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Histopathological Observation of White Spot Syndrome Virus and Infectious Hypodermal and Hematopoietic Necrosis Virus in Shrimp Farms, *Litopenaeus vannamei*, in Bushehr Province, Iran

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ABSTRACT

This study was conducted to identify White Spot Syndrome Virus (WSSV) and Infections Hypodermal and Hematopoietic Necrosis Virus (IHHNV) by using clinical and histopathological sings of cultured shrimp, *Litopenaeus vannamei* in Bushehr farms, from December 2009 to April 2010. Samples were collected from Bushehr shrimp farms and hatcheries. Based on histopathological and gross signs, two viral diseases, WSSV and IHHNV have been detected. The gross signs of WSSV in the moribund samples showed reduced feeding, lethargy, difference in size, reddish coloration of appendages and white plaque on the carapace, while the gross sign of IHHNV exhibited abdominal dorsal changes of the muscles, opaque or milky spot on the cuticles, rostrum deformity syndrome and abdominal segmental abnormalities. The histopathological observations of WSSV showed basophilic Cowdry type A inclusion bodies in all tissues such as gills, haematopoietic tissue, cuticle epithelium, lymphoid organ and connective tissue. However, histologically, the hepatopancreas tissue showed vacuolization of B cells, without inclusion bodies. The histopathological signs observed such as hypertrophy, cellular degeneration and eosinophilic Cowdry type A inclusion bodies in the cells nucleus hepatopancreas, gills, haematopoietic, cuticle epithelium, digestive epithelium, lymphoid organ and connective tissue. Also, histopathological change of the striated muscles of the affected abdominal segmental abnormality showed severe Zenker's necrosis.

Key words: WSSV, IHHNV, gross sign, histopathological observation, *Litopenaeus vannamei*, Bushehr, Iran

INTRODUCTION

Shrimp farms have been expanded rapidly over the last two decades. In 2008, shrimp farming production in the world was reported 3, 281, 253 metric tons and the most commonly cultured species is Pacific white shrimp (*Litopenaeus vannamei*) that included 90% of global cultured shrimp (Afsharnasab *et al.*, 2009).

Over the last decades shrimp pathogens (especially virus) have been expanded throughout shrimp aquaculture industries (Lightner, 1999). It was made a significant economic loses for the shrimp farming industry. So that, annually viral diseases caused millions dollar lost for the shrimp global industry (Wyban *et al*., 1992; Lightner, 1999). *Baculovirus penaei* was the first virus that has been reported from Gulf of Mexico in *Farfantepenaeus duorarum* (Couch, 1974). Although, more than 20 viruses have been identified in Penaeidae shrimps (Lightner, 1999) but four viruses consisted of WSSV, Yellow Head Virus (YHV), Taura Syndrome Virus (TSV) and IHHNV have the major impacts on the shrimp aquaculture (Lightner, 1996; Flegel, 1997).

Among the four viruses; WSSV is the major pathogenic infectious virus in Penaeidae family (Lightner, 1996). For the first time, this virus was reported in *Marsupenaeus japonicus* in Taiwan (Chen, 1995). It was also observed in other species, especially in *Penaeus monodon* in the most of Asia countries and the United States (Lo *et al*., 1999). In Iran the virus was identified in *Fenneropenaeus indicus* from Khozestan farms, that in some years created about 90% mortality within 3-10 days (Afsharnasab *et al*., 2007; Afsharnasab *et al*., 2009).

