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Identification of Hepatopancreatic Parvo-like Virus (HPV) in Cultured *P. semisulcatus* from the Islamic Republic of IRAN

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Abstract: During the period from August 1997 to March 1998, two thousand samples of cultured *Penaeus semisulcatus* postlarvae and subadults were collected from five hatcheries and 20 growout farms distributed in three provinces along the costs of Persian Gulf and the Oman Sea. Based on growth signs, LM and TEM histopathology, Hepatopancreatic Parvo-like Virus (HPV) were identified from the samples. The HPV particles most often appeared in spherical, but occasionally in angular forms. The particles in hepatopancreatocyte sections, averaged 22- 24nm in diameter.

Keywords: Shrimp, *Penaeus semisulcatus*, Hepatopancreatic parvo-like virus, Histopathology, TEM observation.

Introduction

To date, over 15 viruses have been reported from penaeid shrimps world wide (Lightner, 1996). With respect to *Penaeaus semisulcatus* three viruses (MBV, (*Penaeus Monedon* Baculovirus), HPV and IHHNV (Infection Hypodermel and Hematopeitic Necrosis Virus) have been detected which have became a major limiting factor for development of cultured shrimp industry. *Penaeus semisulcatus* has also been reported to be experimentally susceptible to Baculovirus Midgut gland Necrosis Virus (BMN) (Lightner, 1996). With the rapid expansion of the shrimp culture industry, discovery of other viral diseases seems highly probable (Lightner, 1992).

Hepatopancreatic parvo-like virus is small (22-24nm in diameter) DNA-containing parvovirus. The virus morphology, the close association of the nucleolus with the developing inclusion body, and the presence of intranuclear bodies within developing inclusion bodies are similar to cytopathological features reported for parvovirus infections in insects and vertebrates (Lightner & Redman, 1985; Lightner,1996).

The clinical signs of cultured Penaeid shrimp infected by HPV are poor growth rate, anorexia, reduced preening activities, exoskeleton surface fouling by epicommensals, and occasionally, presumed secondary bacterial and fungal infection (Lightner & Redman, 1985; Lightner, 1988; Lightner, 1996).

Conventional diagnosis of HPV is dependent upon the histological demonstration of single prominent basophilic, Feulgen-positive (indicating a high DNA content), intranuclear inclusion bodies in hypertrophied nuclei of hepato-pancreatic tubule epithelial cells. Consequent lateral displacement and compression of the host cell nucleolus and chromatin margination are also prominent characteristics of HPV-infected cells. Early in their development, HPV inclusions are small eosinophilic bodies centrally located within the nucleus and closely associated with the nucleolus (Lightner & Redman, 1985; Lightner *et al*., 1993). A

rapid field diagnostic method for HPV infection was developed using Giemsa stained impression smears of the hepatopancreas (Lightner *et al*, 1993). A few years later, modern techniques such as the use of gene probes in HPV diagnosis have became available after the partial clone of HPV genome by Mari *et al* (1995). Pantoja *et al*. (2000) presented a new method for detection of HPV directly in fecal samples from HPV-infected shrimps, using PCR method.

The present study was conducted to investigate the status of HPV infection and its effect on shrimp industry in Iran.

Material and Methods

During the study period from August 1997 to March 1998, two thousand specimens of cultured *Penaeus semisulcatus*, postlarvae (1400 specimens) and subadults (600 specimens), were collected from 5 hatcheries and 20 growout farms distributed through three provinces along the coasts of the Persian Gulf and the Oman Sea of the I. R. Iran. During the sampling, case history including major environmental parameters such as pH, salinity and temperature, history of disease and other basic farming data or practices were also recorded.

All the specimens collected were transported in a container equipped with aerators to the Aquaculture Department of the Persian Gulf Fisheries Research Centre (PGFRC) in Bushehr province. The samples were maintained in glass-fiber tanks or glass aquarium with disinfected water for 2-3 days. Prior to use, the tanks and equipments were disinfected with 100ppm benzalkonium chloride for 10 minutes. Tanks were provided with filters and aerators. The shrimp were fed commercial feed pellet or *Artemia*. All shrimps were maintained separately and examined by wetmount microscopy as described by Lightner *et al* (1983a). Routine histological and electron microscopical techniques were applied, as described by Lightner (1996).

One group of samples were examined for histochemical studies and special test,

such as Feulgen reaction and Acridine orange staining (AO) to identify the strand the DNA and RNA as described by Hsiung (1973) and Adamas & Bonamei (1991).

