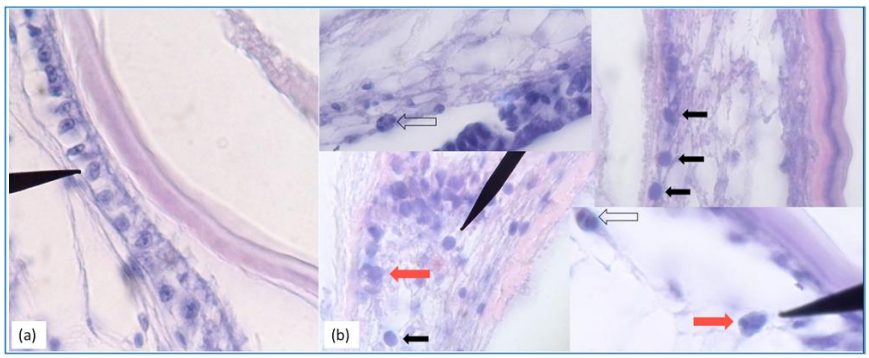

Fig. 5. Comparison of the histology (cross-section) of the stomach of healthy and WSSV infected *L. vannamei.* (a) Stomach of healthy *L.* vannamei showing the normal stomach epithelial cells and muscle layer. (b) Stomachof WSSV infected *L. vannamei* showing hypertrophied nucleus (red arrow), chromatin margination (open arrow)and basophilic inclusion bodies (black arrow). 1000X. H&E stain

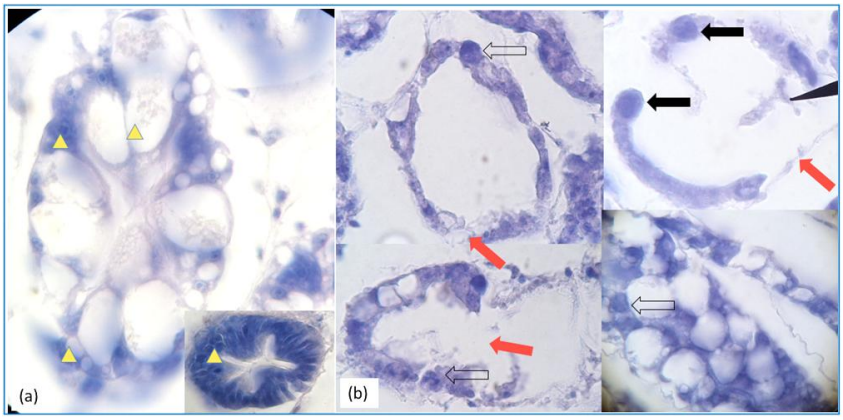

Fig. 6. (a) Hepatopancreas of healthy *L.* vannamei showing normal cell of the tubular epithelium, arrowheads point different cell types of the epitheliun. (b). WSSV infected hepatopancreas of *L. vannamei* showing basophilic inclusion bodies (black arrow), chromatin margination (open arrow) , and disrupted epithelium (red arrow). 1000X. H&E stain

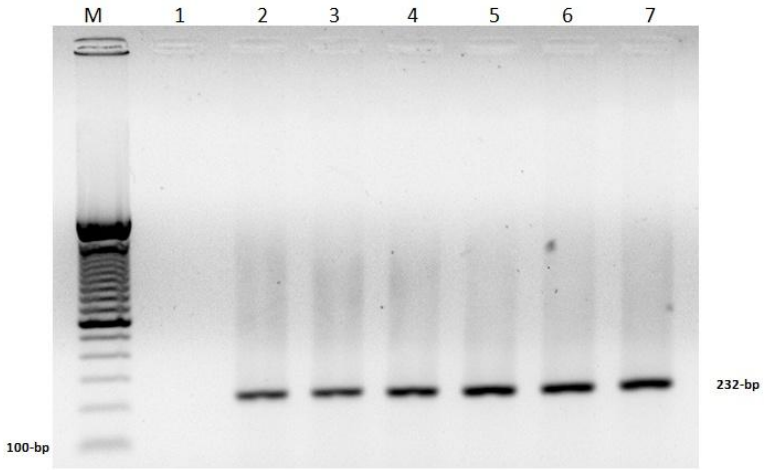

Fig. 7. Polymerase chain reaction results of WSSV infected *L. vannamei.* M-100-bp marker; Lane 1-negative control; Lanes 2-4- stomach; Lanes 5-7- hepatopancreas