IHHNV is a parvovirus, which is the smallest (22 nm) among the mentioned viruses (Bonami *et al*., 1990). The first record of IHHNV was reported in *Litopenaeus stylirostris* from Hawaii farms in 1981 (Lightner *et al*., 1983a, b; Lightner, 1996; Flegel, 1997). This virus has been found in other areas through asymptomatic carriers (Motte *et al*., 2003). IHHNV was routinely found in wild and captured *L. vannamei* without any clinical signs (Mari *et al*., 1993). *L. vannamei* has partially resistant to the virus although lesions on the body and deformities such as Rostrum Deformity Syndrome (RDS) have been observed (Lightner, 1996). When RDS prevalence reached more than 30%, a great loss of 10 to 50% in marketing value has occurred (Wyban *et al*., 1992). Both WSSV and IHHNV have significant impact on shrimp production. WSSV is observed in many shrimp species and crabs, from various geographical areas and accompanying with 100% mortality (Rodriguez *et al*., 2003) that leading to a devastating economic impacts (Lo *et al*., 1999). IHHNV also is one of the most serious viral diseases of farmed penaeid shrimps (Lightner, 2003). It is widely distributed in many countries and has a large range of hosts in many species of cultured penaeid shrimps.

Different methods are used for identifying viral diseases including clinical signs, molecular methods, histopathology and Transmission Electron Microscopy (TEM). In this study WSSV and IHHNV were identified by using clinical signs and histopathology observation in *L. vannamei* farms of Bushehr province.

MATERIALS AND METHODS

The present study was performed in the six grow-out shrimp farms and five hatcheries which located in the Bushehr province, with previous history of WSSV mortality in contrary to IHHNV. Sampling was performed from December 2009 to April 2010. The 150 shrimp samples (juvenile and sub adult) accompanying with slow growth collected from grow-out shrimp farms along the Bushehr coastal area, Persian Gulf (Bandar Rig, Heleh, Shif, Mond and Delvar (I and II) sites) (Fig. 1). In addition, 200 shrimp larvae and post larvae producing from domesticated origin which were randomly selected from hatcheries. Moribund domesticated broodstock samples of *L. vannamei* (average weight 40-45 g) with opaque, whitish abdominal muscles and white, milky spot on carapace body cuticle were obtained from private sectors (87 samples). Individual shrimp were kept on ice and examined grossly for signs of WSSV, IHHNV and other viral diseases. The sub adults and broodstock shrimps were injected with cold Davidson's fixative solution (4°C) into the

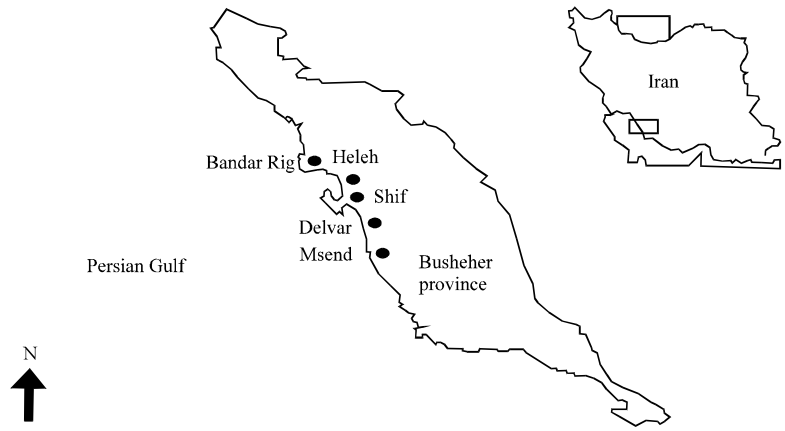

Fig. 1: Location of shrimp farms in Bushehr province

hepatopancreas, gills and 2, 4 and 6 abdominal segmental tissues. All the samples for histopathological examination were immersed with the same fixative for 24-48 h (ratio 1:10). The fixative was discarded and the samples preserved in 50% ethyl alcohol for subsequent histopathological preparation and analysis (Bell and Lightner, 1988). Tissues (hepatopancreas, gills, lymphoid organ, connective tissue, hematopoietic, cuticular epidermis, digestive epithelium and striated muscles) were sectioned at 4-5 µm in thickness and stained with hematoxylin and eosin phloxine (H and E/ph) (Saberi *et al*., 2008; Afsharnasab *et al*., 2009; Fouzi *et al*., 2012). Subsequently gross signs and histopathological studies of WSSV and IHHNV were evaluated using light microscope (CETI; Triton II).