Results

Gross signs and histopathology

High mortality rates, up to 60-70%, were noted in postlarve and juvenile of *Penaeus semisulcatus* infected with HPV in the Abadan Farms, Khuzestan province in spring 1997. In that year, shrimp production recorded a loss of about 700 MT equivalent to about USD15 million. There was no obvious mixed infection. The most striking signs of *P. semisulcatus* affected with this virus were atrophied hepatopancrease (HP), poor growth rate, anorexia, reduced preening activities and consequent increased tendency to surface and gill fouling by epicommensal organisms such as *Zoothamnium* sp. Paraffinized sections stained with Mayer-Bennett H&E revealed that lesions due to HPV infections were localized in HP tubules. No sign of HPV infection was observed in other tissues or organs in the infected specimens examined under light microscope.

Infection by HPV was characterised by deep blue to magenta (basophilic) intranuclear inclusion bodies (IBs) in hepatopancratic tubule epithelium, and especially in E-cells at the distal end of tubules, where mitotic activity was high (Fig. 1). These inclusion bodies begin as small acidophilic (pink) inclusions inside the nucleus, adjacent to the nucleolus. As the inclusion enlarges the nucleus becomes hypertrophied, the chromatin becomes progressively more marginated and the nucleolus is pushed to the side of the nucleus where it sometimes present in the form of small crescent in side view (Fig. 2). In final appearance, the large basophilic inclusion is usually surrounded by an unstained space or empty halo, which separates it from the marginated chromatin. In some cases there was a single inclusion, but two or more were sometimes also found (Fig. 3).

The light microscopic observations revealed the advanced stages of HP

infections in the postlarve and the juvenile of *P. semisulcatus* infected with HPV. The principal feature of the HP lesions observed by light microscopy was the presence of prominent intranuclear inclusion bodies (IBs). These IBs ranged from inconspicuous eosinophilic bodies lying adjacent to the nucleolus and very large usually single, spherical or amorphous, basophilic inclusions that occupied most of the karyoplasm of the hypertrophied nucleus. The IBs were usuallysurrounded by thin empty halo that apparently resulted from shrinkage of the inclusion bodies during the fixation (Fig 3 and 4).

With HPV infection, hemocytic infiltration and encapsulated inflammatory response were seen, along with formation of granulomas (capsules) by numerous hemocytes and layers of fibrous tissue (Fig 5). In advanced stages HPV IBs within the granulomas were disintegrated, this phase, in some samples, was followed by disintegration of the granulomas, nodule formation, melanization and resorption process. The clinical signs of the HPV-infected shrimp were not specific nor age dependent.

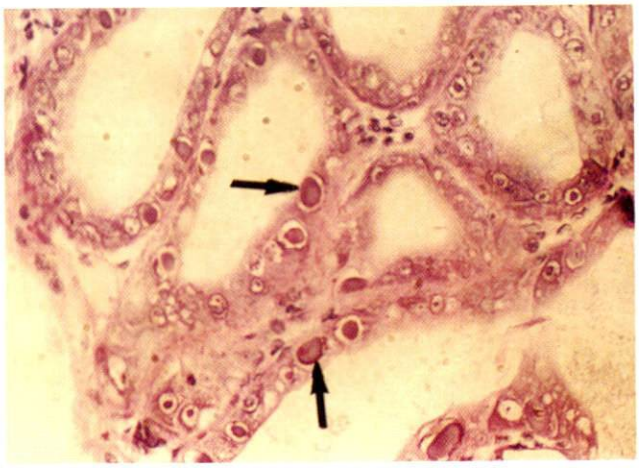

Figure 1: HPV infection in juvenile *Peneaus semisulcatus*. The inclusion bodies characterised by deep blue colour in distal tubule cell (arrows) and especially in E-cell. H&E/phloxine. x 700

