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Observation of Two Viruses in Cultured Giant Freshwater Prawns (*Macrobrachium rosenbergii* de Man) in Thailand

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ABSTRACT

Two viruses in the hepatopancreas (HP) of *Macrobrachium rosenbergii* postlavae (PL) and adult prawns are described. Investigations were carried out by light microscopic examination of HP tissue squashes stained with 0.05 % malachite green and histological sections stained with hematoxylin and eosin (H&E), as well as by transmission electron microscopy (TEM). Histologically, the first pathogen was a baculo-like virus (BLV), recognized by the presence of enlarged nuclei with marginated nucleoli and eosinophilic inclusions that completely filled the nucleus of the HP tubular epithelium. In contrast, the malachite green-stained HP squashes did not show any marginated nucleoli. TEM of the infected HPs revealed the presence of BLV particles of approximately 250-300 nm in enlarged nuclei. The second virus was a hepatopancreatic parvo-like virus (HPLV). Spherical intranuclear inclusions were easily observed in the HP tubular epithelium in both fresh malachite green-stained tissue squashes and H&E-stained histological sections. TEM of infected HPs revealed intranuclear inclusions containing small virus-like particles of approximately 20-24 nm in diameter.

PL (n=200) with percentage infection rates of 20% with the BLV, 50% with the HPLV and 10% with dual infection were stocked into earthen ponds at a density of 60 000 PL/1600 m^2. After rearing for 60 d, the prawns were transferred into earthen ponds at a density of 10 000 prawns/1600 m^2. Sixty days later, (120-day-old) prawns were transferred into earthen ponds at a density of 5000 prawns/ 1600 m^2 and reared until harvesting at day 180**.** The prawns were sampled and divided into small and large-sized prawns at 60, 120, 150 and 180 d for histological studies to determine the viral infections. Results showed that at 120 d, the BLV infection in the small-sized prawns (12.5%) was higher than that of the large-sized prawns (5%). Dual infection with both viruses was 5.0% in small-sized prawns and 2.5% in large-sized prawns. However, at day 150, the BLV pathogen was not observed in either size group. In contrast, the HPLV infection was found in prawns throughout the culture period. The percentage infection rates in small-sized prawns at days 120, 150 and 180 were 22.5, 15.0 and 12.5%, respectively, similar to the infection rates for large-sized prawns of 20.0, 12.5 and 12.5%, respectively. The percentage survival rate and yield from both ponds at harvest were 67-68% and 197-199 kg/1600 m^2, respectively. These results indicated that the infection rates of these two viruses did not increase during the culture period and that the virus infections might have a negative effect on production.

Key words: giant freshwater prawns, *Macrobrachium rosenbergii,* baculo-like virus, hepatopancreatic parvo-like virus

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INTRODUCTION

The giant freshwater prawn (*Macrobrachium rosenbergii* de Man) is an important, farmed species in Thailand and other countries in Asia (New, 2000). In Thailand, *M. rosenbergii* is mainly cultivated in the central provinces, with most of the production used for domestic consumption (Limsuwan and Chanratchakool, 2004). Since 2004, the production of farmed prawns has significantly decreased due to white tail disease problems caused by a combination of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and the extra small virus (XSV) (Sriwongpuk, 2006). During larval rearing, moribund prawns exhibited opaque or whitish abdominal muscles and suffered severe mortality in several countries (Qian *et al*., 2003; Sahul Hameed *et al*., 2004; Bonami *et al*., 2005; Yoganandhan *et al*., 2006). These viruses might be transmitted vertically from infected broodstock to larvae (Sudhakaran *et al*., 2007) or via other crustacean carriers such as wild crabs and shrimp (Sudhakaran *et al*., 2006). Most large-scale hatcheries have been using their own domesticated broodstock reared in growout ponds for producing postlarvae (PL), not only for their farms, but also for business purposes. After using the reverse transcriptase polymerase chain reaction (RT-PCR) assay to screen *M. rosenbergii* broodstock for viruses prior to being transferred into hatcheries for producing larvae, it was found that most broodstock was positive for both *Mr*NV and XSV due to the lack of an effective broodstock biosecurity system.