RESULTS

The gross signs of WSSV infections shrimps showed a reduced feeding, lethargy, difference in size, gathering around the ponds, reddish discoloration of body and appendages (i.e., the antenna, maxillipeds, pereiopods, pleopods, telson and uropods) with presence of white plaque on carapace cephalothorax or diffuse over body cuticle as characteristic feature of WSSV disease (Fig. 2a, b, g and h). These spots were abnormal deposits of calcium salts in cuticle. WSSV infection of some samples was confirmed by histopathological examination. Histopathological changes of hepatopancreas tissues such as vacuolization of B cells and increasing in the number of F and R cells, without intranuclear inclusion bodies Cowdry type A were observed (Fig. 3a). The epithelial pillar cells of secondary lamella of gill tissues showed basophilic intranuclear inclusion bodies Cowdry type A. In addition to fusion formations made between secondary lamella of infect tissue and the cells which exhibited the nucleus hypertrophy and watery and thin cytoplasm (Fig. 3b). Lymphoid organ showed three stages of WSSV infection. At the first stage, the nucleus of cells was hypertrophy and cytoplasm was thin. In the mediate stage, marginated chromatin and the space between nucleus and cell membrane was very thin. In the late stage, the final shape of intranuclear inclusion bodies was observed as similar spheroid cells (Fig. 3c). On the other hand, the number of hyaline cells of hematopoietic tissues in infectious broodstocks obtained from hatcheries accompanying with nucleus pyknosis and karyorrhexis was recorded higher compared to hematopoietic tissues of normal shrimps (Fig. 3d). In the prepared sections with external layers,

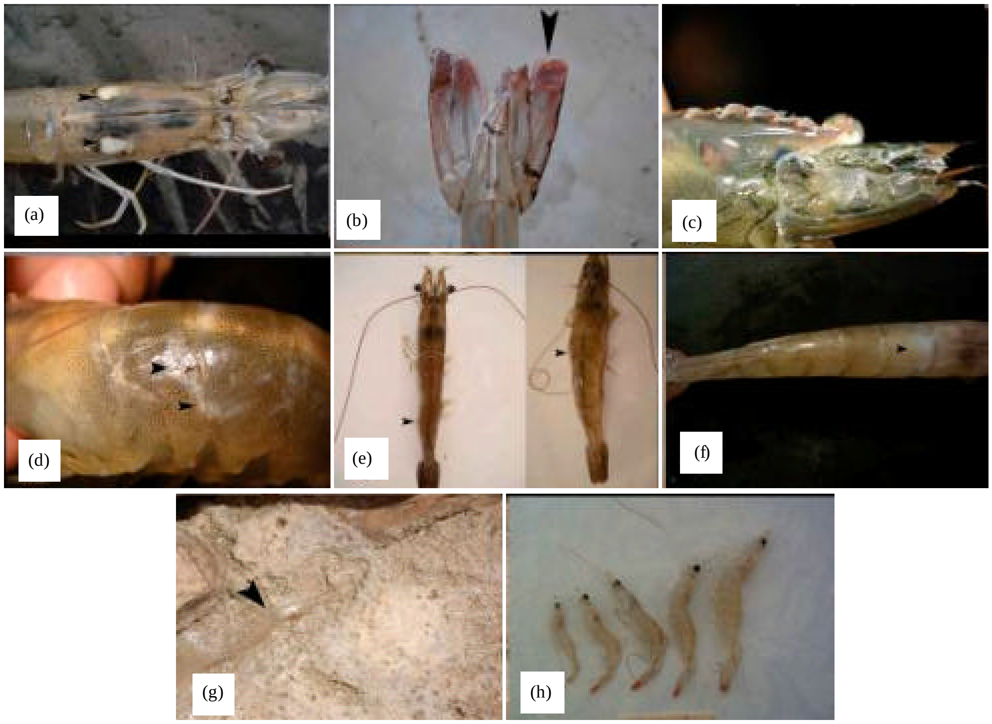

Fig. 2(a-h): Clinical sings in infection shrimps to WSSV and IHHNV, (a) White spots on carapace shrimps, (b) Reddish coloration of appendages (Telson area), (c) Rostrum deformity syndrome (RDS), (d) Milky moles on abdominal segmental, (e) Abdominal abnormalities, (f) Abdominal dorsal muscles opaque, (g) Accumulation of dead shrimps in the pool margin and (h) Difference in size