3.3 Polymerase Chain Reaction

Standardized PCR assay published by [20] was used to confirm WSSV infection in shrimp samples. The expected product size of 232-bp was amplified in all the samples from the stomach and hepatopancreas of infected shrimps (Fig. 7). Representative tissue samples were also extracted from the control shrimp (uninfected with WSSV), and PCR result of this samples were negative. This result confirms successful infection of the experimental shrimp with WSSV and the control group were kept uninfected and remained healthy until the end of the experiment.

4. CONCLUSIONS

The histological sections of hepatopancreas of *L. vannamei* in this study showed cellular abnormalities including basophilic inclusion bodies and chromatin margination, and disrupted epithelium, very similar to the observations found in the cells of other tissues of ectodermal (gill, cuticular epithelium, and digestive epithelium) and mesodermal origin (hematopoietic organ, lymphoid organ, connective tissue and striated muscle). The intense cellular damage further resulted to the disruption of the tubular epithelium of the organ. On the other hand, the cellular abnormalities observed in the stomach of WSSV infected *L. vannamei* in this study include hypertrophied nucleus, chromatin margination and basophilic inclusion bodies

The cellular changes triggered by WSSV infection in the stomach and hepatopancreas of *L. vannamei* maybe one of the reasons of the rapid onset of mortality in infected shrimp stock, considering that these organs play major roles in shrimp survival.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Afharnasab M, Mortezaei R, Yegane V, and Kazemi B. Gross sign, histopathology and polymerase chain reaction observations of white spot syndrome virus in shrimp specific pathogen free *Litopenaeus vannamei* in Iran. Asian J of Animal and veterinary Adv. 2009;4(6): 297-305.
2. Cheng L, Lin WH, Wang PC, Tsai MA, Hsu JP, and Chen SH. White spot syndrome virus epizootic in cultured Pacific white shrimp *Litopenaeus vannamei* (Boone) in Taiwan. Journal of Fish Diseases. 2013; 36:977–985.
3. Claydon K, Tahir RAH, Said HM, Lakim MH, Tamat W. Prevalence of shrimp viruses in wild *Penaeus monodon* from Brunei Darussalam. Aquaculture. 2010; 308:71-74.
4. Lo CF, Ho CH, Peng SE, Chen CH, Hsu HC, Chiu YL, Chang CF, Liu KF, Su MS, Wang CH & Kou GH. White spot syndrome baculovirus (WSBV) detection in cultured and captured shrimp, crabs and other arthropods. Diseases of Aquatic Organisms. 1996;27:215–225.
5. Takahashi Y, Itami T, Kondo M, Maeda M, Fujii R. Electronmicroscopic evidence of bacilliform virus infection in Kuruma prawn (Penaeus japonicus). Fish Pathol. 1994;29:121-125.
6. Wang CH, Lo CF, Leu JH, Chou CM, Yeh PY, Chou HY, Tung MC, Chang CF, Su MS, Kou GH. Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. Diseases of Aquatic Organisms. 1995;23:239–242.
7. Karunasagar I, Otta SK, Karunasagar I. Histopathological and bacteriological study of white spot syndrome of *Penaeus monodon* along the west coast on India. Aquaculture.1997;153:9-13.
8. Hao N, Thuy DT, Loan LDT, Phi TT, Phuoc LH, Duong HHT, Corsin F and Chanratchakool P. Presence of the two viral pathogens WSSV and MBV in three wild shrimp species (*Penaeus indicus, Metapenaeus ensis, Metapenaeus lysianassa*) cultured in the mangrove forest of Ca Mau Province. Asian Fish. Sci. 1999;12:309- 325.
9. Caipang CMA, Sibonga MFJ, Geduspan JS, Amar MJA. An Optimized LAMP assay for detection of white spot syndrome virus

(WSSV) among cultured shrimps in the Philippines. J. Plant and Animal Sciences. 2012;22(24):927-932.