Recently, the Department of Fisheries in the Ministry of Agriculture and Cooperatives gave support to a program for the development of domesticated and genetically selected, specific pathogen-free (SPF) broodstock of *M. rosenbergii*. However, in 2006, during the study on *Mr*NV and XSV infections in cultured *M. rosenbergii*, the authors discovered hepatopancreatic (HP) baculo-like virus (BLV) and hepatopancreatic parvo-like virus (HPLV) infections in PL and adult prawns from several hatcheries and farms. Both viruses occurred in PL without any clinical signs that they had been reared in the same nursery. The objectives of this study were to describe the histopathology of the two HP viruses in PL and the prevalence of these viruses from the PL stage throughout the culture period.

MATERIALS AND METHODS

This research was carried out in an *M. rosenbergii* hatchery and farm located in Ratchaburi province, central Thailand. PL, which were specific negative for the *Penaeus monodon*-type baculovirus (MBV), the hepatopancreatic parvo virus (HPV), *Mr*NV and XSV by PCR and RT-PCR assays, were stocked into a 0.5 ha earthen pond (P) at a density of 60 000 PL/1600 m^2 and reared for 60 d. Prawns were then transferred into two 0.5 ha earthen ponds (P1 and P2) at a density of 10 000 prawns/1600 m^2. Sixty days later, (120-day-old) selected prawns were transferred into two 0.5 ha earthen ponds (P3 and P4) at a density of 5000 prawns/1600 m^2 and reared until harvesting at day 180 (60 d in P3 and P4 ponds).

Identification of BLV and HPLV pathogens from *M. rosenbergii*

A series of studies were conducted to identify the two prawn viruses.

Wet-mounts and histopathology

Wet mounts of squashed HP tissues from PL specimens were stained with 0.05% malachite green and examined under a light microscope. For histopathological studies, PL and adult prawns were fixed in Davidson's fixative and processed for routine histology using hematoxylin and eosin (H&E) staining as described by Bell and Lightner (1988).

Transmission electron microscopy (TEM)

HP tissues of PL and adult prawns of approximately 1 mm^3 were fixed for 2 h at 4°C with 4.5% glutaraldehyde in phosphate buffer and post-fixed in 1% osmium tetroxide in phosphate buffer for 2 h. Tissues were then washed twice with distilled water, dehydrated in a graded series of ethanol from 50 to 100%, and then twice in 100% propylene oxide. Tissues were subsequently embedded in Epon-812 resin by successive 1 h infiltration of 1:1 and 2:1 resin:propylene oxide, and 100% resin. The tissues were polymerized by incubating at 70°C for 48 h in fresh 100% epoxy resin. Semi-thin sections (1μm thick) were stained with 1% toluidine blue and then observed with a light microscope. Ultrathin sections were stained in 2% uranyl acetate and 0.3% lead citrate solutions for examination using a JEOL, JEM 1210 electron microscope at 100 kV.

Prevalence of BLV and HPLV infections

A total of 200 PL, 60-day-old juvenile prawns from the nursery pond, 120-day-old adult prawns from ponds P1 and P2, and 150- and 180-day-old adult prawns from ponds P3 and P4 were randomly sampled for histopathological studies. At each sampling event, prawns were divided into two groups of 20 small-sized and 20 large-sized prawns. All samples were fixed in Davidson's fixative and embedded in paraffin wax, sectioned at 5 μm thickness and stained with H&E (Bell and Lightner, 1988). HP tissues were examined for BLV, HPLV and dual infection with both viruses. The percentages of viral infections from PL, juvenile, small and large-sized prawns at each sampling event were compared.

