Ultrastructural and pathogenesis of *Monodon baculovirus* in SPF shrimp, *Litopenaeus vannamei* imported to Iran

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Abstract

Viral pathogens are major causes of outbreaks in shrimp farms throughout the world. *Monodon baculovirus* has been known to be invasive in 85-100% of the shrimp hatcheries, in early or late stages of shrimp. Three-hundred and sixty juvenile of *Litopenaeus vannamei* with average (±SD) size of 7.99±0.54 g and 3600 post larvae 10-15 were prepared from Shrimp Research Station located in Helleh and 3 hatcheries from Bushehr Province, southern part of Iran, respectively. They were allocated to 9 glass aquariums (50×50×60cm) as 2 groups (postlarvae and juvenile), controls with 3 replications using100 liters well aerated water. They were exposed to MBV as water-born path. Concerning the pathogenesis, three phases were observed. The quantity of intranuclear occlusion bodies was 1-9. Mortality was only observed in postlarvae but not in adult or control groups. It is concluded that MBV can be an invasive pathogen for SPF offspring or postlarvae of *L. vannamei* being imported to Iran.

Keywords: MBV, Ultrastructure, TEM, *Litopenaeus vannamei*

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Introduction

Viral diseases are the major problems of shrimp farms and hatcheries that caused severe outbreaks in last decade (Afsharnasab *et al.*, 2009; Kakoolaki *et al.*, 2011; Lightner, 2011). Although, shrimp culture industry has a short history in Iran, it has been a growing industry as well (Afsharnasab *et al.*, 2009). Based on the expectation and the twenty-year development plan of Iran, at the end of 1400 RIC, about 100,000 acres of lands should be allocated to shrimp industry (Afsharnasab *et al.*, 2006). Due to white spot outbreaks in the most important commercial species of Iran, *Penaeus indicus*, an attempt was made to import a more disease-tolerant species. Therefore, *L. vannamei* was introduced and acclimated to Iranian southern shrimp farms and is cultivated in recent years (Afsharnasab *et al.*, 2006; Kakoolaki *et al.*, 2011). *Monodon baculovirus* (MBV) was first recognized in shrimp in 1977 in Taiwan (Lightner and Redman, 1981). It was then detected in Thailand in 1990 (Fegan *et al.*, 1991). This virus has been known to be the cause of 85-100 percent of mortalities during shrimp larvae, and postlarvae stages of hatcheries (Natividad *et al.*, 2006; de la Peña *et al.*, 2008; Lightner *et al.*, 1983) .MBV has been considered as a notifiable pathogen by the OIE (2006). It has been found in many parts of the world including Australia, India, East Africa, North America, the Middle East (Surachetpong *et al.*, 2005) and spread throughout most of the shrimp farming industries in the world. Although MBV has not been as lethal as white spot syndrome virus (Caipang *et al.*, 2011; Lightner *et al.*, 2012), MBV infection has resulted in economic loss of hatcheries due to weak performance in growth and low survival rate of postlarvae (Lightner *et al.*, 1983). Infected shrimp are susceptible to secondary infections caused by *Vibrio* spp. and protozoan's at poor environmental conditions (Vaseeharan and Ramasamy, 2003) and thereby it may be seen as dual or triple infection (Umesha *et al.*, 2006). The virus is susceptible to environmental condition and therefore shrimp mortality can be fluctuated due to some predisposing factors such as salinity and pH (Afsharnasab *et al.*, 2006). Diagnosis of MBV occlusion bodies in hypertrophied nuclei of anterior midgut epithelium and hepatopancreatic cells has been carried out by light microscope, PCR (Lightner and Redman, 1998) and TEM (Chen *et al.*, 1989).The pathogenesis, distribution and morphology of MBV have been described (Vogt, 1992; Mari *et al.*, 1993; Surachetpong *et al.*, 2005; Afsharnasab *et al.*, 2006;Natividad *et al.*, 2006; Sarathi *et al.*, 2008; Gangnonngiw *et al.*, 2010;Caipang *et al.*, 2011;). (The objective of our study was to ultrastructurally examine and determine the pathogenesis and severity of MBV in the cultured postlarvae and juvenile of SPF (Specific Pathogen Free) *L.vannamei* imported to Iran.

