

## Decontamination of Monodon Baculovirus in Marine Shrimp Eggs using Upwelling Flow-Through System

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### Abstract

*Penaeus monodon*-type baculovirus (MBV) is a horizontal transmission virus disease especially found in marine shrimp. Even though sanitization of MBV contamination in shrimp hatcheries by using clean seawater or chemicals has been recommended, the prevalence of the disease in larvae is still frequently reported. This study is carried out in an attempt to improve previous sanitization methods by using the upwelling flow-through vessel developed for this study. Eggs of *Penaeus monodon* were obtained from infected broodstocks collected from the wild. Decontamination of MBV was performed using three methods. Treatment 1: The eggs were continuously rinsed with chlorinated seawater for 1 min and transferred to a nursing tank. The eggs were allowed to hatch and reared to the postlarval stage in a nursing tank. Treatment 2: The eggs were rinsed with chlorinated seawater for 1 min and hatched in the upwelling vessel. Following hatching, the nauplius larvae were reared to the postlarval stage in a nursing tank. Treatment 3: The eggs were bathed with 20 mg/l iodophore for 30 s and hatched in the upwelling vessel. Following hatching, the nauplius larvae were reared to the postlarval stage in a nursing tank. There was no MBV observed in shrimp at the nauplius stage in all treatments. However, at day 11, when larvae developed to postlarva 1, the infection was found in the shrimp of treatment 1. At day 15 (postlarva 5), MBV was detected in all treatments. At day 20 (postlarva 10), viral incidences were observed in all treatments. This concludes that the upwelling flow-through system developed in this study is able to delay the occurrence of MBV in shrimp larvae. However, it is most likely that horizontal transmission of MBV in *P. monodon* is associated with other means for which the eradication processes, including iodophore bathing and egg cleansing, provide insufficient protection against.

**Keywords:** Decontamination, Monodon Baculovirus, MBV, shrimp egg, upwelling flow-through system

### Introduction

*Penaeus monodon*-type baculovirus (MBV) is found in many parts of the world, from tropical to temperate areas, including the adjacent waters of Australia, East Asia, South East Asia, India, East Africa, North America and the Middle East. Occurrences of MBV have long been reported in Taiwan since 1981 [1], before spreading out to South East Asia and to Thailand in 1991 [2] via trading of shrimp seed. MBV infection causes economic loss, due to reduction of survival and poor growth performance [3], and even more seriously causes reduction in the survival rates of the hatchery-reared larval, postlarval, and early juvenile stages of *P. monodon* [4]. Unfortunately, no treatment has been reported [5]. The virus, when carried in broodstocks, can be transmitted to offspring, via horizontal transmission exclusively by cannibalism and fecal-oral contamination [6]. In hatcheries, oral ingestion of feces of MBV infected broodstock is the main source of MBV infection in larvae [7]. Chen *et al.* [7] suggested that elimination of MBV in larval shrimp can be done by washing fertilized eggs or nauplii with clean seawater, formalin

and iodophore. However, even with this series of eradication processes, infection still occurs in postlarval shrimp. In breeding programs, undetermined effective treatment of MBV is one of the major constraints in obtaining specific pathogen free *P. monodon*, where a large number of expensive broodstock and intensively reared seed have been discarded through quarantine processes [8].

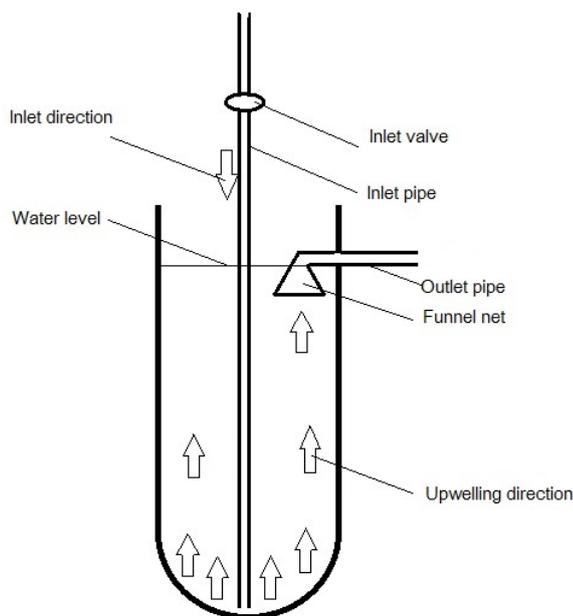
This study was carried out in an attempt to improve the previous sanitization methods by using an upwelling flow-through vessel that has been developed. We presume that the continuous flow-through system can enable removal of virus attached to the outer surface of the eggs during hatching.