RESULTS AND DISCUSSION

Identification of BLV and HPLV

BLV

Samples of hatchery PL and adult prawns showed the presence of enlarged nuclei with marginated nucleoli and eosinophilic inclusions that completely filled the nucleus up to the marginated chromatin (Figure 1) in epithelial cells of the HP tubules, similar to semi-thin sections stained with toluidine blue (Figure 2). In contrast, squash mounts of HP tissues stained with malachite green (Figure 3) showed no clearly

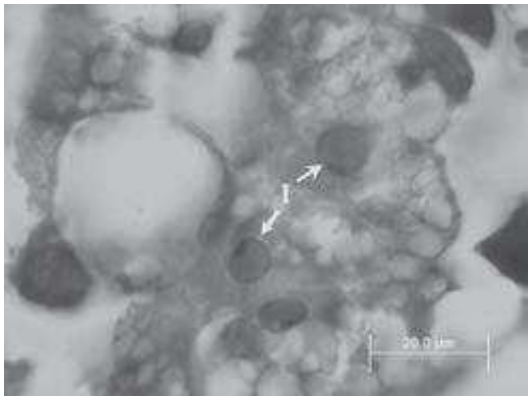

Figure 1 Photomicrograph of a hepatopan-topancreas tissue section of *M. rosenbergii* PL stained with H&E showing indications of baculovirus-like intranuclear inclusion bodies (I).

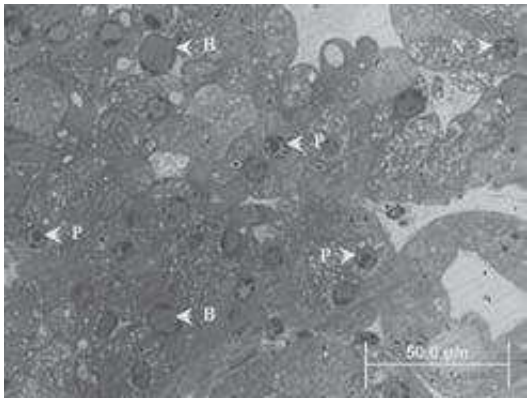

Figure 2 Photomicrograph of a semi-thin section stained with toluidine blue of hepatopancreatic tissue of *M. rosenbergii* PL showing: baculovirus-like intranuclear inclusion (B); early stage of parvovirus-like infected nucleus (P); and normal nuclei (N).

marginated nucleoli as in the histological sections. The inclusions resembled those previously reported for non-occluded baculoviruses in cultivated penaeid shrimp (Lightner, 1996). In Thailand, *M. rosenbergii* PL were investigated and eosinophilic inclusions which completely filled the entire nucleus in HP tubular epithelium were observed (Nash *et al.*, 2003). TEM of the HP of infected prawns revealed the presence of rod-shaped, enveloped virions of approximately 250-300 nm in enlarged nuclei with marginated chromatin (Figure 4A,B) These were similar to typical baculovirus virions found in other shrimp species (Lightner, 1993; Flegel, 2006).

HPLV

Malachite green-stained HP tissue squashes from the PL showed the presence of spherical intranuclear inclusions in the HP tubular epithelium (Figure 5). HP tissues of PL stained with H&E revealed medium to large eosinophilic to lightly basophilic intranuclear inclusions in the tubular epithelium (Figure 6).

In most cases, both large and medium-sized inclusions were observed in the same PL.

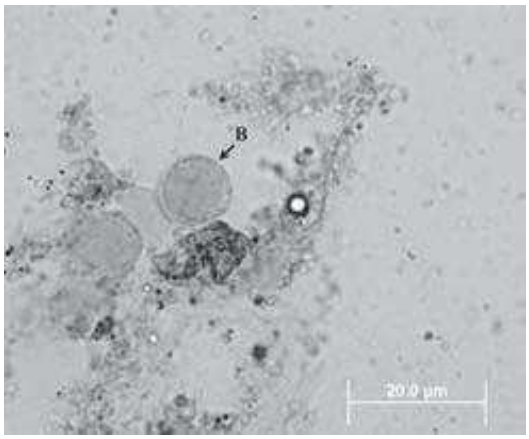

Figure 3 Photomicrograph of a squash-mount of hepatopancreatic (HP) tissue of *M. rosenbergii* PL stained with malachite green showing baculovirus-like intranuclear occlusion (B).