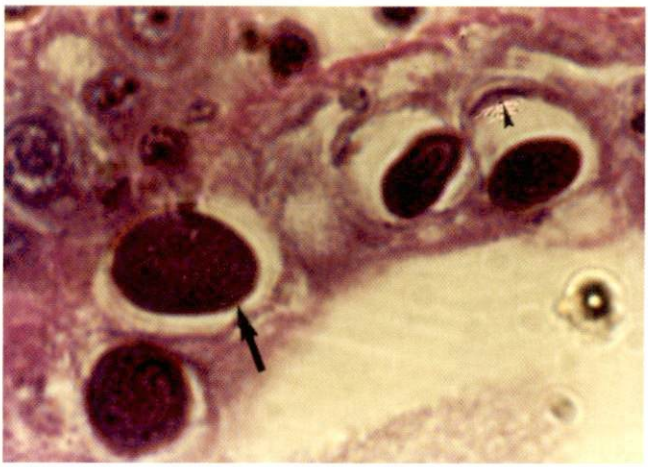

Figure 2: The enlarge inclusion bodies in juvenile *Peneaus semisulcatus* showing the hypertrophied nucleus (arrow). The chromatin becomes progressively more marginated and the nucleolus is pushed to the side of the nucleus where it sometimes presents the form of small crescent in the side (arrowhead). H&E/Phloxine. x 1,800

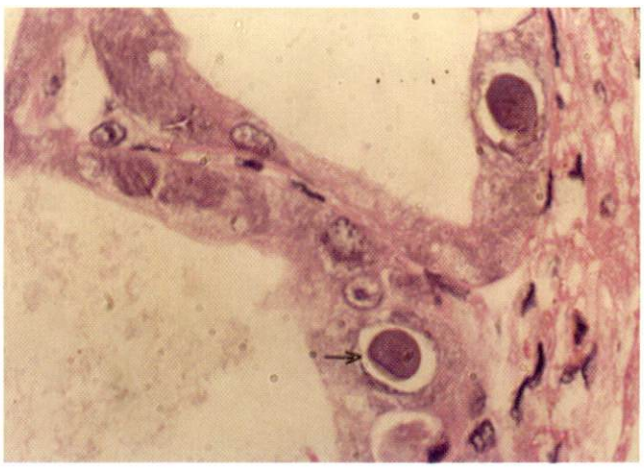

Figure 3: Advance stage of HPV infection showing hypertrophied nucleus, large well develed intranuclear inclusion bodies, basophilic inclusion bodies. Note to the inclusion body surrounded by empty halo space (arrow). H&E/Phloxine. x 1,800

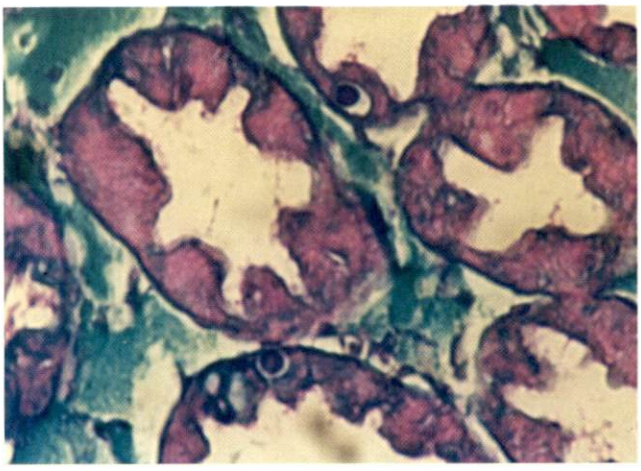

Figure 4: Feulgen staining of inclusion body of HPV infection in *Peneaus semisulcatus*. The violet to red inclusion bodies indicate that it is a DNA viruse. x 700

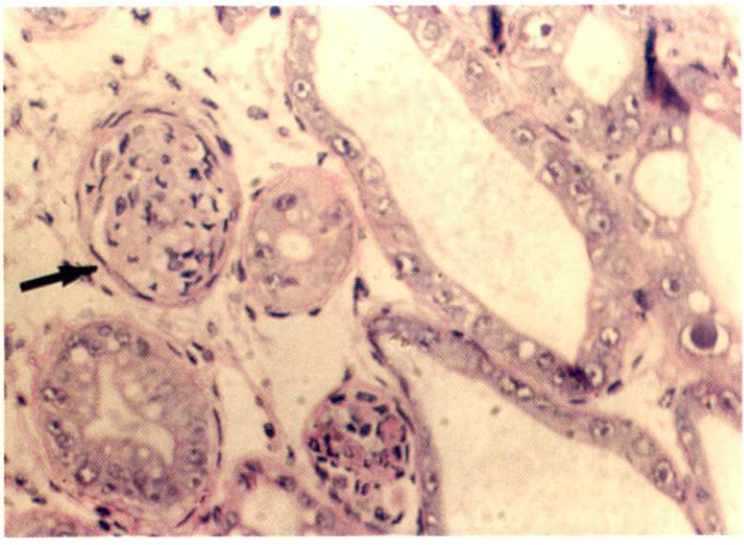

Figure 5: The hepatopancraetic tissue showing granulomas (arrow). H&E/Phloxine. x700

The various stages of HPV infestation were identified in *P. semisulcatus*. Granulomatous reaction, also was seen in the HP tubules of HPV-infected *P. semisulcatus*, indicating secondary infection.