## Materials and methods

### Upwelling flow-through vessel

Two upwelling flow-through vessels used as MBV eradication device were made. Each vessel was made of a fiberglass cylinder of 21 cm diameter and 45 cm height with a rounded bottom (**Figure 1**). The cylinder had an outlet pipe (2 cm diameter) attached to a side 14 cm below the cylinder's top. The inner end of the outlet pipe was a closed end, connected with a funnel net of 100 micron mesh size. This was to retain the eggs within the cylinder during the operation of the device.

Upwelling was done by feeding water into the vessel through a tin inlet pipe of 1 cm diameter. The pipe was located in the center, extending to the bottom of the cylinder. The opened end of the inlet pipe was located 1 cm above the bottom. The total operating water volume of the cylinder was 10,500 ml.



**Figure 1** An upwelling flow-through vessel of 10,500 ml operating volume, showing components and flow directions (drawing not to scale).

### Decontamination of MBV

The experiment was carried out in the quarantine unit of the Walailak University Shrimp Excellence Center, Thailand. MBV infected eggs of *P. monodon* were obtained from wild broodstock. The eggs were collected from spawning tanks using a scoop net of 100 micron mesh size. The eggs were washed and once again filtered using a net of 300 micron mesh size to remove fecal pellets and debris. The eggs were

then used for MBV decontamination examination. The eradication feasibility was examined using 3 different methods, as follows. Treatment 1: The eggs were continuously rinsed with chlorinated seawater for 1 min and transferred to a nursing tank. The eggs were allowed to hatch and rear to the postlarval stage in that nursing tank. Treatment 2: The eggs were rinsed with chlorinated seawater for 1 min and hatched in the upwelling vessel. Following hatching the nauplius larvae were reared to the postlarval stage in a nursing tank. Treatment 3: The eggs were bathed with 20 mg/l iodophore for 30 s and hatched in the upwelling vessel. Following hatching the nauplius larvae were reared to the postlarval stage in a nursing tank. The experiments were repeated 3 times on different occasions according to the availability of broodstock. A total of 95,200 - 112,000 eggs were used in each treatment replication. Larvae were reared in the nursing tank at a density of 8-9 shrimp/l. Larvae of the protozoa stage were fed exclusively with diatom of *Chaetoceros* sp. Larvae of the mysis stage were fed with a mixture of the diatom and *Artemia* nauplii. Larvae of the postlarval stage were fed exclusively with *Artemia* nauplii. The diatom was obtained from cultivation of high cell-density cultures, propagated in laboratory conditions. *Artemia* nauplii were obtained from the encapsulated cyst. Water quality prior to use was measured in terms of oxygen concentration, pH, alkalinity, and total ammonia and nitrite concentrations (**Table 1**).

**Table 1** Physicochemical properties of the water used in the experiments.

Water temperature (°C)	Salinity (ppt)	DO (mg/l)	pH	Alkalinity (MgCaCO <sub>3</sub> /l)	Total ammonia (mg NH <sub>3</sub> N/l)	Nitrite (mg NO <sub>2</sub> N/l)
27.5 - 29.4	30 - 32	6.12 - 6.43	7.6 - 8.3	120 - 160	0.02 - 0.10	0.05 - 0.10

#### Examination of MBV

Examination of MBV contamination in shrimp was done in the stages of egg, nauplius, postlarva 1, postlarva 5 and postlarva10, and also in *Artemia* nauplii. A real-time PCR technique was utilized, following the method developed by Wuthisuthimethavee *et al.* [9] with some modifications. Samples of 0.1 g tissue were homogenized with a 1 ml extraction buffer (100 mM Tris-HCl, pH 8; 100 mM EDTA, pH 8; 250 mM NaCl, 1 % SDS and 100 µg proteinase K/ml extraction buffer) in 1.5 ml microcentrifuge tube, and incubated at 65 °C for 1 - 2 h. Total DNA was extracted using phenol: chloroform: Isoamyl alcohol. The aqueous (upper) layer (approximately 500 µl) was transferred to a new tube, and mixed with an equal volume of cold absolute ethanol. The mixture containing the DNA was stored in a freezer (-20 °C) for 24 h, and then centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was discarded, leaving the visible (precipitate) pellet in the tube. The total DNA pellet was washed with 1 ml 70 % ethanol and centrifuged at 13,000 rpm for 5 min at 4 °C. The ethanol was discarded, and the pellet was air dried for a few minutes. The dried pellet was resuspended in 100 µl of 1X TE buffer and stored at -20 °C until use.

