Experimental pathogenicity of shrimp, *Penaeus vannamei* exposed to monodon baculovirus (MBV)

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Received: May 2013 Accepted: June 2014

Abstract

The objective of our study was to explain the histopathological changes of monodon baculovirus (MBV) in hepatopancreas and midgut tissues of the cultured *Penaeus vannamei*. Five-hundred and forty juvenile of *P. vannamei* with average size of 7.99±0.54 g and 3600 post larvae $_{10\text{-}15}$ were distributed to 18 glass aquariums (50×50×60cm) with 100L well aerated water per each aquarium as water borne MBV (group A) and food borne MBV (Group B) and one control group (C), in triplicates. Also, 3600 post larvae were dedicated for water borne exposure (D) based on one time immersion exposure in 24 h without water exchange and the untreated group was studied as control in triplicates. The specific pathological sign of MBV was observed as a multiple intranuclear eosinophilic occlusion bodies (OBs) in hepatopancreas and midgut tissues. Our result indicates that the severity of the MBV infection is more considerable in post larvae than juvenile stage and confirms that MBV can be an invasive pathogen for shrimp culture industry in Iran.

Keywords: *Monodon baculovirus*, *Penaeus vannamei*, Intranuclear eosinophilc occlusion body, Pathogenicity

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Introduction

Viral diseases are the major problem of the shrimp farms and hatcheries and have caused severe outbreak in Iran in the last decade (Afsharnasab *et al*., 2009; Kakoolaki *et al*., 2011; Lightner, 2011). Among the pathogens, MBV is very important economically (Lightner and Redman, 1998). MBV was firstly identified in 1997 (Lightner and Redman, 1998) and reported to be originated from Taiwan with wide geographic distribution. The Monodon is present as enzootic in penaeids of the Indo-pacific coasts of Asia water (Rajendran *et al*., 2012). It was reported from hatcheries (de la Peña *et al*., 2008) through diagnostic and quality assessments and grow-out ponds (Fegan *et al*., 1991; Flegel, 2006). All the life stages are susceptible to MBV, with the exception of egg and nauplii (OIE, 2006). MBV has worldwide spread throughout most of the shrimp farming industry in Asia (Lightner *et al*., 2012). A distinct clinical sign of MBV is associated with whitish discoloration of midgut that reveals in zoa, napulii and early days of post-larval stages (Afsharnasab, 2007). In addition, shrimps infected with MBV are susceptible to secondary infections caused by *Vibrio* spp and protozoans in poor environmental conditions (Vaseeharan and Ramasamy, 2003) and thereby it may cause dual or triple infection. Afsharnasab *et al*. (2006) found that the virus keeps its pathogenicity in *P. semisulcatus* whenever it is exposed to different degree of salinity from 5 ppt up to 40 ppt. Lethargic and anorexic shrimps are observed among the infected animals (Lightner, 1992; Afsharnasab, 2007). The infected larval shrimps were suffering from overcrowding stress, slow growth and stoppage of fundamental activities (Vickers *et al*., 2000) that ultimately led to significant economic loss (Flegel, 2006). As mentioned by Lightner (1996) in histopathology the acidophilc occlusion bodies are the specific sign for detection of the MBV in tissues from hepathopancrease and midgut areas. MBV transmission is associated with vertical and horizontal pathways (Lightner, 1996; Afsharnasab, 2007). Persistent infection in penaeid hosts of MBV and *P. monodon* broodstocks infected with MBV shows excretion of MBV-contaminated faeces during spawning, thereby contaminating the eggs and passing the virus to the next generation (Lightner, 1996) and injection of the infected tissues (OIE, 2006). Prevalence of MBV is varied from 1 up to100 percent in wild to cultured shrimp, respectively (OIE, 2006; Afsharnasab, 2007). Lightner (1996) listed very few pathogens of shrimp for which there are no methods to detect sub-clinical infections (hepatopancreas parvovirus, HPV) and some pathogens can only be detected following stress-testing (e.g. MBV).