Histochemical Studies

The inclusion bodies were Feulgen-positive and ranged from small inconspicuous, eosinophilic, amorphous bodies lying in the karyoplasm adjacent to the nucleolus in an otherwise normal nucleus (Fig 4), the inclusions stained was violet to red, indicating the presence of DNA. Acridine orange combined with nucleic acids in cells by salt linkages and by other cohesive forces, when examined with a fluorescence microscope (blue light is adequate) resulted in fluoresces green indicating that HPV IB mainly contain single-stranded DNA (Fig 6). The methylene blue stained sections showed hepatopancreas tubules being heavily infected with HPV. Affected cells possessed a single prominent basophilic intranuclear inclusion body in a hypertrophied nucleus (Fig 7).

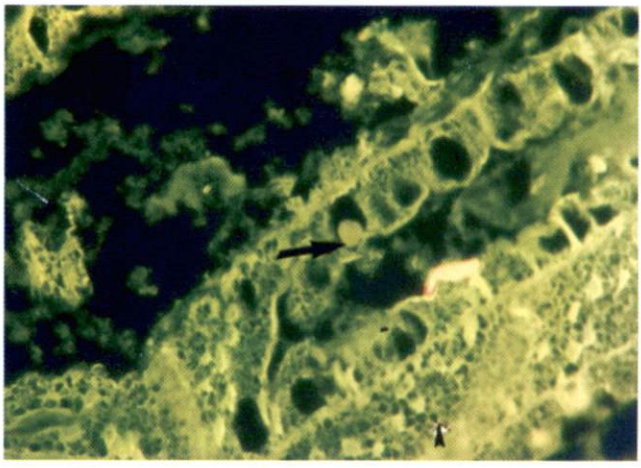

Figure 6: Fluorescent micrograph of AO staining of HPV-infected hepatopancreatic tissue. The intranuclear inclusions were yellow-green (arrow). The normal nuclei are smaller and their nuclear chromatin were displayed as a numerous yellow dots (arrowhead). x700

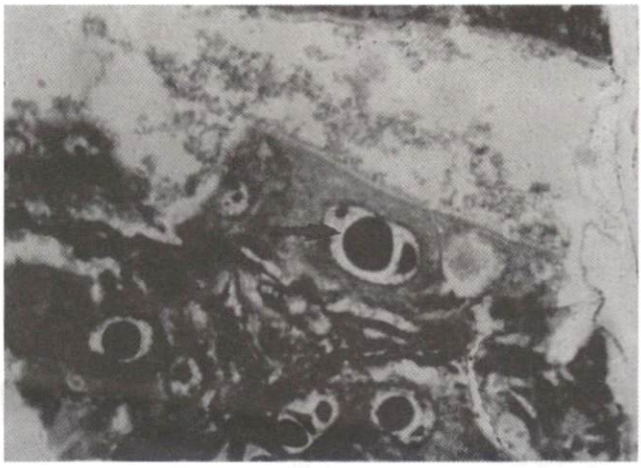

Figure 7: Methylene blue stained section showing hepatopancreas tubules heavily infected with HPV. Dense basophilic HPV inclusion bodies (arrow) are present along with markedly hypertrophied nuclei. x700

Ultrastructural cythopathology and virus morphology

Figures 8 to 11 show the TEM observations on various stages of HPV-infected hepatopancreatocytes. At the first stage, the nucleus of the affected cells were slightly hypertrophied, the chromatin marginated to the nucleus periphery and degenerated into scattered patches and gradually transformed into loose granules and fibrillars. These transformed nucleolar granules (TNG) were specially granulated, and were bigger than the original nucleolar subunits. The low dense nucleolus was enlarged and flattened, to proliferate into spherical particles on its periphery (Fig. 8). At the second stage, the nucleolus was pushed to the periphery of nucleus by growing virogenic stroma, while the degenerated chromatin was still marginated (Fig. 9). At this stage the cytoplasmic changes were displayed by endoplasmic reticulum (ER) dilation and dilated lysozomes appeared as angular viral particles.