Purified plasmids containing the genomic sequences of MBV were used as positive controls. The PCR reaction mixture containing the amplified 135 bp products of MBV was purified and cloned into a pGEM-T Easy Vector (Promega, Madison, WI), and transformed into *Escherichia coli* DH10B competent cells. A recombinant clone was identified as a white colony on an X-gal and IPTG plate. The plasmid DNA was purified using a High Pure Plasmid Isolation Kit (Roche), and this DNA was used as a PCR positive control template. Ten-fold serial dilutions of positive control plasmid DNA template were used to construct a standard curve for real-time PCR analysis. A set of standard curves was constructed using seven dilutions of each control plasmid to determine the copy number of MBV infection in shrimp (1×10<sup>6</sup> to 1 copy per reaction). The standard curves were evaluated in duplicate to demonstrate reproducibility. The specific primers and probe used for the real-time PCR are shown in **Table 2**.

**Table 2** Specific primers and probe sequence for MBV detection.

Primers/probe	Nucleotide sequence
MBV_F	5'-ACT ACC ATA AGC TAG CAT ACG TCC TTT-3'
MBV_R	5'-AAA GGT CAG CAA AAA ACA CTC AAT A-3'
MBV_probe	5'-ACC CTC TAC CGA TAT GGT ATC AAT GTC TGG AGT T-3'

A real-time PCR assay was performed using a 7300 Real-time PCR System (Applied Biosystems, USA). The amplification reactions were performed in volumes of 20  $\mu$ l, with 2  $\mu$ l of template DNA (10 ng/ $\mu$ l), 0.5  $\mu$ M of each MBV primer F/R, 0.4  $\mu$ M MBV probe, 0.25 mM dNTP mix, 2  $\mu$ l of 10x buffer (+Mg<sup>2+</sup>) (final concentration 1x), and 0.1 Fast Taq DNA polymerase (Roche, USA). The real-time PCR amplification consisted of 1 cycle at 94 °C for 3 min, then 94 °C for 30 s; 60 °C for 1 min, repeated 40 - 50 cycles. Amplification, detection, and data analysis were performed with a Mastercycler Realplex.

#### Statistical analysis

All analyses were performed in triplicate. Data of shrimp survival rates and percentages of hatching were subjected to analysis of variance (ANOVA). A comparison of means was carried out by Duncan's new Multiple Range Test (MRT). Analysis was performed using a SPSS package (version SPSS 10 for Window, SPSS Inc).

### Results and discussion

#### Hatching viability using upwelling flow-through vessel

Two upwelling vessels were used, each accommodating proximately 100,000 eggs, or at a density of proximately 9.5 eggs/ml. The upwelling flow rate of water was adjusted to a speed of 42 l/h, which was sufficient only to suspend the individual egg in water column (i.e., it was not wash out of the vessel). It was found that the flow rate of 42 l/h was not adequate to float most of the eggs. This made some eggs settle on the bottom. A low hatching rate was obtained at 39.5 $\pm$ 7.8 % due to the envelop surrounding the eggs [10], which obstructed gas ventilation and consequently reduced hatching viability. The flow rate was then readjusted to 102 l/h to float all individual eggs. However, with this flow rate, the eggs were drained out of the vessels. The vessel was then modified by attaching a funnel net to the outlet pipe, to retain the egg within the vessel. The hatching rate was increased to 77.5 $\pm$ 4.9 % (9.5 eggs/ml) compared to the hatching rate of 81.0 % (10 eggs/l) in the nursing tank. This hatching rate is acceptable for commercial hatcheries as recommended by the FAO (> 75 %) [11]. The flow rate of 102l/hr was then used for MBV eradication.

#### Decontamination of MBV

The results showed that MBV was not detected in the eggs and nauplii right after treating with all treatments (**Table 4**). This suggested that washing of the egg surface only by using clean seawater was sufficient to remove MBV, compared to eggs bathed with 20 mg/l iodophore. However, the hatching viabilities of eggs were different among the treatments. The hatching rates of eggs in the upwelling vessels (76.3 $\pm$ 9.3 %) were not different from those of eggs hatched in the rearing tanks (72.7 $\pm$ 14.6 %), whereas iodophore at 20 mg/l, as recommended by Chen *et al.* [7], showed an adverse effect in reduction of the hatching viability of the eggs (63.7 $\pm$ 15.8 %). Within 2 days after hatching, MBV contamination was not observed in nauplii in both those that hatched through the upwelling flow-through vessels and through the nursing tanks. This indicates that simply washing the infected eggs with clean seawater is sufficient to eradicate MBV, providing that the virus only attaches to the egg outer surface and is not embedded in the egg mass.

