Iranian Journal of Fisheries Sciences 4(1), 25-44 (2004)

Identification of *Penaeus monodon* Baculovirus (MBV) in Cultured *Penaeus semisulcatus* in Islamic Republic of Iran

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Abstract: Shrimp aquaculture has a short history in I.R Iran. Farmed shrimp production grew slowly from 1992 until 2000 when a number of large farms started to produce. In 2000, the total production was about 4,500 MT and it was estimated that more than 7,000 MT would be produce by the year 2001. From August 1997 to March 1998, two thousand samples of cultured *Penaeus semisulcatus* postlarvae and subadults were collected from 5 hatcheries and 20 growout farms, distributed in 3 provinces along the coasts of the Persian Gulf and the Oman Sea. Based on, gross signs, histopathology, LM and TEM, *Penaeus monodon* baculovirus (MBV) from samples is recorded. The MBV is a rod-shaped baculovirus, DsDNA virus with the virion diameter of 300±75 nm. The target organ of virus is hepatopancreas and midgut epithelium.

Key Words: Shrimp, *Penaeus semisulcatus*, *Penaeus monodon* baculovirus, Histopathology, TEM observation.

Intoduction

Since 1992, farms for shrimp culture have established, after the Kolahi shrimp development and Training center was established in Hormozgan province as a resulte of an earlier UNDP-funded project. In Iran, the growout earthen shrimp ponds are usually, 0.7 to 1 ha and 1 to 1.5 m in deep. The seawater supply for the

farms is either pumped or flows in by natural tidal flow. Ponds are rarely equipped with aerators and the initial stocking density of postlarvae (PL12) is usually between 200,000 to 220,000 per hectare. The average production per hectare is 1,500-2,000 kg.

To date, over 15 viruses have been reported from Penaeid shrimps worldwide (Lightner, 1996). With respect to *Peneaus semisulcatus* three viruses (MBV, HPV, IHHNV) have been detected which have become major limiting factors for development of shrimp cultivation industry. *P. semisulcatus* has also been reported to be experimentally susceptible to BMN (Lightner, 1996).

Penaeus monodon baculovirus (MBV) is a rod–shaped virus possessing a relatively thin multilaminar envelope around a dense nucleocapsid (Lightner & Redman, 1981). The virions are 75 ± 4 nm in diameter and 324 ± 33 nm in length (Lightner *et al.*, 1983). Presumably, DNA of the MBV virus is consistent in morphology with members of the subgroup A, genus *Baculovirus* (Brock *et al.*, 1983; Lightner *et al.*, 1983). *Penaeus monodon* baculovirus was first reported in Mexico affecting shrimp imported from Asia. It was also detected in hatcheries located in the Indo-Pacific, Mediteranean, The Philippines, Taiwan, Malaysia, Tahiti, Singapore, Kuwait, Kenya, Israel and Italy (Villasante & Puent, 1993; Lightner, 1996). Often with severe mortality, MBV infects hatchery reared larval, postlarval, juvenile and adult of *P. monodon, P. merguiensis, P. semisulcatus, P. indicus, P. plebejus, P. pencillatus, P. esculentus*, *P. kerathurus* and possibly *P. vannamei* (Lightner *et al.*, 1983; Johnson & Lightner, 1988; Lightner, 1996). The clinical signs of MBV disease include lethargy, anorexia, and suppressed preening activity. The affected shrimps show retarded growth, grey blue to bluish black coloration, shell disease and microbial epibiotic fouling (Brock *et al.*, 1983).

Material and Methods

During the period of August 1997 to March 1998, two thousands of cultured *Penaeus semisulcatus* postlarvae (1400 specimens) and subadults (600 specimens) were collected from 5 hatcheries and 20 growout farms distributed in 3 Iranian provinces along the northern coasts of the Persian Gulf and the Oman Sea. During the sampling, major environmental parameters, such as pH, salinity and

temperature, as well as history of diseases and other basic farming data or practices were recorded.

Samples collected were transferred into containers with aerators to the Aquaculture Department of the Persian Gulf Fisheries Research Center (PGFRC) in Bushehr province. The samples were maintained in fiber glass tanks (300 L) or glass aquarium (100 L) with disinfected water for 2-3 days. Prior to use, the tanks and equipments were disinfected with benzalkonium chloride at dose rate of 100 ppm for 10 minutes (LeBlanc & Overstreet, 1990). Tanks were provided with filters and aerators. The shrimps were fed with commercial feed pellet or *Artemia*. All shrimps were maintained separately and examined as follow:

Wet- mount microscopy and gross examination of live shrimp: Ten to 20 animals were examined by wet-mount microscopy. Fresh squash smear of mainly hepatopancreas (HP), lymphoid organ (LO), gills, appendages, faeces and body surface especially external foci, were observed under microscope. In addition, 0.05% aqueous solution of malachite green was often used for enhancing the examination (Lightner *et al.*, 1983). Preliminary diagnosis was made for viruses, which can induce prominent dense inclusions. In addition, gross examination for clinical signs, multiple infections with other pathogens were also observed by this procedure.