the epithelial cells of cuticle in infected shrimps, which showed hypertrophy and basophilic intranuclear inclusion bodies, were detached from epidermis (Fig. 3e). In these specimens, characteristic WSSV basophilic intranuclear inclusion bodies and nucleus pyknosis of the cells were abundant in connective tissues of the general body; cuticle, appendages and hepatopancreas (Fig. 3f). Histopathological signs of striated muscles indicated hypertrophy and dense nucleus which were higher in infected broodstocks in compare with post larvae (Fig. 3g). The hypertrophied columnar cells and intranuclear inclusion bodies were observed in mid gut, where some same cells were ruptured and released inclusion bodies to the lumen of gut (Fig. 3h). Significantly, all these samples were showed WSSV infectious in histopathological study.

Clinical signs of IHHNV infected shrimps such as opaque, whitish abdominal muscles and white, milky moles on body cuticle (abdominal segmental junction) and shrimp with different size, slow growth and Rostrum Deformity Syndrome (RDS) were observed in samples. In addition cuticular deformities were found in the third to sixth abdominal segments and tail fan in sub adults grow-out shrimp farms and broodstocks hatcheries (Fig. 2c, d, e, f and h). Histopathological changes of the post larvae, sub adults and broodstocks infected tissues, especially in the hepatopancreas, cuticular epithelium, gills, connective tissues, hematopoietic tissues and digestive epithelium were