Materials and methods

Virus preparation

MBV was collected from a *Penaeus semisulcatus* hatchery in Bushehr Province located in south of Iran where MBV outbreak occurred in 2011. The samples were frozen and stored at -80°C until the experiment was begun. The origin of the shrimp for this study was the imported SPF offspring of *L. vannamei.*

Shrimp and experiment protocol

Three-hundred and sixty juveniles of *P. vannamei* with an average ($\pm$SD) size of 7.99$\pm$0.54 g and 3600 post larvae 10-15were prepared from the Shrimp Research Station located in Helleh site and 3 hatcheries, respectively, from Bushehr Province, southern Iran in 2012. Based under controlled conditions, they were transferred to the Iran Shrimp Research Center for PCR, laboratory and experimental examinations. Based on the primary result of the PCR, no sign of viral infection, white spot syndrome virus (WSSV), (MBV) and Hepatopancreatic parvo-like virus (HPV), were observed.

Shrimps were acclimated to new condition at the pH of 7.3, and salinity of 40 ppt with well oxygenated water, for 1 week before the study was begun. They fed with commercial feed during the experiment. Oxytetracycline at 20 ppm was used to prevent bacterial infections. All 360 juvenile shrimps were equally allocated to 9 glass aquariums (50$\times$50$\times$60cm) as 2 treatments groups: 1) water-born path exposure group (Group A), as control group (C1), and 3600 PL were dedicated for 2) water-borne path exposure (Group B) with control group (C2) in 3 replications, each aquarium with 100 liter well aerated water . At first, shrimps in the treatment groups A and B were exposed to MBV within 24 h without water exchange. The exposed shrimps were then washed with clean water, the examinations carried out and analyzed after 7 days post exposure (hour post exposure), mortalities were recorded daily.

PCR and histopathological examinations

Nested PCR, and histopathological samples were collected from the shrimp showed abnormality at 0, 12, 24, 48, 96 and 120 h. After the first cases observed, three moribund shrimps in each replication are sampled in order to transfer to histopathology and PCR labs of Iranian Shrimp Research Center and Veterinary Office of Bushehr Province. A small section of hepatopancreas tissue from each of the 3 samples of juvenile shrimps was placed in Eppendorf micro tubes containing ethanol 70% for PCR technique, using IQ2000 MBV commercial kit (Afsharnasab *et al.*, 2006) as well as 3 whole body PL from each replication. The remained parts of hepatopancreas tissues and midgut of the shrimps were placed in tubes containing Davidson's fixative. They were then transferred to the lab. After 48-72 hours davidson's fixatives were discarded and replaced with ethanol 70% in the lab. Tissue sections were then stained with Hematoxylin and Eosin (H & E) and examined to determine the histopathological changes using a light microscope (Lightner, 1996).

Transmission Electron Microscopy (TEM)

Study on prepared samples of shrimps exposing to the virus was continued for transmission electron microscopy following Afsharnasab (2007). MBV-infected hepatopancreas tissues of *L. vannamei* were fixed in 2.5% glutaraldehyde at 4ºC for 24 h, and then replaced in 0.2M cacodylate phosphate buffer (pH 7.4) at the same temperature and duration. The tissue was then dehydrated in graded acetone and embedded in the resin mixture. Approximately 1-µm thick section were prepared from tissue block and stained with 1% methylene blue for 1 minute at 60ºC. Tissue showing the desired degree of destruction and the occlusion bodies were then thin sectioned (50-80 nm) using diamond knives on a ultra microtome. Section mounted on a copper grid were stained with uranyl acetate and lead citrate and viewed under electron microscopy CM 10 Philips in Faculty of Veterinary Medicine, Shiraz University.

Severity of infection (SOI)

Grading the severity of infection was analyzed based on pathological findings and is described in table 1.

Table 1:Severity of infection in different conditions of the treatments.