The objective of our study was to explain the histopathology changes of MBV among the cultured *P. vannamei*.

Materials and methods

Virus preparation

The source of MBV were infected shrimp, *P. semisulcatus* collected from a hatchery in Bushehr province located in south of Iran where MBV outbreak had occurred in 2011 and the samples were frozen and stored at -80$^{o}_{c}$ until the experiment was begun.

Shrimp and experiment protocol

Five-hundred and forty juvenile of *P. vannamei* with average size of 7.99±0.54 g and 3600 post larvae $_{10\text{-}15}$ were prepared from Shrimp Research Station located in Helleh site and 3 hatcheries from Bushehr Province, southern part of Iran, respectively.

The samples were transferred under controlled conditions to Iran Shrimp Center for PCR, laboratory and experimental examinations. Based on the primary result of the PCR no sign of infection with the viruses, white spot syndrome virus (WSSV), MBV and Hepatopancreatic parvo-like virus (HPV), were observed.

The samples were acclimatized with new condition at pH 7.3, 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 probable bacterial infections. All, 540 juvenile shrimps were equally allocated to each 18 glass aquariums (50×50×60cm) with 100 liter well aerated water per each aquarium as water borne (group A) and food borne exposure (Group B) and one control group (C), in triplicates. Also, 3600 post larvae (PL) were dedicated to water borne group (D) based on one time immersion exposure in 24 h without water exchange and the untreated group as control was also used all in triplicates. The exposed shrimps were then washed with clean water, mortalities were recorded daily.

Wet mount of fresh samples

Wet mounting was the additional examination to investigate the occlusion bodies in hepatopancreas and gut tissues. Rapid diagnosis of MBV infection was carried out through wet-mounts of squash preparations of hepatopancreas and midgut stained with 0.05% aqueous malachite green in order to observe the intranuclear-spherical occlusion bodies at diameter range of less than 0.1 μm to nearly 20 μm, using light microscopy following the method described by Lightner (1996).

PCR and histopathological examinations

Nested PCR and histopathological study of samples performed at 0, 12, 24, 48, 96 and 120 hpi (hour post inoculation) on the shrimps showed abnormality. After the first cases observed, the 3 moribund shrimp from the each replication were sampled to transfer to histopathology and PCR labs of Iranian Shrimp Research Center and Veterinary Office of Bushehr province. A small section of hepatopancreas tissue of each 3 samples of juvenile shrimps were placed in Eppendorf micro tubes containing ethanol 70% for PCR technique using IQ2000 WSV commercial kit (Afsharnasab *et al*., 2006) as well as 3 PL whole body for each replication, D or E. The remained part of hepatopancreas tissues and midgut of the shrimp were placed in tubes containing Davidson's fixative. They were then transferred to the lab. The tubes containing Davidson's fixative were discarded and replaced with ehanol 70% after 48-72 h in the lab. Tissue sections were then stained with Hematoxiline and Eosine (H & E) and examined to determine the histopathological changes using light microscopy (Lightner, 1996).

Results

Results of the occurrence of the MBV antigen within the samples of each treatment and control are shown in Fig. 1 (based on the IQ2000, MBVcommercial kit).