For HP tissue of adult prawns, only small to medium-sized inclusions (Figure 7) were observed and stained more eosinophilic than those found in PL. The inclusions in the E-cells from the PL specimens were very similar to those seen in HP of black tiger shrimp (*Penaeus monodon*) in Thailand infected with HPV (Limsuwan, 2000;

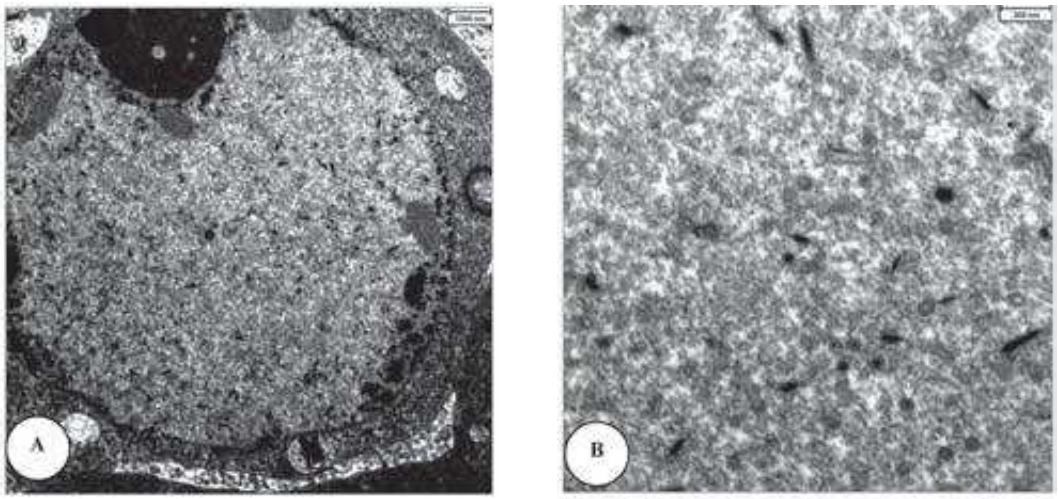

Figure 4 Transmission electron micrographs of baculovirus-like intranuclear inclusion in PL specimens of *M. rosenbergii* showing virion morphology (arrows). (A) Low magnification electron micrograph. (B) High magnification electron micrograph.

Flegel, 2006). Semi-thin sections of the HP stained with toluidine blue revealed deep blue inclusions compared with light blue inclusions for BLV in the same PL (Figure 2). TEM of affected nuclei revealed intranuclear inclusions which contained small viral-like particles of approximately 20-24 nm in diameter (Figure 9B), similar to those reported for other parvoviruses in penaeid shrimp (Lightner, 1993; Flegel, 2006). Some of these inclusions appeared to contain small bubble-like structures (Figure 9A). The HP inclusions observed in these specimens resembled those previously reported for HPV in *M. rosenbergii* from farms in Malaysia (Lightner, *et al.*, 1994).

Figure 5 Photomicrograph of a squash-mount of hepatopancreatic (HP) tissue of *M. rosenbergii* PL stained with malachite green. (Parvovirus-like intranuclear inclusions=P, normal nuclei=N).

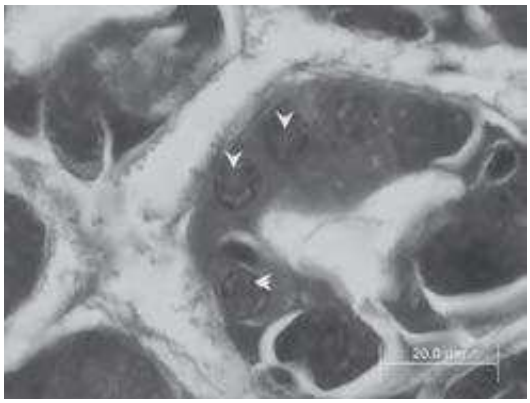

Figure 6 Photomicrograph of hepatopancreatic tissue of PL stained with H&E showing parvo-like virus intranuclear inclusions (arrows).

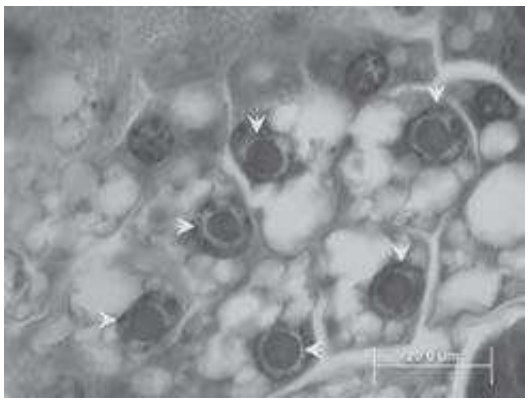

Figure 7 Photomicrograph of H&E stained hepatopancreatic tissue of an adult prawn showing parvovirus-like intranuclear inclusions (arrows).