Histopathological and histochemical techniques:

Larval and early post larvae were directly immersed in the Davidson's fixative, while Juveniles and adult shrimps were injected with 1–10mL (depending on the size of shrimp) of Davidson's fixative into hepatopancreas, region anterior to hepatopancreas, anterior abdominal and posterior abdominal regions. A large share of fixatives was injected into the cephalothorax region and posterior abdominal region. The amount of fixative used varied from 5–10% of body weight. After the injection, the cuticle from sixth abdominal segment to the rostrum was cut with a sharp scissors, without damaging the internal organs. The specimens were immersed in 10 volumes of fixative (i.e., tissue of 1 mL volume require) for 24 hr. For large animals (up to 20 gr.), tissue was fixed up to 72 hr. After fixation tissue was transferred to 50% ethyl alcohol for storage. The fixed tissue was processed through normal routine methods and embedded in paraffin and sectioned at 5-6 μm

and stained with hematoxylin and eosine/phloxine (H&E/Pheloxine)(Bell & Lightner, 1984; Adams & Bonamei, 1991).

Section from the blocks, which had shown viral infections in H&E/Phloxine staining, were also utilized for the histochemical examination. Acridine orange (AO) staining combined with DNAse & RNAse digestion methods was used for recognition of single stranded (Ss) and double stranded (Ds) DNA or RNA nucleic acid types of viruses (Luna, 1968; Disbrey & Rack, 1970; Hsiung, 1973, Adams & Bonami, 1991). Preparations of AO stained specimens were viewed under a fluorescent microscope (Nikon Fluophot) with blue excitation filter at 495-nm wavelength.

Transmission electron microscopy:

Ten specimens were subcollected to prepare ultrathin section for virus morphological and ultrastructural cytopathology studies by using TEM. Specimens were injected with cold 4% glutaraldehyede in 0.2 M cacodylate phosphate buffer (pH 7.4) and then the cephalothorax was dicected into small pieces (1-2 mm^3) which would be representatives of LO, HP, gills, midgut, epidermis with associated cuticle, as well as other tissues or organs. They were fixed in the same fixative at 4°C overnight, postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1-2 hours. The tissue was then dehydrated in graded acetone and embedded in the resin mixture. Approximately 1-µm thick sections were prepared from each tissue block and stained with 1% methylene blue for approximately 1 min at 60°C. Blocks showing the desired degree of tissue destruction and/or the presence of nuclear inclusion bodies were then thin sectioned (50-80) nm using diamond knives on a ultramicrotome. Sections mounted on a copper grid were stained with uranyl acetate and lead citrate (Hayat, 1986; Adams & Bonami, 1991). The prepared sections were viewed under CM 10 Philips transmission electron microscope at Electron Microscope Unit (EMU), Faculty of Veterinary Medicine, Shiraz University.

Virus identification was based on the gross signs, histopathological and cytopathological characteristics of the target tissues, virus morphology and its biochemical reaction during examination.

Results

Gross Signs and Histopathology

In juveniles of *P. semisulcatus* affected by MBV, partial anorexia, observation of dead shrimp in feeding trays, and rise of large numbers of lethargic shrimps to the water surface may been seen. In the affected ponds, dead shrimps were also seen to sink along the perimeter of the dikes. Gross observations showed black appearance of gills in the moribund shrimps.

Histological investigations, using H&E/Phloxine stained specimens, did not show any pathological signs of MBV in any other organ except the HP and anterior midgut. MBV was seen to spread quickly, resulting in high larval mortalities. Effect on adults were less severe. In serious infections, the virus ruptured hepatopancreatic cells, allowing occlusion bodies to pass through the midgut and excreted in the feces. The staining of hepatopancreas and feces with 0.05% aqueous malachite green showed occlusion bodies (OBs) under light microscope (Fig. 1).

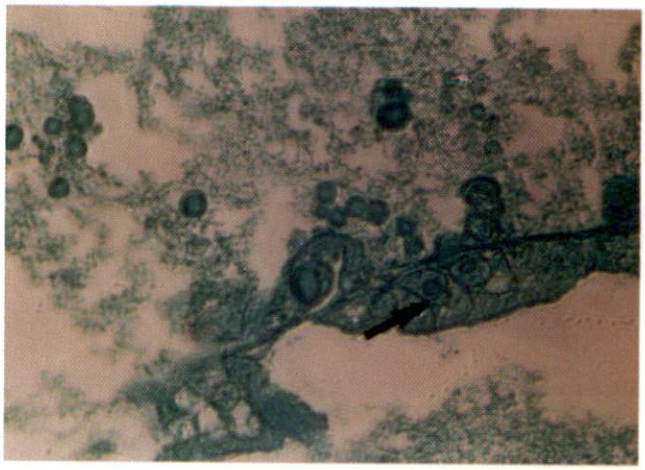

Figure 1: Wet mount view of tissue squash preparation of the hepatopancreas (HP) from *Penaeus semisulcatus* post larva with MBV infections. Some of the HP cells display multiple generally spherical intranuclear occlusion bodies (arrow) that are diagnostic for MBV. 0.05% malachite green. X.1,800

Characteristic eosinophiliic intranuclear inclusion body (so called occlusion bodies) in hepatopancreas cells, without significant tissue damage or only with mild to moderate vacuolated degeneration was seen in the early stage (Fig 2). The

basophilic accumulation often had one or two thicken areas, the larger of which may have been the remains of the nucleolus. The size of HP was greater than normal size. Thus the profile of the nucleus was given a signet ring appearance (Fig. 3). The nucleus presented a weeding ring appearance when the deep blue compressed nucleolus was cut in side view like a signet stone set in the blue, circular band of marginated chromatin.