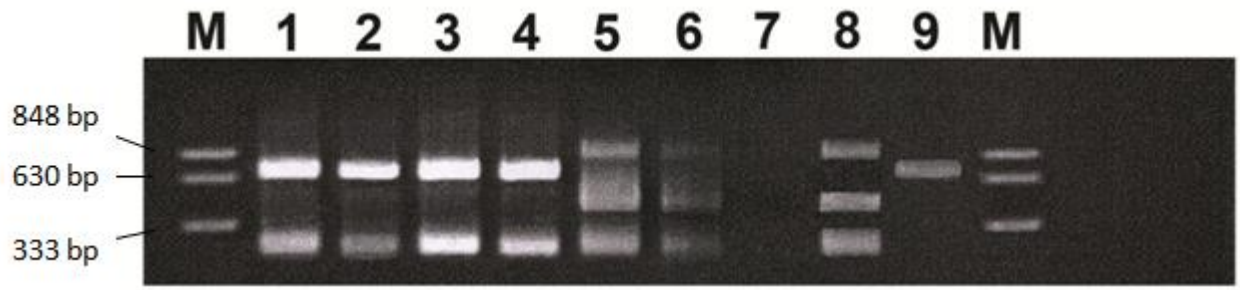

Figure 1: PCR result for MBV; Lane M: Marker, Lanes 1, 2: Water borne exposure of juvenile shrimp; Lanes 3, 4: Food borne exposure of juvenile shrimp; Lanes 5, 6: Water borne exposure of PL shrimp; Lane 7: Negative control; Lane 8: Positive control; Lane 9: Negative Sample.

Histopathological and wet mount examinations were useful to confirm the PCR results in our study. Histopathological sections of apparently healthy post larvae did not show the presence of occlusion bodies. However, they were positive for MBV by nested PCR showing 225 bp and 444 bp bands. The MBV infection was firstly identified by wet mount of juvenile fresh samples. Fig. 2 shows the wet mount result for the hepatopancreas. MBV histopathological finding was observed with difficulty in the early stage of PL or juvenile shrimp due to absence of occlusion body in our LM observations (Fig. 3) although in some cases hypertrophied nucleus hepatopancreas cells with eosinophilic occlusion bodies were observed. Diagnostic procedure was continued until the occlusion bodies in the nucleus of hepatocytes were observed. Histopatholical observations showed mild to marked degenerative changes in hepatopancreas cells. Approximately, single to multiple intranuclear occlusion bodies (3-9) were observed in each infected hepatopancreas cell (Figs. 4-7).

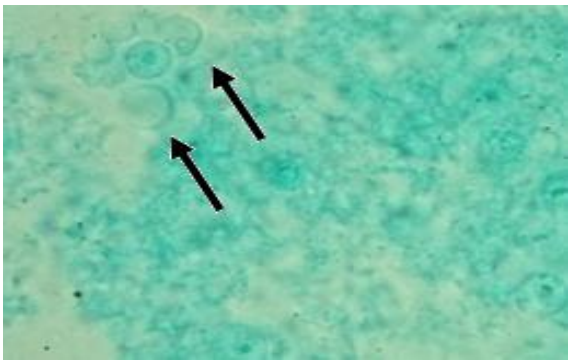

Figure 2: Photomicrograph of MBV occlusion bodies (arrow signs) in the hepatopancreatic tissue smear from a PL by normal light microscopy, a drop of Malachite green × 100.

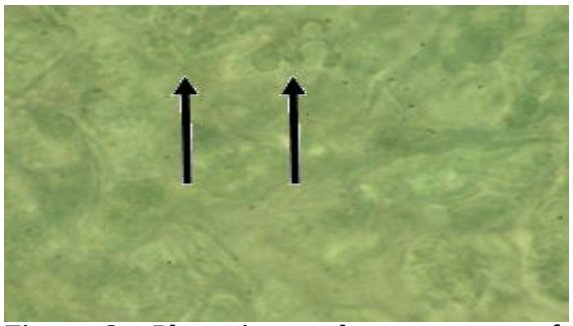

Figure 3: Photomicrograph wet-mounts of squash preparations of hepatopancreas tissue smear from a juvenile shows the occlusion bodies (arrows) by normal light microscopy (LM), a drop of Malachite green × 100.