During the third stage, severe hypertrophy of the nucleus was observed due to

enlargement of the intranuclear IB. The nucleolus was compressed between the large IB and the nuclear membrane. The nucleolus was in crescent shape. At this stage, the highly degenerated chromatin was still visible (Fig 10). Finally, at the fourth stage, because of the large number of electron-dense virus particles, the virogenic stroma was highly electron-dense. The nuclear chromatin was mostly disappeared. Light microscopy showed that most of the affected hepato-pancreatocytes were degenerated. The cell membrane had completely disrupted and the cellular properties including IBs were separated from the basal membrane where the myoepithilial cells and the associated contractile were fibrillar merged with it (Fig 10).

The developing IB was composed of an electron-dense fine granular material (the virogenic stroma) and viral particles. The profiles of sectioned HPV particles most often appeared spherical, but occasionally particles appeared angular (Fig 11). The particles in hepatopancreatocyte sections, HPV profiles averaged 22-24nm in diameter.

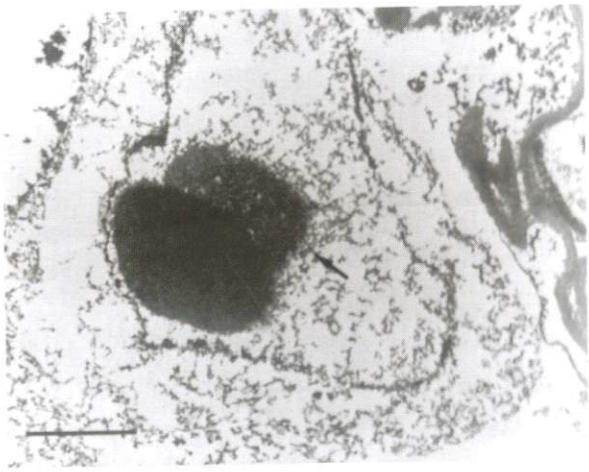

Figure 8: Stage 1 of HPV infection, showing slightly hypertrophied nucleus, and hypertrophied nucleolus (arrow) attached to the marginated chromatin. Lead citrate and uranyl acetate. Scale bar = 3µm

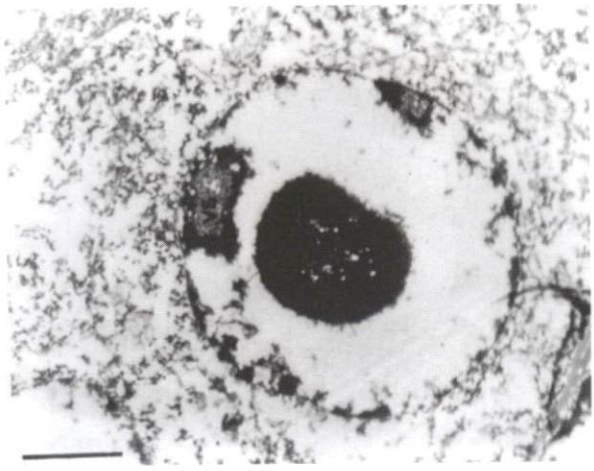

Figure 9: Stage 2 of HPV infection, the nucleolus is pushed to the periphery of nucleus by the growing virogenic stroma (VS). The virogenic stroma of the IB appeared to be mottled. Lead citrate and uranyl acetate. Scale bar = 3μm.

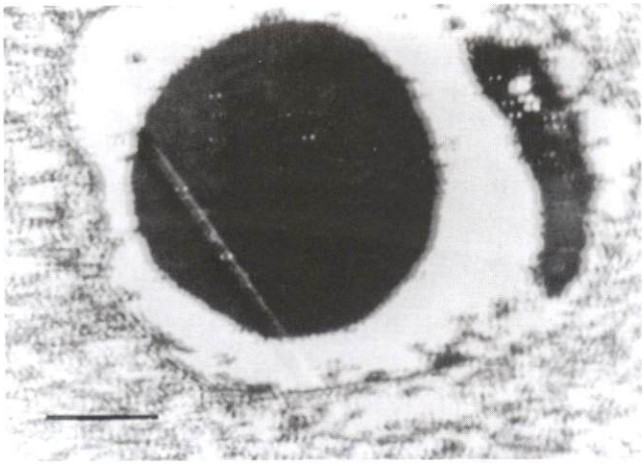

Figure 10: Electron micrograph of stage 3 of HPV infection, marked hypertrophy of the nucleus due to enlarged intranuclear IB. The nucleolus was compressed between the large IB and the nuclear membrane. Lead citrate and uranyl acetate. Scale bar = 1.9μm