The intermediate or second stage had much the same appearance, but in these the nucleus was more hypertrophied and contained one or more weakly eosinophilic occlusion bodies, presumed to be in an early stage of development. The cytoplasm revealed increased numbers of free ribosome's, proliferation of membranous labyrinth membranes (ML), and formation of polyhedral at or near the endoplasmic reticlum (Fig. 4). Cells in the third stage had greatly hypertrophied nuclei with several larger eosinophilc occlusion bodies. The cytoplasm of such cells was often more basophilic than the normal cells, and was markedly reduced due to hypertrophy of the nucleus. The occlusion bodies from stage 3 cells could be easily demonstrated in squashed tissue using phase or bright field microscopy (Fig 4 and 1). In infected shrimp, free polyhedral occlusion bodies released from lysed, infected cells could be found in the lumens of the hepatopancreatic tubules (Fig 5).

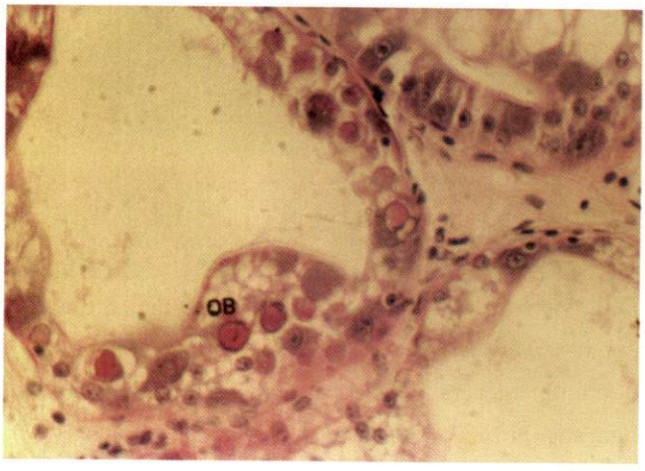

Figure 2: Hepatopancrease of *Penaeus semisulcatus* with MBV infected cells in the developmental stages (stage 1) and (stage 2). Developing occlusion bodies (OB) are visible in the nuclei. H&E/Phloxine. X. 700

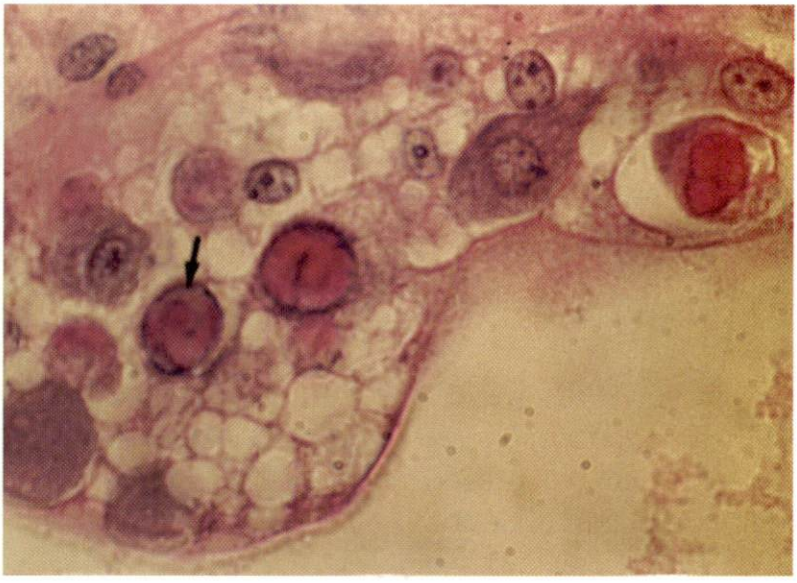

Figure 3: Hepatopancreas of *Penaeus semisulcatus* infected with MBV. The profile of the nucleus was a signet ring appearance(arrow). H&E/Pheloxine. X. 1,800