SOI index	Pathological finding
0	No signs of infection and tissue damages found
1	Considerable damages found but no sign of disease
2	Mild to moderate-tissue damages found
3	Moderate to severe-tissue damages found
4	Severe -tissue damages found

Results

MBV infection was recorded in epithelial cells of midgut and hepatopancreas cells of the infected postlarvae and juveniles (Fig. 1).
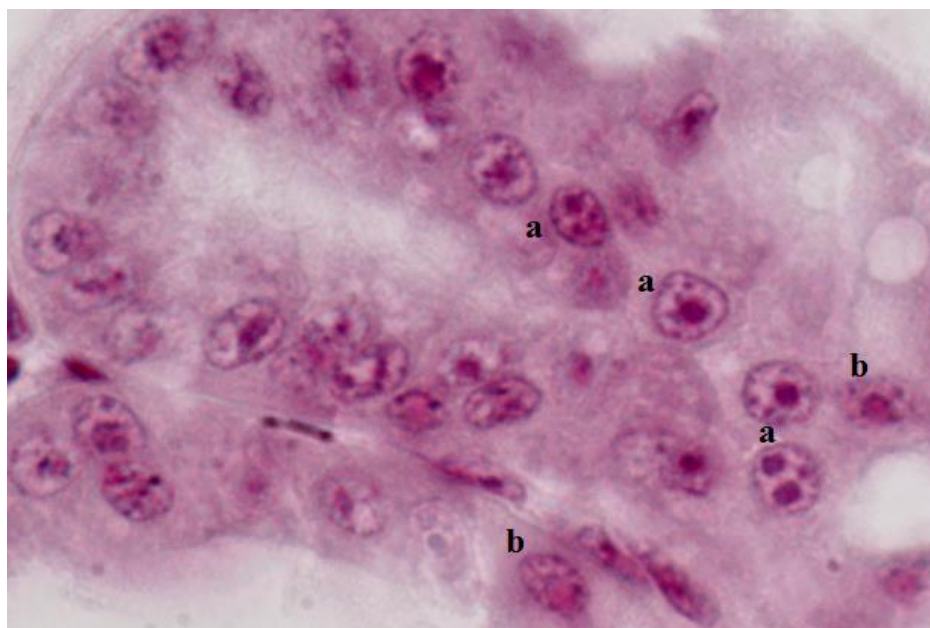
Figure 1: Photomicrograph of MBV infection found in hepatopancreas of PL. with hypertrophied nucleus (a) and eosinophilic occlusion bodies (b) H & E, ×1000.

Confirmations for the MBV of each treatment and control are shown in Figure 2 (based on the IQ2000, MBV commercial kit).
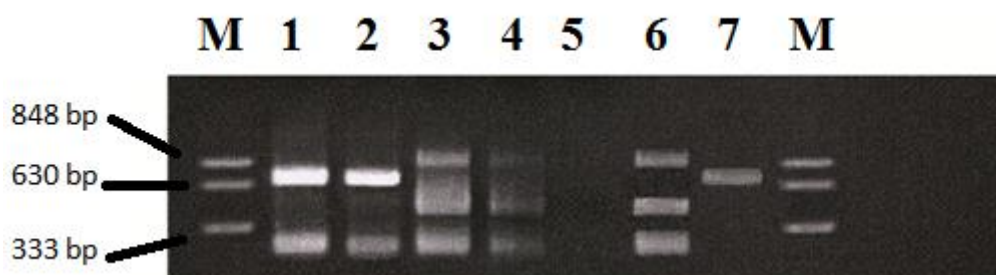

Figure 2: PCR result for MBV; Lane M: Marker, Lanes 1, 2: Water borne exposure of juvenile shrimp; Lanes 3, 4: Water borne exposure of PL shrimp; Lane 5: Negative control; Lane 6: Positive control; Lane 7: Negative Sample.

The clinical signs such as losing the appetite, lethargy and irritability were observed in adult shrimps. Nevertheless, the signs in postlarvae include lethargy, anorexia, blanching the midgut, unusual and swirling behavior, unilateral swimming in the water column.

According to our result, mortality was observed only in postlarvae but no mortality was observed in adult or control groups. The mortality for each group and control is given in Table 2.