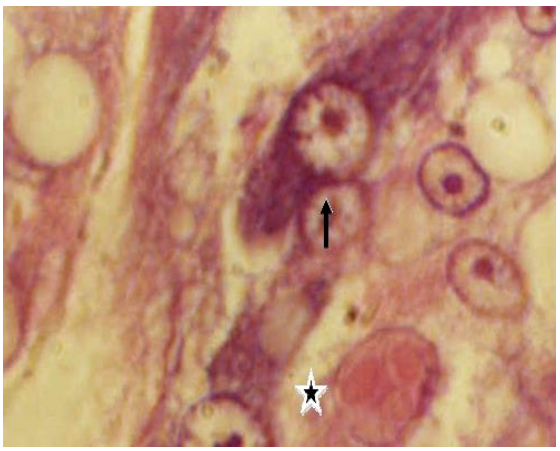

Figure 4: Photomicrograph of MBV infection found in PL. hypertrophied nucleus (arrow) and ring form one (asterisk). H & E, ×1000.

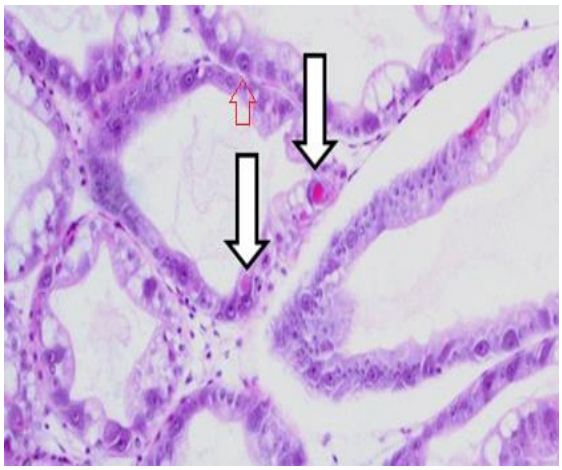

Figure 5: Photomicrograph of MBV infection found in PL. Hypertrophied nucleus (red arrow) and eosinophilic occlusion bodies (black arrows) H & E, ×1000.

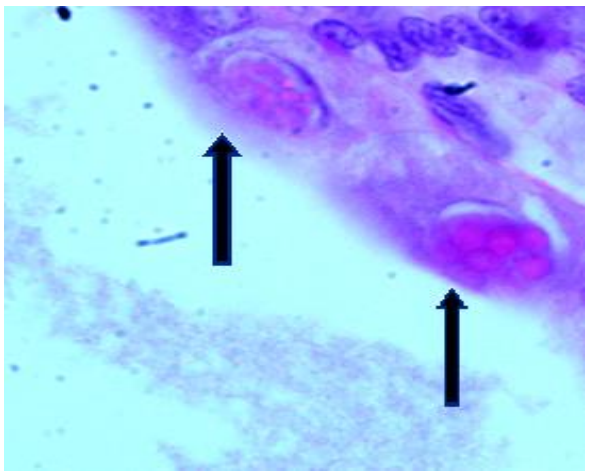

Figure 6: Eosinophilic intranuclear occlusion bodies (arrows) in the hepatopancreas of shrimp juvenile. H & E, ×1000.

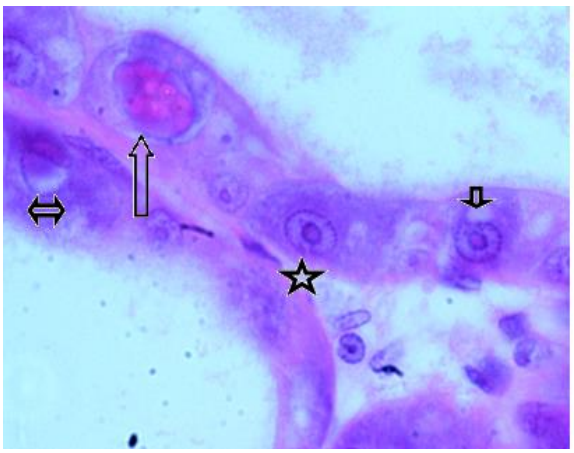

Figure 7: Photograph shows a complex of MBV phases in hepatopancreas of juvenile shrimp; hypertrophied nucleus (astriks), Chromatin migration (small arrow), translocation of nucleolus (two-sided arrow), intranuclear occlusion bodies (arrow). H & E, ×1000.