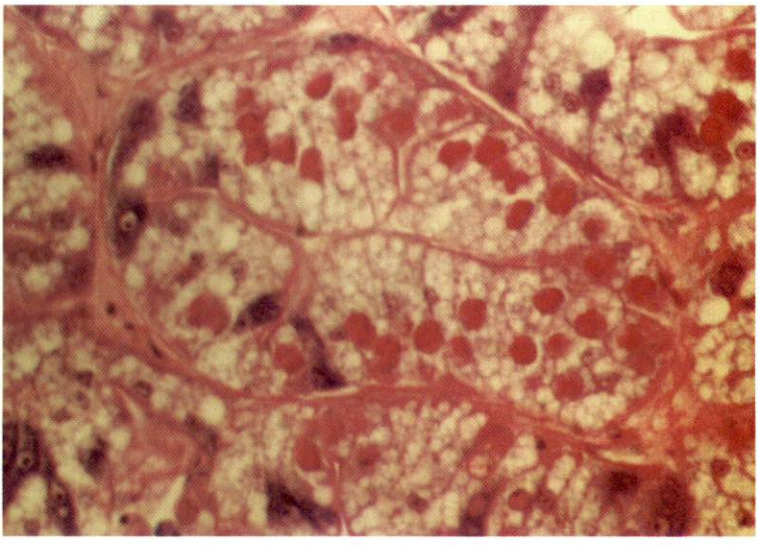

Figure 4: Hepatopancreas cells of *Penaeus semisulcatus* in advanced (stage 3) MBV infection. Nuclear changes include increased hypertrophy appearance of many growth of occlusion bodies. H&E/Pheloxine. X. 1,800

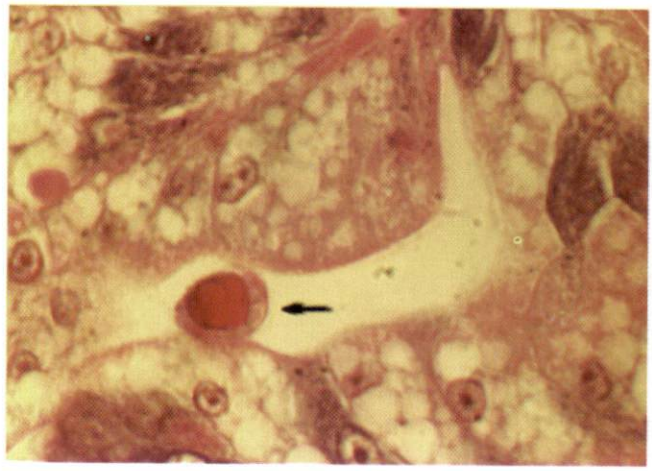

Figure 5: Hepatopancreas (HP) of *Penaeus semisulcatus* with MBV infected cells. The free polyhedral occlusion were released from infected cell in the lumens (arrow) of the HP. H&E/Pheloxine.X.700

Histochemical Studies

Methylene blue stained sections showed typical histopathology of MBV, with enlarged nuclei thinly surrounded by cytoplasm or disintegrating cytoplasm. Infected nuclei examined in this study contained 1 to 5 occlusion bodies (OBs) (Fig.6). Acridine orange staining by fluorescent microscope showed the karyoplasm and OBs in yellow-green color, the cytoplasm was orange-red and the lumens in black (Fig. 7).

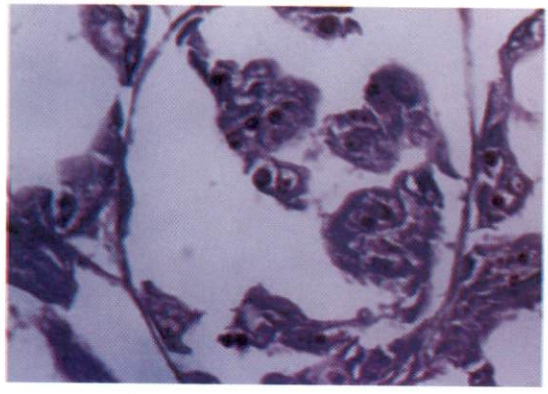

Figure 6: Methylene blue stain hepatopancreas cell showing typical histopathology of MBV infection, with enlarged nuclei thinly surrounded by cytoplasm or disintegrating cytoplasm. .X 700

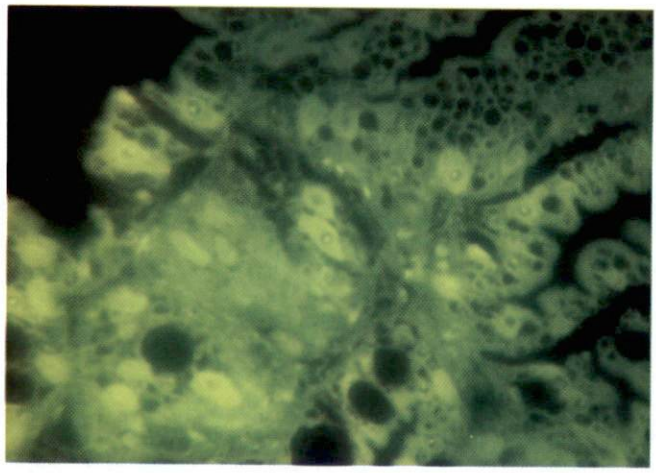

Figure 7: Acridine orange stained section hepatopancreas cell observed by fluorescent microscope showing the karyoplasm and OB in yellow-green. The cytoplasm is in orange-red and lumens are in black. X. 1,800