Table 2: The comparison of mortality value for each treatment and determined post exposure days.

	1	2	3	4	5	6	7
A	0	13	21	31	31	13	4
C1	0	0	3	2	7	4	1
B	0	0	0	0	0	0	0
C2	0	0	0	0	0	0	0

1-7 are the post exposure days. A, C1, B and C2 are treatments and controls.

Electron microscopic (EM) studies of the virus pathogenesis were examined and fourth phases were observed. Firstly, the cells showed a slight hypertrophy, some nucleolus deformed and then migrated to the nucleus membrane. Chromatin were decomposed and seen as bright spots in the center of the cell (Fig. 3). In the second phase, one or more small occlusion bodies were visible in the hypertrophied nucleus. At this point, the nucleolus as well as the nucleus was under going to be destroyed. Chromatin migration to the nucleus membrane completed (Fig. 4). In the third and fourth phase, the cell nucleus was quite hypertrophied. It filled the cytoplasm, entirely and occlusion bodies performed, subsequently (Fig 5, 6).
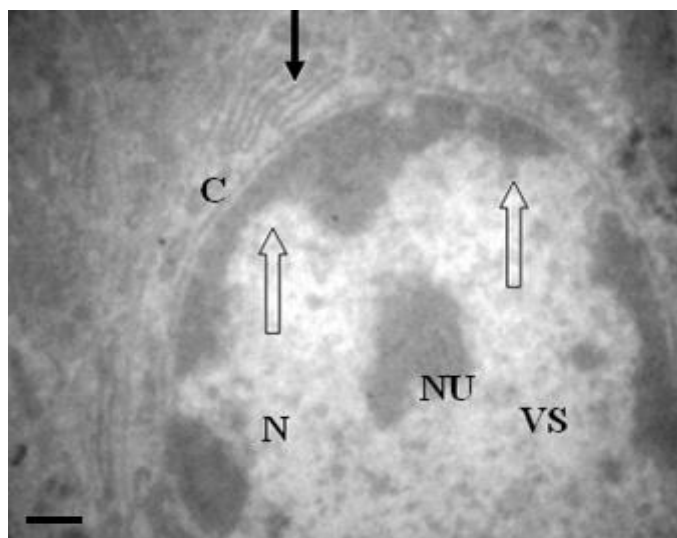

Figure 3: Transmission electron microscopy micrograph of a section through a MBV infected hepatopancreas cell of PL of *L. vannamei* showing the first stage of MBV disease which Hypertrophied nucleus (N), and the nucleolus(NU) migration of chromatin to the nucleus membrane(white arrow) and virogenic stroma (vs) are identified. Dense cytoplasm(C) within Endoplasmic Reticulum(ER, black arrow) is seen. Lead citrate and uranyl acetate. Bar: 1.8μm.
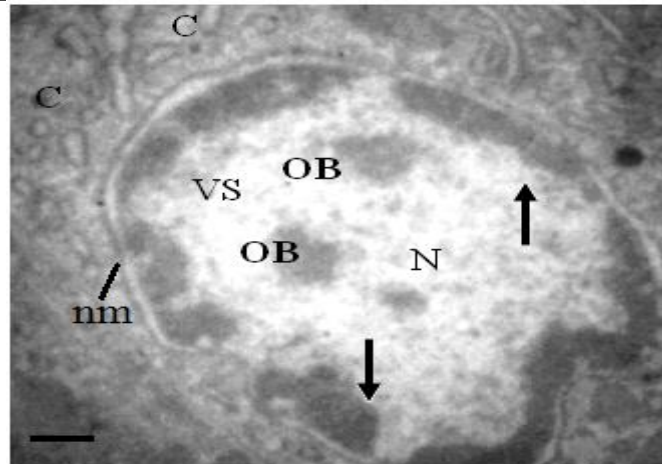
Figure 4: Transmission electron microscopy micrograph of a section through a MBV infected hepatopancreas cell of PL of *L. vannamei* showing the second stage of the disease. The chromosomes migrated (black arrow) completely to the nucleus membrane (nm) and accumulation of virus and cellular proteins resulted in occlusion bodies(OBs)in the center of hypertrophied cell are identified. Cytoplasm (c) with ribosome and Endoplasmic Reticulum were observed. In addition, virogenic stroma that is responsible for replication of virus (VS) appeared in nucleus. Lead citrate and uranyl acetate. Bar: 1.8μm
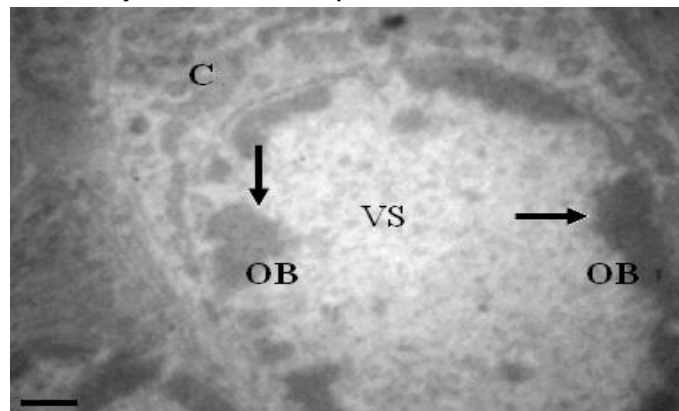