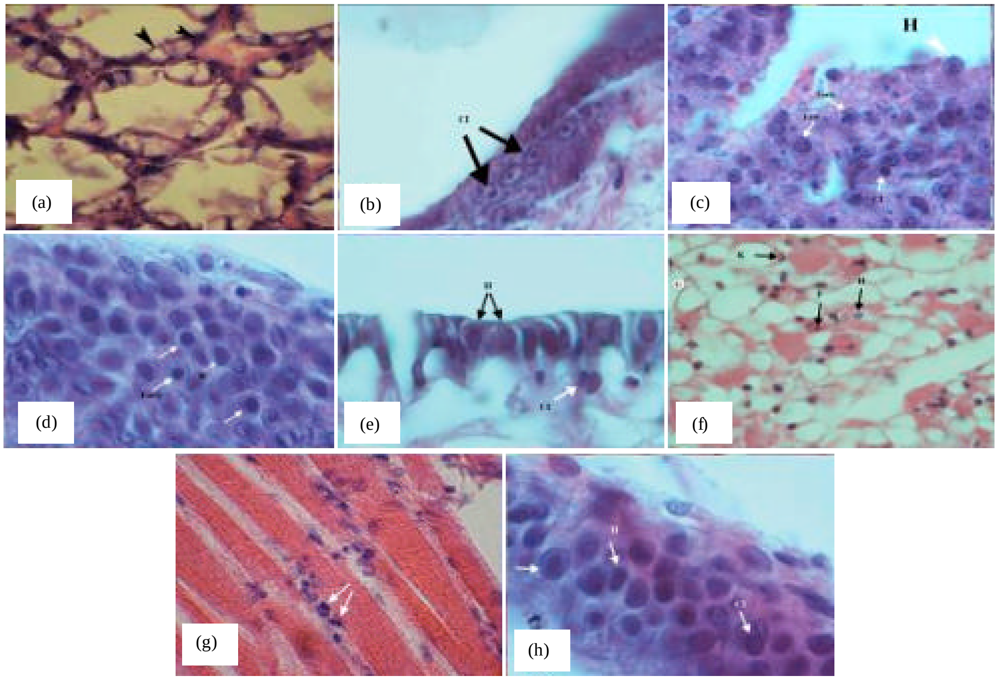

Fig. 3(a-h): Histopathological observations of WSSV infection in tissue shrimps include nucleus hypertrophy (H), cellular degeneration (CD), intranuclear inclusion bodies Cowdry type A (CI) and nucleus pyknosis (P) and karyorrhexis (K) in *L. vannamei* (H and E\Ph 1000X), (a) Vacuolization of B cells in the hepatopancreas tissue and increase F cells (arrowheads), (b) Intranuclear inclusion bodies (arrowheads) in the gill tissue, (c) Nucleus hypertrophy, early, late and Cowdry type A inclusion bodies in the lymphoid organ (arrowheads), (d) Intranuclear inclusion bodies in hyaline cells of the hematopoietic tissue (arrowheads), (e) Nucleus hypertrophy and large basophilic intranuclear inclusion bodies (arrowheads) in the cuticle epithelium, (f) Nucleus hypertrophy, large basophilic intranuclear inclusion bodies and nucleus pyknosis and karyorrhexis (arrowheads) in the connective tissue, (g) Nucleus hypertrophy and dens nucleus (arrowheads) in the striated muscles and (h) Large basophilic intranuclear inclusion bodies (arrowheads) in the digestive epithelium

characterized by widespread cellular degeneration, severe nuclear hypertrophy and margination chromatin (Fig. 4a-g). Development of cells hypertrophy that observed in the necrotic tissues was different in the viral infection stages. Eosinophilic intranuclear inclusion bodies Cowdry type A exhibited in numerous hepatopancreas epithelial cells (Fig. 4a). The tissues section showed eosinophilic enlarged nuclei, often accompanying whit cloudy inclusion surrounded by marginated chromatin. Muscular atrophy, associated with reddish discoloration of the cuticles was prominent. Histologically, the striated muscles of the affected abdominal segmental abnormality showed severe Zenker's necrosis (Fig. 4h). All tissue exhibited nucleus pyknotic and karyorrhectic in advanced infectious stages A. The presence of IHHNV was confirmed by histopathological observation.