Ultrastructural Cytopathology and Virus Morphology

Transmission electron microscopy (TEM) observations showed three stages in the pathogenesis of MBV disease in the affected hepatopancreatocytes (HPCs). In the first stage nuclei of the affected hepatopancreatic epithelial cell were hypertrophic. Some of the chromatin disintegrated into lucent granular and fibrillar stroma in the central area, while the rest marginated to the nuclear border into an electron-dense zone. The electron-dense zone at the nuclear margin subsequently transformed into a vacuolisation form, where the vacuoles mixed together with the virogenic stroma, microfilaments and occasionally some virus particles (Fig. 8). A proliferation of smooth endoplasmic reticulum (ER) was noted in the cytoplasm of stage 1 cells, especially within foci forming a membranous labyrinth (Fig. 9). Also noted was a decrease or absence of secretary granules, vacuoles, and lipid storage droplets. The cytoplasm and associated organelles of stage 1 cells otherwise retained a normal appearance.

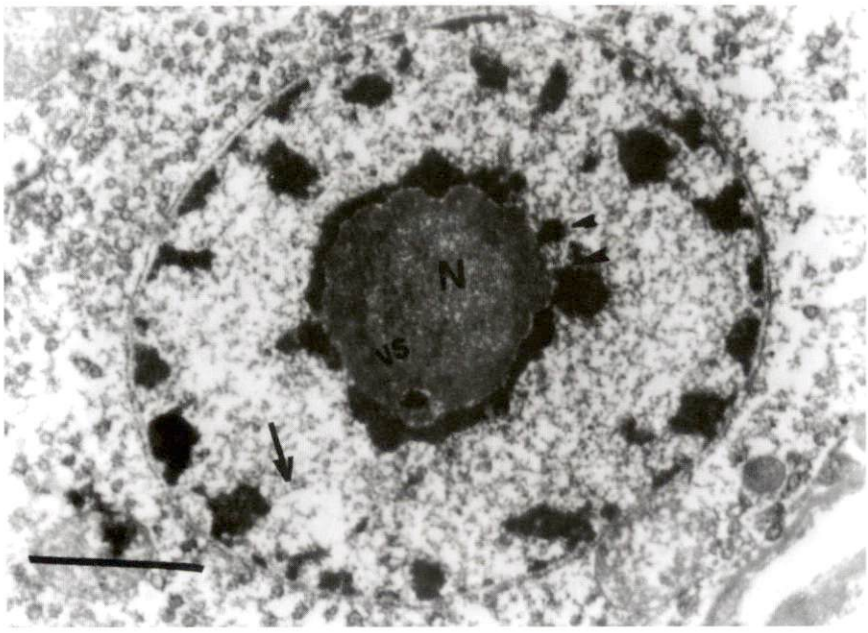

Figure 8: Electron micrograph of stage 1 MBV infected hepatopancreatocyte. The nucleus is slightly hypertrophied, has a central rarefaction of chromatin, some chromatin margination and a peripheral migration of the nucleolus (N). The cell has a signet-ring appearance. The electron-dense zone formed by marginated chromatin, which subsequently transformed into a vacuole (arrows). Note the dense virogenic stroma (VS), few viral particles (arrowheads), and electron-lucent granular and fibrillar stroma in the central area of the nucleus. Lead citrate and uranyl acetate. Bar: 1.9μm.

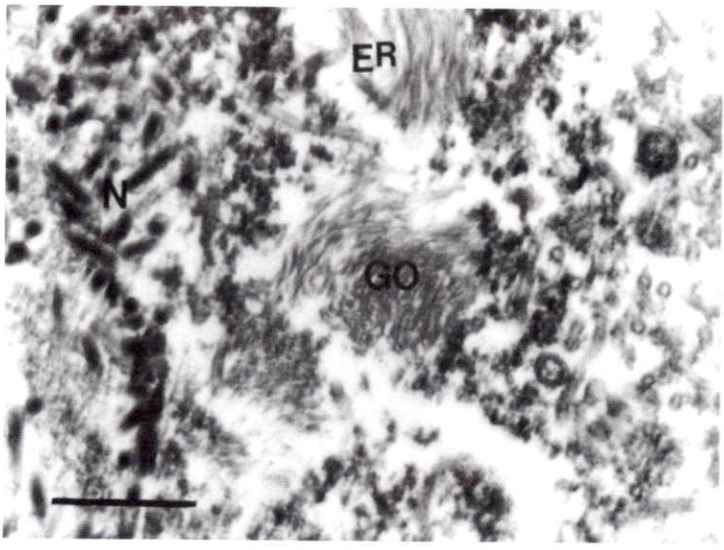

Figure 9: Electron micrograph of hepatopancreatocyte with stage 1 MBV infection. Early stages of virus are evident in the nucleus (N). The cytoplasm contains a conspicuously enlarged Golgi complex (GO) which appears to be the origin of membranes of a granular endoplasmic reticulum (ER). Lead citrate and uranyl acetate. Bar:500nm.