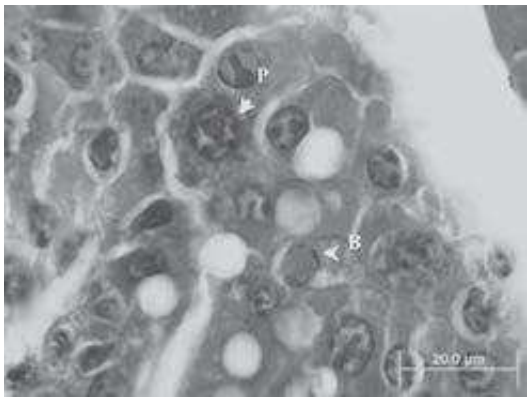

Figure 8 Photomicrograph of H&E stained hepatopancreatic tissue of PL showing tubule epithelial cells with a parvo virus -like infected nucleus (P) and a baculovirus-like infected nucleus (B).

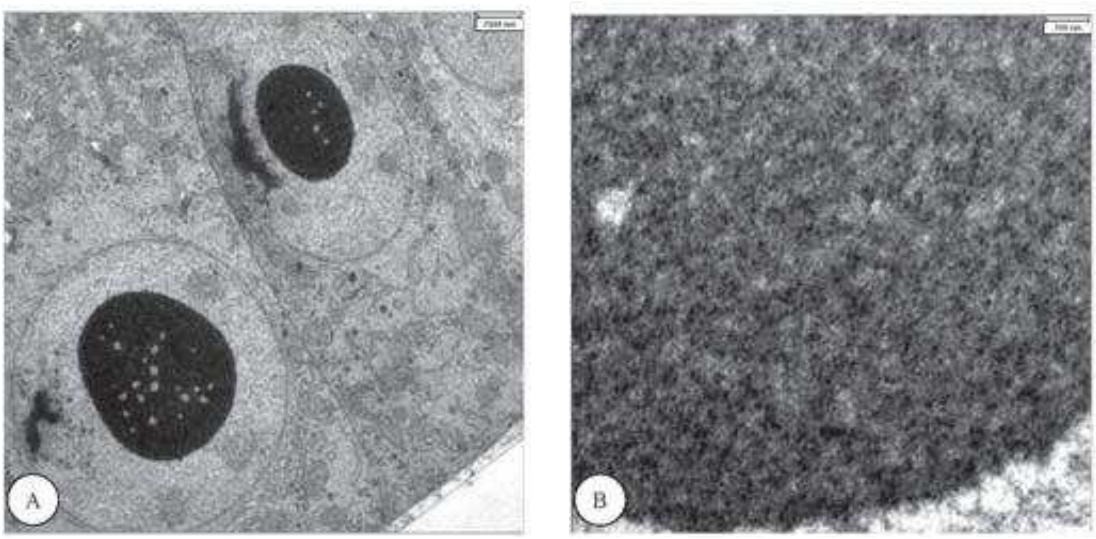

Figure 9 Transmission electron micrographs of hepatopancreatic parvovirus-like intranuclear inclusion in hepatopancreatic tubule epithelial cells of a PL specimen of *M. rosenbergii*. (A) Low magnification electron micrograph. (B) High magnification electron micrograph.

Prevalence of the BLV and HPLV

Percentage infection rates of both viruses in PL (n=200) were determined by evaluating the histopathological studies. Results for the PL were 20% with BLV, 50% with HPLV and 10% with dual infection (Table 1). At day 60, both small and large-sized juvenile prawns in the nursing earthen ponds (P) showed similar percentages of infection from BLV, HPLV and dual infection (Table 2), and the percentage infection rates were lower than the infection rates during the PL stage. At day 120, prawns from both P1 and P2 had similar percentages of infection from both viruses. No BLV infection was observed in P3 and P4 at day 150 and 180. However, HPLV was observed from both ponds and the two different-sized prawns. This study indicated that the infection rates of these two viruses did not increase during the growout period. Moreover, these two viruses were not associated with growth retardation in cultured *M. rosenbergii*. In contrast, stunted *P. monodon* in growout ponds were related to HPV or dual infection with MBV and HPV (Flegel *et al*., 1999; Vasinamaykin, 2005; Joradol, 2005) .