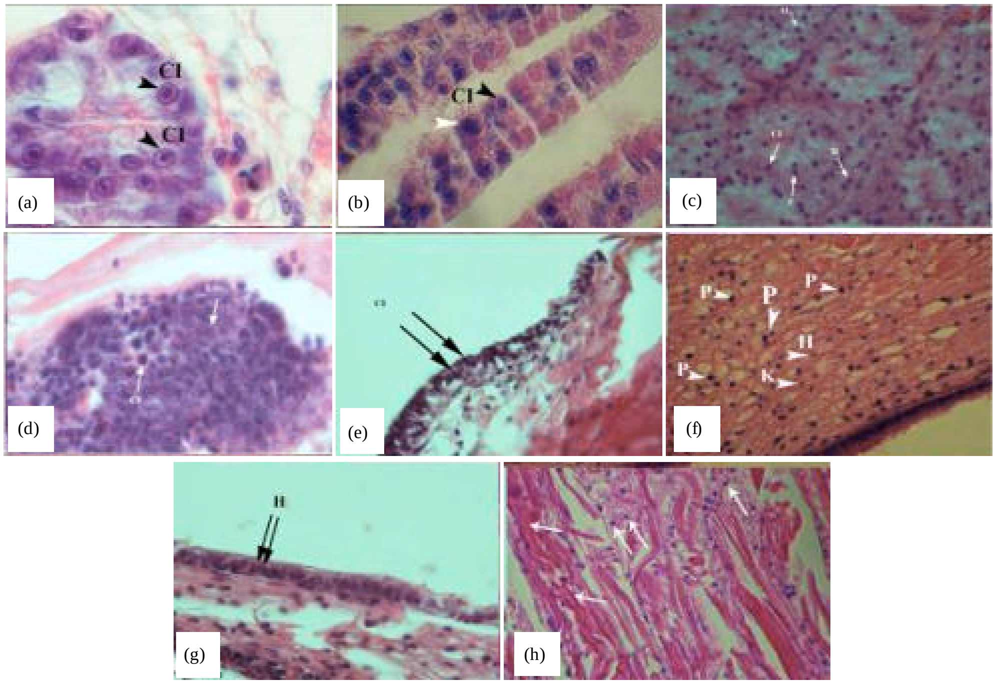

Fig. 4(a-h): Histopathological observations of IHHNV infection in tissue shrimp include nucleus Hypertrophy (H), Cellular Degeneration (CD), Cowdry type A intranuclear Inclusion bodies (CI) and nucleus Pyknosis (P) and Karyorrhexis (K) in *L. vannamei* (H and E\Ph 1000X), (a) Large eosinophilic intranuclear inclusion bodies Cowdry type A in the hepatopancreas tissue (arrowheads), (b) Intranuclear inclusion bodies (arrowheads) in the gill tissue, (c) Nucleus hypertrophy in the lymphoid organ (arrowhead), (d) Intranuclear inclusion bodies and nucleus pyknosis and karyorrhexis (arrowheads) in the haematopoietic tissue, (e) Nucleus hypertrophy and intranuclear inclusion bodies (arrowheads) in the cuticle epithelium, (f) Nucleus hypertrophy, intranuclear inclusion bodies and nucleus pyknosis and karyorrhexis (arrowheads) in the connective tissue, (g) Intranuclear inclusion bodies (arrowheads) in the digestive epithelium and (h) Nucleus hypertrophy and Zenker's necrosis (arrowhead) of the striated muscles

DISCUSSION

To detect viral diseases of Penaeidae shrimps, different methods are being used such as clinical signs, histopathology, molecular methods (Polymerase Change Reaction) and Transmission Electron Microscopy (TEM). Viral acute infections associated with mass death (100%) and without clinical symptoms (Lightner, 1996; Lightner, 1999). On the other hand, the genetic mutations due to geographic isolation may not be a suitable molecular method so by histopathological method, tissue damages caused by the pathogenic viruses were studied (Bell and Lightner, 1988; Lightner, 1996).

WSSV infection was created by Nimaviridae (Chen 1995). Other names for WSSV are Systemic Ectodermal and Mesodermal Baculovirus (SEMBV) (Wongteerasupaya *et al.*, 1995), Rod-shaped

Virus of *Marsupenaeus japonicus* (RV-PJ), Penaeid Rod-shaped DNA Virus (PRDV) (Inouye *et al.*, 1996) Hypodermal and Hematopoietic Necrosis Baculo-like Virus (HHNBV) of *Fenneropenaeus chinensis*(Sreenivasa Rao *et al.*, 2007). This virus is capable to infect at least 78 species, mainly decapods crustaceans including marine and fresh water shrimps, crabs, crayfish and lobster (Lightner, 1996; Flegel, 2006) and some arthropods can be the source of virus (Lo *et al.*, 1999). It is rarely observed in non-penaeidae species, except *Macrobrachium rosenbergii* and *Orconectes punctimanus* (Peng *et al.*, 1998).

The broodstocks *L. vannamei* collected from hatcheries of Bushehr province, showed typical symptoms of white spots on the inner surface of the carapace and shell similar to symptoms described by Afsharnasab *et al.* (2009). But the shrimps were normally feeding and no deaths were occurred, which may be due to accommodation of host with the virus (Afsharnasab *et al.*, 2009). Juvenile and sub adult shrimps accompanied with severe mortality due to high virulence of virus but were not observed white spots on their carapace. Other signs were feeding reduction, as Saberi *et al.* (2008) reported the reddish body coloration on the moribund shrimp. Virulence studies show that *L. vannamei* has little resistance to WSSV, so when the virus has a high virulence, it could be associated with mass death (100%) (Wang *et al.*, 2000).