At day 11, when larvae developed to postlarva 1, infection was observed, firstly found in the shrimp of treatment 1 (eggs rinsed with clean seawater, hatched and reared in the nursing tanks). At day15 (postlarva 5), MBV was detected in shrimp in all treatments of some replicates. At day 20 (postlarva 10),

the incidence of MBV was observed in shrimp in all treatments of every replicate. The survival rates (%) of P-10 in treatments 1, 2, and 3 were  $12.3 \pm 8.6$ ,  $14.7 \pm 3.1$ , and  $13.3 \pm 8.5$ , respectively ( $p > 0.05$ ) (**Table 4**).

The results of this experiment suggested that the washing of eggs by using clean seawater was sufficient to remove MBV, providing that the pathogen is transmitted horizontally, and attached solely to the outer surface of the egg. The upwelling flow-through system may enable removal of virus associated with egg albumen (if existing) during hatching resulting in extending the time of viral incidences. Horizontal transmission of MBV has been declared by several studies [6,7,12], and it has not been proven that transmission can also be vertical from broodstock to offspring [13]. However, the study of Sankar *et al.* [14] suggested that the most likely means of transmission of MBV is vertical. Chen *et al.* [7] found that, when eggs were initially disinfected using iodophore at a concentration of 20 mg/l for 30 s, MBV was not initially observed until the postlarva 12 stage, which is compatible to the results of this study. However, cross contamination due to handling was unlikely to be occurring in this study, given that the life food organism was clean and the rearing facilities were well disinfected. Furthermore, the experiments were carried out in a highly cautious disease control unit and operated by skilled workers. Contamination from feeding material was also negligible, because there was no MBV detected in Artemia. The study of Sarathi *et al.* [13] has also proven that Artemia exposed to MBV by immersion and oral administration is not a vector for MBV transmission when fed to *P. monodon*.

**Table 3** Hatching viability of shrimp eggs in different experiments using upwelling flow-through vessels.

Treatments	Percent hatch
Treatment 1: Eggs rinsed with clean seawater, hatched in nursing tanks	$76.3 \pm 9.3^a$
Treatment 2: Eggs rinsed with clean seawater, hatched in upwelling vessels	$72.7 \pm 14.6^a$
Treatment 3: Eggs bathed with 20 mg/l iodophore, hatched in upwelling vessels	$63.7 \pm 15.8^b$

Values presented with the same letter are not significantly different ( $p > 0.05$ ).

**Table 4** Occurrences of MBV in various shrimp stages of 3 experiments; (+) indicates positive test and (-) indicates negative test.

Tested samples	Treatment 1	Treatment 2	Treatment 3
Eggs from infected broodstocks	(+) (+) (+)	(+) (+) (+)	(+) (+) (+)
Decontaminated eggs (day 0)	(-) (-) (-)	(-) (-) (-)	(-) (-) (-)
Nauplius (day 2)	(-) (-) (-)	(-) (-) (-)	(-) (-) (-)
Postlarva 1 (day 11)	(+) (+) (-)	(-) (-) (-)	(-) (-) (-)
Postlarva 5 (day 15)	(+) (+) (-)	(+) (-) (-)	(-) (-) (+)
Postlarva 10 (day 20)	(+) (+) (+)	(+) (+) (+)	(+) (+) (+)
Survival rates of P-10 (%)	$12.3 \pm 8.6^a$	$14.7 \pm 3.1^a$	$13.3 \pm 8.5^a$

Values presented with the same letter are not significantly different ( $p > 0.05$ ).

**Treatment 1:** Infected eggs rinsed with chlorine disinfected seawater, hatched, and reared to the postlarval stage in nursing tanks.

**Treatment 2:** Infected eggs rinsed with chlorine disinfected seawater, hatched in upwelling vessels, and reared to the postlarval stage in nursing tanks.

**Treatment 3:** Infected eggs were bathed with 20 mg/l iodophore, hatched in upwelling vessels, and reared to the postlarval stage in nursing tanks.

## Conclusions

The upwelling flow-through vessel developed in this study can be potentially developed on a larger scale for commercial hatcheries, in terms of providing good hatching rates and being less labor intensive. However, the system is not viable in removal of MBV in the infected egg. Cleaning of MBV contaminated eggs could be simply done by using clean seawater, providing that the pathogen is solely horizontally transmitted. However, it is most likely that horizontal transmission of MBV in *P. monodon* is associated with other means for which the eradication processes, including egg washing, provide insufficient protection against. It is suggested that the use of MBV infected prawns as broodstocks should be avoided.

## Acknowledgements

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