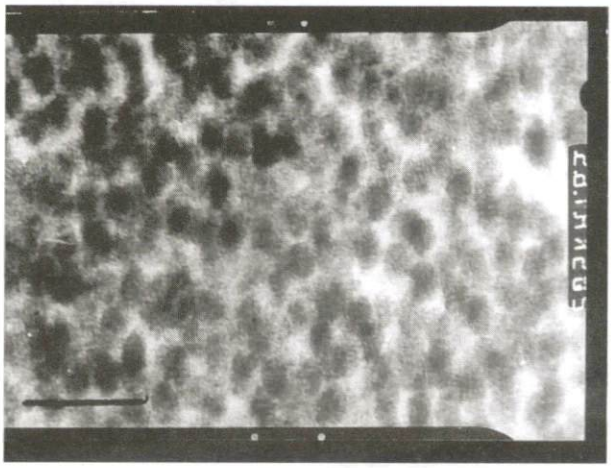

Figure 11: High magnification of HPV particles. Virions are usually spherical with a capside, but some profiles are clearly angular. Lead citrate and uranyl acetate. Scale bar = 136 nm

Discussion

Hepatopancreatic parvo-like virus (HPV) related diseases are reported in *Penaeus chinensis (orientalis)* from China, *P. merguiensis* from Singapore, *P. semisulcatus* from Kuwait and *P. monodon* from the Philippines (Lighter & Redman, 1985). Its natural host range also includes *P. vannamei, P. esculentus, P. indicus, P. stylirostis, P. schmitti, P. japonicus* and *P. penicillantus* and its known geographical distribution corresponds to the Indo-Pacific area (China, Korea, Taiwan, Philippines, Malaysia, Singapore, Indonesia, Thailand and Australia) Africa (Kenya), the Middle East (Israel and Kuwait), and the America (Brazil, Ecuador, Mexico and Hawaii) (Lightner & Redman, 1992; Lightner, 1993; Lightner, 1996). With the wide distribution of HPV, the present finding on the occurrence of HPV in the I. R. Iran is, thus, not surprising. Since there are no imports of *P. semisulcatus* into Iran and all brood stocks used for production of post larvae are collected from the Persian Gulf, it is highly likely that the wild

caught shrimp are the source of HPV infection. It will be interesting to investigate the prevalence of the infection in wild populations.

Lightner and Redman (1985) reported that the gross signs of HPV infection included atrophy of the hepatopancreas, poor growth rate, anorexia, reduced preening activity and consequent increased tendency for surface and gill fouling by epicommensal organisms, and secondary infections by opportunistic pathogens like *Vibrio* spp. and *Fusarium solani*. Mortalities accompanied by these signs most often occur in the juvenile stages. These observations were similar to our findings for HPV infection in *P. semisulcatus*. Secondary infection with*Zoothamnium* sp. and epicommensal gill fouling was found in the present study indicating that shrimp infected by HPV was susceptible to opportunistic organisms. These findings were also similar to the findings of Mallolahy (1988) and Majdynesab (1998) who worked on *P. semisulcatus* sp. and found *Zoothamnium* sp.and *Vibrio* sp.

The light microscopy findings for HPV infection were similar with the findings of Lightner and Redman (1985) who reported the presence of prominent intranuclear inclusion bodies in hepathopancreatic tubule epithelial cells. These inclusion bodies (in paraffin section) were often surrounded by a thin empty halo.

In the present study, the developing inclusion bodies were similar to the immature viral particles (empty capsids) in terms of particle sizes. These findings suggest that the nucleolus material possibly took part in the viral replication process, in which the transformed particles derived from nucleolus might provide the viral structural precursors most likely as procapsids. Similar findings were seen in insects infected with a densovirus of Parvoviridae, in which the nucleoli underwent hypertrophy that was accompanied by a segregation of its fibrillar and granular components and finally disappeared completely. The phenomenon was regarded to be associated with the viral replication and transcription (Tijssen & Arella, 1991).