In intermediate stage of infection (stage 2) cytoplasm revealed increased number of free ribosome's, proliferation of membranous labyrinth membranes and formation of polyhedra at or near the ER. Nuclear changes included increased hypertrophy, appearance of many enveloped virions, and appearance and growth of occlusion bodies to occlude many mature virions (Fig. 10 and 11). In cytoplasmic changes mitochondria were swollen with broken crests, and then become autophagic vesicles, which had residuals of the crests. Increase of free ribosome's, loss of ribosome's attached to ER, and most strikingly, proliferation of membranes of the cytocavitary system to from massive membranous labyrinth that eventually lead to complete loss of cellular integrity and structure, and eventual lysis or fragmentation of infected cells were seen in cytoplasmic changes (Fig. 11).

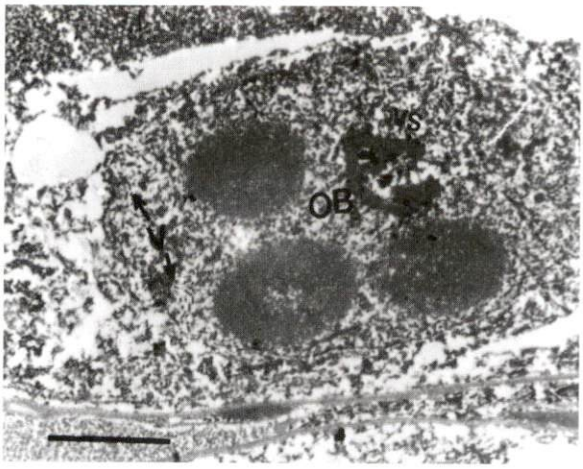

Figure 10: A high magnification electron micrograph of the nucleus of stage 2 MBV-infected hepatopancratocyte. Developing occlusion bodies (OB), membrane of envelop material and empty capside are present centrally, and more peripherally located are denser areas of virogenic stroma (VS) with associated complete virions (V).Lead citrate and uranyl acetate. Bar: 1.9μm.

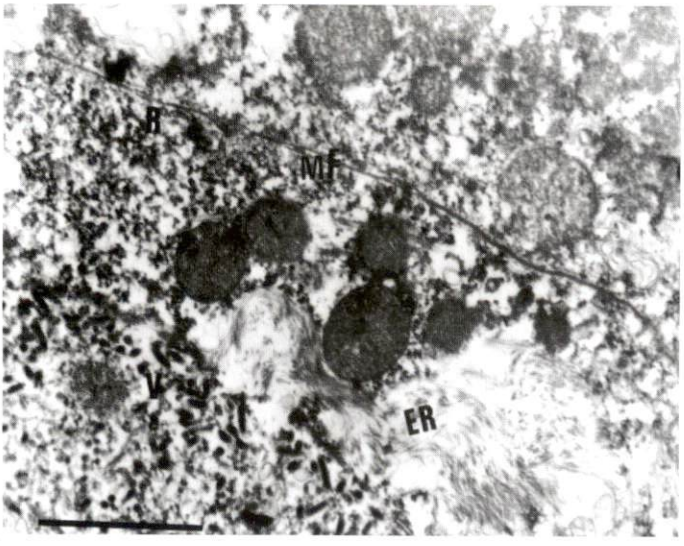

Figure 11: Electron micrograph the basal portions of stage 2 MBV. Numerous virus (V) bodies are present, especially near membranes of the endoplasmic reticulum (ER). Free ribosome (R) are present as patches of microfilaments (MF). Lead citrate and uranyl acetate. Bar: 1.3 μm.

In stage 3 or advance infection of MBV the nuclei were completely hypertrophied and filled the capacity of the cells, leaving a thin layer of cytoplasm. There were several large matured OBs. This was followed by a marked increase in cytoplasmic density caused by super abundance of free ribosome's, and the presence of numerous bundles of microfilaments and large membranous labyrinth. Completed virions were abundant, both occluded and free within the nucleus, especially in those perinuclear areas where the virogenic stroma had been the dens in the earlier stages (Fig. 12).

Typically complete virus particles, were common in the occlusions. Occluded virions were not oriented to the axes of the polyhedrin arrays and seemed to be embedded in random fashion in the matrix (Fig. 12). Virions that were free in the nucleoplasm also showed no pattern of orderly organization into clusters, arrays, etc. The virions, both free in the nucleoplasm or occluded within the inclusion bodies, were rod shaped, and possessed a relatively thin multilaminar envelop around a dense nucleocapside. The nucleocapsid of the virions measured 38 ± 5 nm in diameter by the 246 ± 18 nm in length and virions average 74 ± 6 nm in diameter by 324 ± 25 nm in length (Fig. 13 and 14).