Nuclear hypertrophy and cellular degeneration of the cells from ectoderm and mesoderm origin were commonly observed in moribund shrimp. Wang *et al.* (2005) reported that *L. duorarum* showed different tolerance with virus virulence in different geographical regions. Initial studies showed that clinical signs observed in *M. japonicus* and *P. monodon* infected to WSSV, after 3 days, made high mortality percentage (70-100%). Also there were differences in intensity virulent WSSV in *Fenneropenaeus indicus* compared to other species (especially *L. vannamei*) which it may be due to different sensitivity of the species, their defensive mechanism and environmental factors of the studied area. Afsharnasab *et al.* (2009) and Granja *et al.* (2003) showed that apoptotic cells reduce virus replication and control disease in *L. vannamei*. Apoptosis plays a critical role in development and maintenance of multicellular organisms. It has also been described as an anti-viral mechanism in both insects and vertebrates. In fact, to resistance against the immune system and to increase their outbreak, some viruses such as *Baculovirus* sp. produce anti-apoptotic molecules (Granja *et al.*, 2003). Histopatholgical studies implies existence intranuclear inclusion bodies Cowdry type A in cells tissue target such as gills, lymphoid organ, hematopoietic tissue, cuticular epithelium, digestive epithelium, striated muscles and connective tissues which advanced stages were observed basophilic (H and E/Ph) (Perez *et al.*, 2005; Afsharnasab *et al.*, 2009). Lack of polyhydrogenic materials in WSSV structure caused basophilic color inclusion bodies but some of viruses (i.e., *Parvovirus*) existence polyhydrogenic material in the structure caused an eosinophilic color inclusion bodies (Afsharnasab *et al.*, 2009). Cowdry type A, basophilic, intranuclear inclusion bodies surrounded by marginated chromatin in hypertrophied nuclei of cells in tissues of ectodermal (gills, cuticular epithelium and digestive epithelium) and mesodermal origin (hematopoietic organs, lymphoid organ, connective tissue and striated muscle) (Perez *et al.*, 2005). However, in white-spot syndrome the Cowdry A inclusions represent an early stage of viral infection. Once infecting of these nuclei undergo further degeneration and finally develop into prominent eosinophilic and pale basophilic type inclusions.

Cells of the hepatopancreas has never shown to be infected with WSSV but enlargement and fragility of hepatopancreas tissue of contaminated shrimps, microscopic observations indicated vacuolization tissue which can be due to increased hemolymph from this organ to promote system of immunity cell (Lightner, 1996; Afsharnasab *et al.*, 2009). Similar histopathological changes were observed among infected cultured Penaeid shrimps such as *F. chinensis, M. japonicus, F. indicus, F. merguiensis* and *P. monodon*. These observations coincided with the reports described by Wongteerasupaya *et al*. (1995) and Inouye *et al*. (1996). In addition, LOS (Lymphoid Organ Spheroids) was clearly observed in lymphoid organ that was infected by WSSV. LOS usually appears when shrimp was able to control or respond the infection to pathogenic agent in survival or chronic states (Lightner, 1996; Hasson *et al*., 1999). Nuclear pyknosis and karyorrhexis were observed in advance stages of WSSV and other viral severe infections, such as YHV, TSV, Lymphoid Organ Vacuolization Virus (LOVV) (Lightner, 1996). Existence of nuclear pyknosis and karyorrhexis in the hematopoietic tissue and lymphoid organ were accompanied with losses in tissue structure that implied attack viral infection (Rodriguez *et al*., 2003). However, in investigated samples, these lesions were associated with severe WSSV infection. Pantoja and Lightner (2003) have observed nuclear pyknosis and karyorrhexis in shrimps that were infected by WSSV experimentally. In addition, pyknosis and karyorrhexis were observed in the hematopoietic tissue of *F. duorarum* with exposure to WSSV experimentally (Wang *et al*., 2005).