Bonami & Lightner (1991) and Lightner (1992) showed four crustacean paroviruses: infection hypodermal and hematopoietic necrosis virus (IHHNV), Lymphoidal parvo–like virus (LPV), Hepatopancreatic parvo–like virus (HPV) and PC84 virus. The first three are detected from penaeid shrimps and PC84 virus from the crab, *Carcinus mediterraneus*.

Hepatopancreatic parvo-like virus differs from the other penaeid viruses or virus –like structures and was thus suggested to be parvoviruses by Foster*et al.* (1981) and Lightner *et al.* (1983b). IHHNV induces prominent Cowdry typeA eosinophilic, usually Feulgen-negative intranuclear IBs in the tissue of ectodermal and mesodermal origin (Lightner *et al.*, 1983_b; Bell and Lightner, 1984; Bonami *et al.*, 1990), while, LPV induces unique eosinophilic to basophilic spherical intranuclear IBs in the affected cells, and the infected shrimp exhibit multinucleated giant cell in hypertrophied lymphoid organ (Owens *et al*, 1991). On the other hand, PC84 virus is present at both the nucleus and cytoplasm of connective tissues in the crab, *C. mediterraneus* (Mari & Bonami, 1988; Bonami & Lightner, 1991; Mari *et al.*, 1995). Thus, it is obvious that the virus found in the present study differs from IHHNV, LPV and PC84 virus. However, it resembles HPV in terms of histopathology, viral morphology and nucleic acid contents. The similarities include the following aspects: (1) This virus causes single prominent basophilic, Feulgen-positive intranuclear IB only in the hepatopancreatic tubule epithelial cells, which are the characteristic of HPV (Lightner & Redman, 1985); (2) Both viruses are spherical to icosahedral shaped particles with similar size, e.g. 22-24 nm for HPV (Lightner & Redman, 1985) and 22-24 nm in the present study, and (3) They possess the same nucleic acid content of ssDNA. Therefore, the virus found in the present study is classified as hepatopancreatic parvo-like virus (HPV), under the family Parvoviridae. In this context, this report confirms the presence of HPV in the cultured *P. semisulcatus* in Iran.

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under the family Parvoviridae. In this context, this report confirms the presence of HPV in the cultured *P. semisulcatus* in Iran.

Parvoviruses are known from vertebrates and invertebrates, and the available information on the invertebrate parvoviruses has been reviewed by Kelly (1981). HPV displays similarities in the cythopathology in shrimp hepatopancreatocytes with those reported for densonucleosis virus (DNV) in *Galleria mellonella* (Lepidoptera) larvae (Kurstak *et al.*, 1977), and canine parovirus (CPV) (Paradiso *et al.*, 1982). The principal similarities of HPV and its cytopathology to DNV and CPV includes particle diameter of 22-24 nm, DNA content (from Fuelgen reaction), intranuclear replication in a virogenic stroma that is closely associated with host cell nucleolus, and the presence of intranuclear bodies and microfibrils in the virogenic stroma (Lightner & Redman, 1984).

In the present study, HPV was also identified in *P. semisulcatus* by methylene blue staining, and found to be similar in appearance to HPV infected cells as demonstrated in routine H&E stained paraffin sections. Mature HPV intranuclear inclusion body was basophilic in both methylene blue staining and H&E staining. Because the H&E methods is quite labour intensive technique, the methylene blue staining technique is a rapid method that provides relatively good diagnostic tool to the detect HPV infections and, therefore is recommend for rapid diagnosis in hatcheries and farms. However, this technique should be used cautiously, since the number of inclusion bodies is low during the early stages of infection, so there is a possibility that these may not be detected using investigation with methylene blue. This technique could, however, be used with confidence in heavily infected specimens.

In the present study the occurrence of hemocytic infiltration, encapsulation and melanization without the presence of other pathogen, indicate that these response were probably caused by HPV infection. In addition, histopathology revealed that some of the heavily HPV-infected individuals did not show any moribund sign. All

reason why the HPV-infected shrimp showed no acute mortality in growout ponds Nevertheless, histopathology revealed that severe HPV infection could induce extensive necrosis and collapse of hepatopancreatic tubules. The disfunction of the organ is indicated, and lethal prognosis should be an expected event in association with poor environmental factors. Previous reports on HPV infection by Lightner & Redman (1985) and Lightner *et al.* (1993) did not mention the presence of infiltration responses.

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