10. Flegel TW. Major viral diseases of black tiger prawn (*Penaeus monodon*) in Thiland. World J. Microbiol. Biotechnol. 1997;13:433-442.
11. Mayo MA. A summary of taxonomic changes recently approved by ICTV. Archives ofVirology 2002;147:1655–1663.
12. van Hulten MC, Witteveldt J, Peters S, Kloosterboer N, Tarchini R, Fiers M, Sandbrink H, Lankhorst RK, Vlak JM. The white spot syndrome virus DNA genome sequence. Virology. 2001;286(1): 7-22.
13. Yang F, He J, Lin X, Li Q, Pan D, Zhang X, Xu X. Complete genome sequence of the shrimp white spot bacilliform virus. J Virol. 2001;75:11811-11820.
14. Wang YG, Hassan MD, Shariff M, Zamri SM, Chen X. Histopathology and cytopathology of white spot syndrome virus (WSSV) in cultured *Penaeus monodon* from peninsular Malaysia with emphasis on pathogenesis and the mechanism of white spot formation. Diseases of Aquatic Organisms. 1999;39,1–11.
15. El-Shahidy MS, El-Gamal RM, Dessouki AA, Thabet RY, Abdelwahab AS, Abd-Eldaim MM. Detection of White Spot Syndrome Virus in Cultured Penaeid Shrimp using Histopathological Observation and Polymerase Chain Reaction. SCVMJ. 2015;20(1):213-226.
16. Pazir MK, Afharnasab M; Jalali Jafari B, Sharifpour I, Motalebi AA, Dashtiannasab A. Detection and identification of white spot syndrome virus (WSSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) of *Litopenaus vannamei* from Bushehr and Sistan and Baloochestan provinces, Iran, during 2009-2010. Iranian Journal of Fisheries Science. 2011;10(4):708- 726.
17. Pazir MK, Afharnasab M, Niamaymandi N, Khadem H, Akbarpour E, and Zendebudi AA. Histopathological observation on white spot syndrome virus and hematopoietic necrosis virus in shrimp farms, *Litopenaeus vannamei,* in Bushner Province, Iran. Asian Journal of Animal Sciences. 2012;6(5):209-219.
18. Ramadevi KRLS, Shyamasundari K, Hanumantha RK. Observation on the hepatopancreas of *Ocypoda platytarsis* (Milne-Edwards) (Crustacea, Brachyura), Bolletino Di Zoologia. 1990;57:3:261-265.
19. Liu F, Li S, Yu Y, Zhang C, Li F. Antennal gland of shrimp as an entry for WSSV infection, Aquaculture. 2021;530:735932.
20. Flegel TW. Detection of major penaeid shrimp viruses in Asia, a historical perspective with emphasis on Thailand. Aquaculture. 2006;258:1-33.
21. Domínguez-Borbor C, Betancourt I, Panchana F, Sonnenholzner S, Bayot B. An effective white spot syndrome virus challenge test for cultured shrimp using different biomass of the infected papilla. Methods X. 2019;6:1617-1626.
22. Gurr E. Staining Animal Tissues, Practical and Theoretical. Leonard Hill (Books) Ltd, London; 1962.
23. Muegue MFS, Geduspan JS, and Caipang CMA. Optimization of PCR protocols for detection of Viral pathogens in shrimp aquaculture in the Philippines. European Journal of Experimental Biology. 2013;3(6): 270-275.
24. Hemambika M, Raj RP. An ultrastructural study of the hepatopancreas of Indian white prawn *Penaeus indicus* H. Milne Edwards. The Fourth Indian Fisheries Forum Proceedings. 24-28, November, 1996. Kochi. 1999;291-294.
25. Lightner DV, Hasson KW, White BL, Redman RM. Chronic toxicity and histopathological studies with Benlatee, a commercial grade of benomyl, in Penaeus Íannamei_Crustacea: Decapoda.. Aquat. Toxicol. 1996;34:105–118.
26. Chang PS, Lo CF, Wang YC, and Kou GH. Identification of white spot syndrome virus associated baculovirus (WSBV) target organs in the shrimp *Penaeus monodon* by in situ hybridization. Diseases of Aquatic Organisms. 1996;27:131–139.