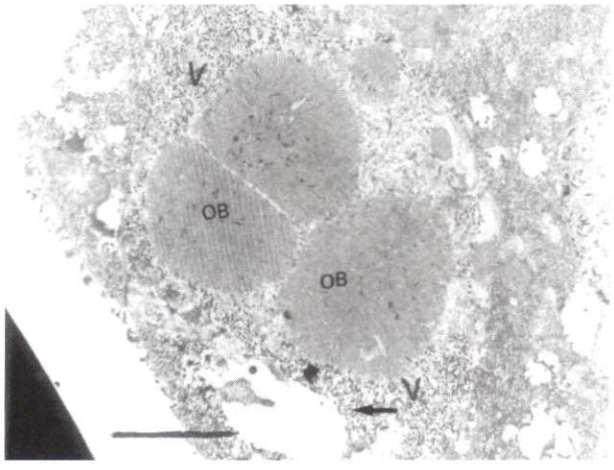

Figure 12: Electron micrograph of stage 3 of MBV infected hepatopancreatocyte. Conspicuous occlusion bodies (OB) and masses of free virions (V) are present in the markedly hypertrophied nucleus of the infected cell. The thin ring of cytoplasm is made dense by markedly numerous free ribosome's. Lead citrate and uranyl acetate. Bar: 1.9μm.

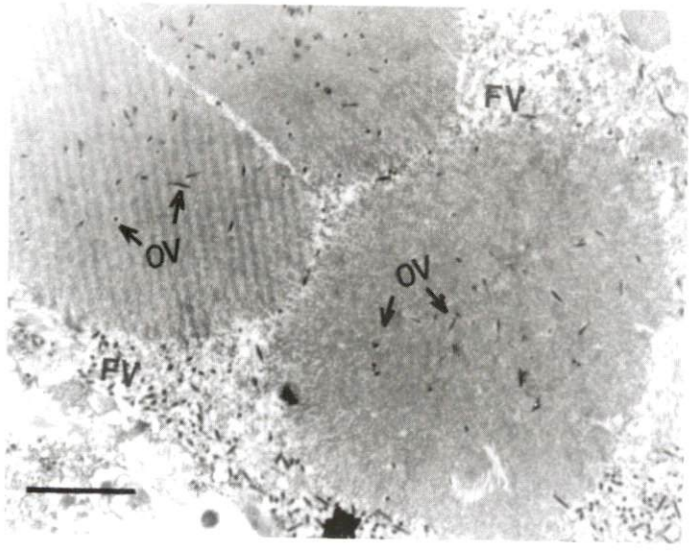

Figure 13: A high magnification electron micrograph of a stage 3 MBV infection. Both free (FV) and occluded (OV) virions are shown. Occluded virions show no orientation to the axes of the paracrystalline structure of the occlusion body. Lead citrate and uranyl acetate. Bar: 2μm.

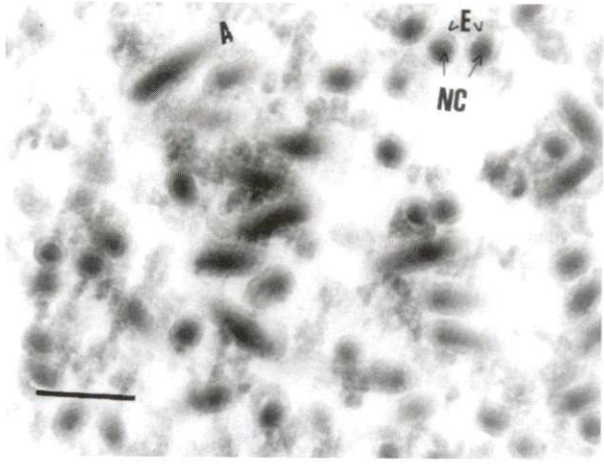

Figure 14: Electron micrograph of high magnification of an aggregation of non-occluded MBV. Evident in both cross and longitudinal sections of the virions is a dense nucleocapsid (NC) and an outer envelope (E). Apical protrusions (A) of envelope material is clearly shown on a number of the virions in longitudinal section. Lead citrate and uranyl acetate. Bar: 270nm.

Discussion

The baculoviruses are classified in the family Baculoviridae, which is divided into three subgroups: 1) nuclear polyhedrosis viruses (NPVs), 2) granular virus (GV), and 3) nonoccluded baculoviruses. From subgroup NPVs two baculoviruses have been reported for shrimps and prawns: *Baculovirus penaei* (BP) and *Penaeus monodon* baculovirus (MBV) (Couch, 1991).

Lightner (1996) reported two strains of MBV: 1) MBV-type (which is now known as PmSNPV) viruses are type-A occluded baculovirus (for single enveloped nuclear polyhedrisis virus from *P. monodon*) or MBV and 2) PBV (plebejus baculovirus). The first type is reported from the Indo-Pacific and S. E. Asia from *P. monodon, P. indicus* and *P. merguiensis* and the second type is reported from Australian shrimp. Furthermore Sano *et al.* (1981) reported a type-C baculovirus from *P. japonicus* from Japan and Korea and named it baculovirus midgut (hepatopancreas) gland necrosis virus (BMNV) or Type C Baculovirus (TCBV).