Figure 5: Transmission electron microscopy micrograph of a section through a MBV infected hepatopancreas cell of PL of *L. vannamei* showing the third stage of the MBV disease. Chromatin migrated completely to the nucleus membrane and occlusion bodies (OBs) are also fully drawn in to the membrane. Virogenic stroma (vs) is seen, too. Lead citrate and uranyl acetate. Bar: 1.8μm.
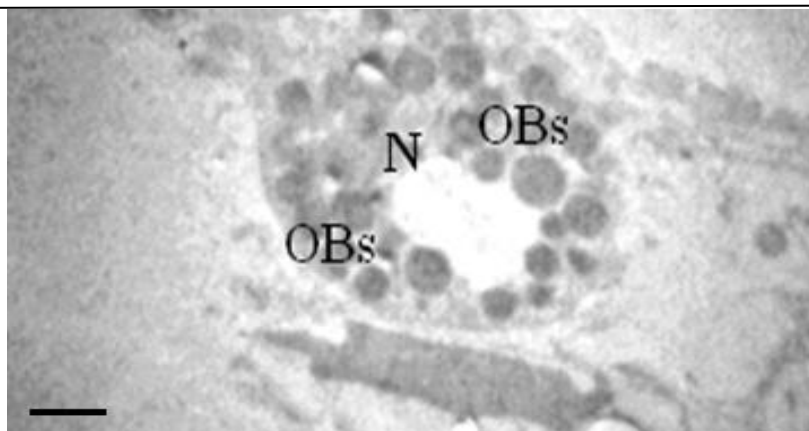
Figure 6: Transmission electron microscopy micrograph of a section through a MBV infected hepatopancreas cell of PL of *L. vannamei* showing the fourth stage.of the disease. Rounded occlusion bodies (OBs)were visible and the nucleus occupied the cytoplasm, entirely. Lead citrate and uranyl acetate. Bar: 1.8µm.

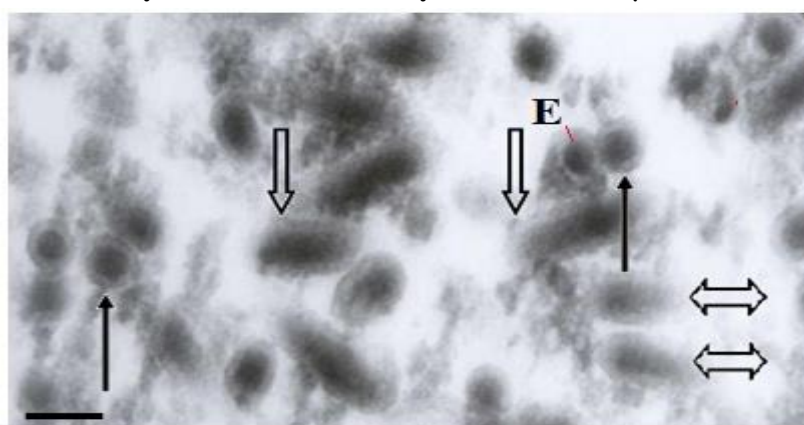**Figure 6:** Transmission electron microscopy micrograph of a section through a MBV infected hepatopancreas cell of PL of *L. vannamei* showing rod shaped viruses existing in an occlusion body with outer and inner envelopes (E). Virus cross section (black arrow) and longitudinal sections of virus (white arrow) and the distal part of the virus that has not completed Yet (double arrows). Lead citrate and uranyl acetate. Bar: 290nm.