There are milky moles on the surface of external cuticles (abdominal segmental) and opaque dorsal muscles in some sub adult shrimps accompanied with rostrum and abdominal segmental deformity. It was implicated IHHNV infection, but not observed death, because *L. vannamei* is resistant to the virus and act as a carrier (Lightner, 2003). *L. vannamei* led to transmission IHHNV to other parts has accompanied by economic losses (Lightner, 1999). IHHNV is one of the smallest viruses of penaeidae shrimps that identified in early 1980s and closely related to mosquito brevidensoviruses (Yang *et al*., 2007). Virus can infect shrimps during larval stages to adult and throughout vertical transmission or consume infected shrimps by healthy shrimps and potentially contact contaminated water (Motte *et al*., 2003). IHHNV usually infects ectodermal, mesodermal and rarely endodermal (Flegel, 1997). Some published reports describe that IHHNV has an affinity for various tissues and is rarely detected in endoderm derived tissues (Lightner, 1996; Tang and Lightner, 2006). However, if shrimps affected after post larval stage (sub adult), they could show symptoms such as rostrum deformity, curly antennae, carapace blistered, cuticle deformity and stunting (Tang and Lightner, 2006). RDS observed in *L. vannamei* by IHHNV infection experimental after 30 days (Singhapan *et al*., 2004). Usually RDS can affect in nursery period and growth stages of *L. vannamei* that associated with great economic damage, including irregular growth and difference in size (Lightner, 2003).

This study indicated that IHHNV had an affinity in the hepatopancreas of post larval of *L. vannamei*. Histopathological changes are formation of eosinophilic intranuclear inclusion bodies in the hypertrophied nuclei with marginated chromatin and cellular degeneration but in the present study affected sub adult showed growth retraction and deformities of the third to sixth abdominal segments and RDS, without eosinophilic intranuclear inclusion bodies formation. Eosinophilic intranuclear inclusion bodies Cowdry type A is due to existence of polyhydrogenic material in IHHNV structure (Rodriguez *et al*., 2003; Afsharnasab *et al*., 2009). These lesions were similar to those of RDS described in *P. monodon, M. rosenbergii* and *L. stylirostris* with IHHNV infection by Hsieh *et al*. (2006). So the presence of IHHNV from both hatcheries and grow-out shrimp farms (*L. vannamei*) indicated that this virus could slowly growth and severe difference in size which may be due to feeding reduction. Histopathological studies of gills, hematopoietic tissue, cuticular epidermis, digestive epithelium, lymphoid organ and connective tissue of infected shrimps were evidence of hypertrophy, cellular degeneration and formation intranuclear inclusion bodies that in advanced stages were together with nuclear pyknosis and karyorrhexis. Also, histopathological changes in the striated muscles of infected shrimp exhibited severe Zenker's necrosis. Muscular lysis was sometimes found in affected fiber accompanied with inclusion bodies while Hsieh *et al.* (2006) reported no observation inclusion bodies in striated muscles of *M. rosenbergii*.

Lightner (1996) reported HPV infections in *L. vannamei* by parvo or parvo-like viruses. He described that gross signs of HPV may not be specific, but in severe infections may include an atrophied heptopancreas, reduce growth rate and anorexia. The characteristic lesion of HPV infection is presence of prominent basophilic or eosinophilic intranuclear inclusion bodies associated with cap formation in the hypertrophied nuclei of hepatopancreatic tubular epithelial cells (Lightner, 1996). But the result of this study is quite different, where neither basophilic intranuclear inclusion bodies nor cap formation were observed.

White spots were observed on the cuticle surface of infected shrimps in both, WSSV and IHHNV. In WSSV infection, white spots were only observed on internal and external of surfaces carapace while in IHHNV infection, white plaque observed on the third to sixth abdominal segments. Cowdry type A inclusions were found in infected shrimp with WSSV similar to the appearance of intranuclear inclusions caused by IHHNV. Quere *et al.* (2002) also reported that inclusion bodies of WSSV could be easily mistaken as by of IHHNV but histopathological findings (H and E\Ph) showed that intranuclear inclusion bodies Cowdry type A were as basophilic in WSSV infection while in IHHNV infection observed as eosinophilic (Afsharnasab *et al.*, 2009). Infected hepatopancreas tissues by WSSV showed severe vacuolization that was due to high activity it, but Cowdry type A inclusion intranuclear was not observed.

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