After the prawns were harvested at day 180, the yield from both P3 and P4 showed similar percentage survival rates (Table 3). The percentages of small-sized prawns were lower than those of large-sized prawns due to the cannibalistic behavior of this species. The prawn farmers transferred only male prawns from P1 and P2 to P3 and P4 for the final growout period, thus the small-sized prawns might have been eaten by the larger ones during the molting period. The yield

Table 1 Percentage of BLV, HPLV and dual infection (BLV and HPLV) by histological examination from PL.

Sample (n=200)	BLV infection	HPLV infection	Dual infection	Uninfected	Total
Number of PL	40	100	20	40	200
Average (%)	20	50	10	20	100

from this study was only 197-199 kg/1600 m^2 compared with 305 kg/1600 m^2 reported by Panthong (2005). This study differed from that of Panthong (2005) in that both small and large-sized prawns were randomly transferred into P3 and P4, but in Panthong's study only large-sized prawns were transferred into the final growout ponds.

CONCLUSIONS

BLV and HPLV in PL and adult *M. rosenbergii* were identified. Histopathology of BLV revealed enlarged nuclei with marginated nucleoli and eosinophilic inclusions that completely filled the nuclei of the HP tubulular

Table 2 Percentage of BLV, HPLV and dual infection (BLV and HPLV) in giant freshwater prawns during the culture period.

Pond	Days	Size	Average weight (g)	Number of prawns (n=20)	Percentage of BLV infection	Percentage of HPLV infection	Percentage of dual infection
P	60	small	2.83 ± 0.67	20	15	30	15
		large	6.80 ± 0.77	20	10	25	10
P1	120	small	10.15 ± 0.65	20	10	25	5
		large	19.63 ± 1.0	20	5	25	5
P2	120	small	9.25 ± 0.92	20	15	20	5
		large	18.93 ± 1.22	20	5	15	0
Average	120	small	9.70 ± 0.91		12.5	22.5	5.0
		large	19.28 ± 1.15		5.0	20.0	2.5
P3	150	small	30.13 ± 1.13	20	0	15	0
		large	42.48 ± 1.52	20	0	10	0
P4	150	small	31.15 ± 1.41	20	0	15	0
		large	45.35 ± 1.29	20	0	15	0
Average	150	small	30.64 ± 1.41		0	15.0	0
		large	43.91 ± 2.01		0	12.5	0
P3	180	small	41.18 ± 0.91	20	0	20	0
		large	69.25 ± 1.69	20	0	10	0
P4	180	small	40.48 ± 1.11	20	0	5	0
		large	68.93 ± 1.77	20	0	15	0
Average	180	small	40.83 ± 1.06		0	12.5	0
		large	69.09 ± 1.69		0	12.5	0

Table 3 Production of giant freshwater prawns at harvest time.

Pond	Days	Size	Average	Production		Number of prawns		Survival
			weight(g)	kg/1,600 m^2	%	prawns	%	rate %
P3	180	small	41.18	55.27	28	1,342	39.54	67.89
		large	69.25	142.11	72	2,052	60.46	
			Total	197.38	100	3,394	100	
P4	180	small	40.48	54.88	27.56	1,356	39.32	68.96
		large	68.93	144.23	72.44	2,092	60.68	
			Total	199.11	100	3,448	100	

epithelium. TEM of HP tissues showed BLV particles of approximately 250-300 nm in enlarged nuclei. HP tissue of prawns infected by HPLV revealed medium to large eosinophilic to lightly basophilic intranuclear inclusions. TEM of affected nuclei revealed HPLV particles of approximately 20-24 nm in diameter.

These two viruses did not increase from PL to adult prawns during the 180-day culture period. Moreover, the percentage infection rates of these viruses from both small and large-sized prawns were similar. Further studies are needed to compare the survival rates of virus-infected and non-infected PL during the growout period.

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