Table 3: Severity of infection index in juvenile and postlarvae in different days.

Days	SOI index postlarvae	SOI index juvenile
1	0	0
2	1	0
3	2	0
4	3	1
5	4	2
6	4	2

Discussion

In this study, the pathogenesis of MBV in the imported SPF shrimp, *L. vannamei* post larvae and adult was evaluated through exposing the shrimp to the contaminated water. The occurrence of intra nuclear spherical occlusion bodies confirmed MBV presence in *Penaeus monodon* and *P. indicus* as described by (Lightner and Redman, 1998; Ramasamy and Brennan, 2000), as well as our findings for *L. vannamei*. Despite histopathological changes in hepatopancreas and midgut, no evidence for MBV was found in other examined tissues supporting the observations of Lightner *et al.* (1983)and Ramsey and Brennan (2000). Light microscopic examinations showed that the histopathological changes were kicking off by nuclear hypertrophy, chromatin migration and nucleolus displacement, which are exactly compatible to the result of Afsharnasab *et al.* (2009). In earlier studies conducted by Lightner and Redman(1981) and Afsharnasab (2007) infected shrimps showed clinical signs such as lethargy, anorexia, blanching of midgut and immobility. As Brock *et al.* (1983) and Paynter *et al.* (1992) confirmed, MBV disease occur in mysis and post larvae ranging about 3-20 mm in length. This result is compatible to our findings, but no mortality found in juvenile group in our study, perversely. Contrary to the findings by Viker *et al.* (2000) and similar to the results of other researchers (Brennan, 2000; Manivannan *et al.*, 2002;Claydon *et al.*, 2010 and

Ramasamy)that mentioned the number of occlusion bodies were 1-2 and 1-12, respectively, our findings showed that the quantity of intra nuclear occlusion bodies is 1-9.The average (±SD) length and diameter of the virus were 320±4 nm and 72±2 nm (n=15), respectively, supporting the findings by Sano *et al.* (1984) and Ramasamy and Brennan (2000). It should be noted that with the exception of the juvenile group, no losses in adult shrimps (treatment and control) were observed. The accumulative mortality in both juvenile groups and the control were 18.83 percent and 2.38 percent, respectively. The mortality begun 2 days after exposure in post larvae and its trend increased subsequently but concurrent mortality was constantly low for the control group. It seems that MBV is a moderate to severe disease for the late post larvae, *L. vannamei* in contrary to WSSV (White Spot Syndrome Virus) that is invasive pathogen for each phase of growth in the same species (Afsharnasab *et al.*, 2007; Kakoolaki *et al.*, 2011; Lightner, 2011).This finding is confirmed by SOI results (Table 3)that showed the severity of the infection for the juveniles and postlarvae reached 2 and 4 after 6 days post exposure, respectively, suggesting that juvenile shrimp can tolerate MBV. Since the infected Epithelium of midgut and hepatopancreas, and virions along with occlusion bodies were constantly shed into the feces of infected shrimp (Lightner *et al.*, 1983) it led us to predict that juvenile and or broodstocks played as carriers in region, contaminating with

MBV. Despite MBV being controlled by several quarantine and prophylactic programs through egg disinfecting methods, many reports have stressed that the high occurrence of MBV in shrimp hatcheries is continued (Flegel *et al.*, 2001; Afsharnasab, 2007).

It is concluded that MBV can be an invasive pathogen for SPF postlarvae of *L. vannamei* importing to Iran.

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