MBV was first identified in *P. monodon*, causing moderate to high mortality rates in infected postlarvae, juveniles and adults (Lightner & Redman 1981; Lightner *et al.*, 1983). Since the latter report, MBV or MBV-like infections have been diagnosed in samples of cultured postlarvae and juveniles *P. merguiensis, P. semisulcatus, P. indicus, P. esculantus, P. merguiensis, P. plebejus, P. penicillantus* and *P. kerathurus*, and possibly *P. vannamei* (Lightner, 1996).

In the present study the histopathology, viral morphology, TEM observation and gross signs of MBV infected shrimp from Iran was similar to those of the type of MBV reported by Lightner *et al.*(1983) from S. E. Asia. The principal clinical sign of MBV was the presence of a single or multiple, generally spherical occlusion bodies in the hepatopancreas and midgut epithelial cells. MBV can cause moderate to severe infection of the hepatopancreas and anterior midgut of all stages (except egg, nauplius, and protozoea 1 and 2 stages) of susceptible penaeids. Serious epizootic in the larval, postlarval, and/or juvenile stages of *P. monodon* have been linked to MBV (Lightner, 1996).

Penaeus monodon baculovirus found in our study was a rod-shaped baculovirus, DsDNA virus and the virion size is 300±75 nm in diameter. This virus, which is typically different from the other shrimp baculovirus such as BP and BMN. *Baculovirus Penaei* (BP), the first to be described in a non insect host, produces no gross signs of infection in adult or larval penaeid shrimps. However, characteristic tetrahedral occlusion bodies (OBs) in hepatopancreatic cells may occasionally be observed directly in infected, transparent larval shrimp. Diagnosis of infection in most cases is dependent upon fresh squash examination of tissue sections. Heavy infections may result in appearance of characteristic occlusion bodies in feces (Couch, 1991). *Baculovirus Penaei* is a rod shape virus, 269.6 ± 20.7 nm in length with a nuclocapsid diameter of 50.3 ± 4.8 (Couch, 1974; Brock *et al.*, 1983). Although until now the BP has not been reported from *P. semislcatus.*

Penaeus monodon baculovirus in the present study is also different from Baculovirus midgut gland necrosis (BMN) or type C baculovirus. BMN may be easily confused with MBV, especially at early or low grade of infection in which occlusion bodies are not easily stinguished. BMN can also experimentally infect *P. semisulcatus*. BMN can be identified by the appearance of hypertrophied hepatopancreatic tubule epithelial cells that contains a single eosinophilic to pale basophilic, irregularly shaped inclusion body that fills the nucleus of the infected cell. Affected nuclei also display diminished nuclear chromatin, marginated chromatin, and absence of occlusion bodies that are characteristic of infection by the occluded (SNPV-type or type-A) baculoviruses (Lightner, 1996). BMN disease is caused by a rod-shaped viral agent identified as baculovirus subgroup C (non-occluded, rod-shaped nuclear viruses). The non-envelope nucleocapside measures 36 by 250 nm, envelope virions measuring about 72 by 310 nm (Sano *et al.*, 1984; Lightner, 1996).

Vickers *et al.* (2000) described the cytopathology, virogenesis and replication of monodon baculovirus (MBV) in *P. monodon* from Australia that some how is

different from our findings. He reported electron-dense unenveloped nucleocapsids, not previously described for MBV, in the cytoplasm and attached to the nuclear envelope of infected hepatopancreatocytes. These nucleocapsids comprise a missing link in the published literature on the replication cycle of MBV by providing evidence for the means by which the viral genome travels from the plasma membrane of the hepatopancreatocyte to the nucleus. Features similar to those of MBV from other areas, but not previously reported for MBV from Australia include empty capsids attached to the nuclear pore, central filaments in developing capsids, capsides partly filled with nucleic acid, and filaments in subapical envelop expansions.

The molecular studies on DNA of MBV and protein OB have been done in several laboratories. Vickers *et al.* (1992) have been dealing with detection of MBV using PCR, in which the primers successfully primed the amplification of the MBV polyhedrin gene. Based on their results, Vickers *et al* (1992) attempted to clone the amplified DNA and construct a MBV polyhedrin gene probe, which could be used in highly sensitive diagnostic test for MBV detection in shrimp. In contrast, Spann and Lester (1996) found adults of *Metapenaeus bennettae*, infected with monodon baculovirus (MBV) giving negative results with an in suit hybridization test using a DNA probe from MBV, and *Penaeus monodon* postlarvae, experimentally exposed to the virus, was not infected.

Since 1992 the Iranian government has identified the southern provinces along the Persian Gulf and Sea of Oman as important sites for development of shrimp culture. To date 100,000 ha of lands have been allocated for shrimp farming. Furthermore, the government of I.R.Iran is promoting pond culture of penaeids (*P. semisulcatus* and *P. indicus*) using large tracks of marginal agricultural land on the eastern shores of the Caspian Sea in Golestan and Mazenderan provinces as a means of providing alternative employments in the north of country. Investigation on shrimp diseases especially on identification of diseases sign is useful to monitor

the regional disease situation and draw up prevention scheme, while it also is essential for quarantine practices.

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