In the later phase of infection, many vacuolated cells accompanying with abundant eosinophlic intranucleus occlusion bodies were seen in hepatopancreas (Fig. 8). In this phase, clinical signs of infected juveniles included lethargy, anorexia and decrease of irritability threshold but anorexia, white discoloration of midgut and swirly swimming were seen in the post larvae.

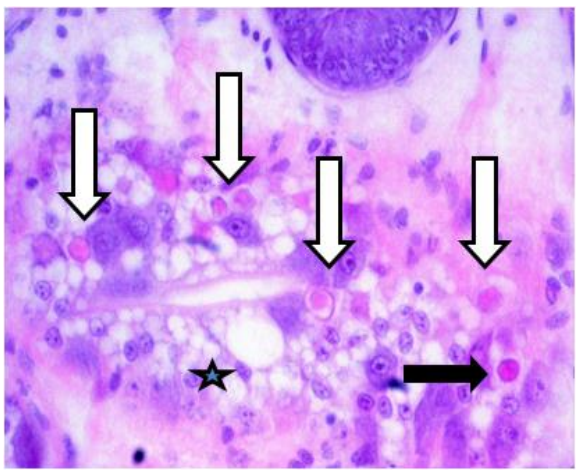

Figure 8: Photograph shows later phase of disease; many complex occlusion bodies (hollow arrows) accompanying with numerous vacuolated cells (asterisk) and a cell within one occlusion body (black arrow). H&E ×1000.

Discussion

The main goal of our study was to examine the histopathological changes in MBV disease. Occlusion-bodies observation of MBV in wet mount can lead us to doubt the infection, primarily. MBV release within feces was seen in the outbreaks (Sankar *et al*., 2011). Based on the finding of Lightner (1996) two other buclouviruses being invasive for shrimp are MBMN and BP. Against these two viruses that included basophilic intranuclear occlusion bodies the occlusion bodies of MBV are eosinophilic. According to the former finding and our PCR result, it seems the virus of our study was MBV.

Lightner (1983) confirmed that occlusion bodies of MBV ultimately released to the water with the feces and resulted in higher prevalence of MBV disease in the farms near the discharge area. Similar to the result of Paynter *et al*. (1992) our result showed the severity of the infection was more considerable in PL than juvenile stage. They showed that MBV occur in the age ranging from $Mysis_3$ up to $PL_{20.}$ On the contrary, Rajendran *et al*. (2012) reported all the stages, late larvae, PL and young juvenile are more susceptible to MBV. Vijayan *et al*., (1995) found that hypertrophy of hepatopancreas cells is the primary histopathological change for MBV. Similarly, results of clinical signs in our study was in accordance with that of other researchers (Lightner, *et al*., 1983; Paynter *et al*., 1992). Although intranuclear occlusion bodies were also seen in juvenile and adult of the shrimp but no clinical signs were observed. It could be due to the immunity level improving in developed stages of the shrimp life. It seems that cell types of hepathopancreas can be contaminated with MBV disease (Figs. 7, 8). According to the findings of Natividad et al. (Natividad *et al*., 2006) there are two ways to decrease the rate of MBV in hatcheries; washing eggs and feeding broad stocks with supplementary diets. No inflammative reaction due to hemocyte infiltration or nodule formation was observed in the shrimp of our treatments and controls similar to Sundaraj *et al*. (Sundaraj *et al*., 1996).

It is concluded that MBV can be the cause of mortality and histopathological changes in the shrimp *P. vannamei*, particularly in late PL and young phases. The results confirmed that MBV could be pathogenic in *P. vannamei*, which is the main species of shrimp culture industry in Iran.

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