

## **Prevalence of White Spot Syndrome Virus (WSSV) and Monodon Baculovirus (MBV) Infection in *Penaeus monodon* Postlarvae in Vietnam**

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### **ABSTRACT**

A survey on the nature and prevalence of white spot syndrome virus (WSSV) and monodon baculovirus (MBV) infection in *Penaeus monodon* postlarvae collected in hatcheries in the centre and the south of Vietnam was conducted from mid December 2001 to mid May 2002. A total of 471 samples were collected and subjected to nested polymerase chain reaction (PCR) analysis for WSSV. Of these, 388 samples were also tested for MBV using rapid staining with Malachite green. The prevalence of WSSV infection in postlarvae collected from hatcheries and nurseries was 20.6% (95% CI: 17.1-24.6%), whereas 46.4% (95% CI: 41.4-51.5%) prevalence was found in samples tested for MBV. In addition, it was found that the prevalence of WSSV infection fluctuated according to sampling month and was the highest in February (37.7%; 95% CI: 28.7-48%) while the prevalence of MBV infection remained the same throughout sampling period. Samples from the central region, which supplies approximately 80% of postlarvae for grow-out ponds in southern provinces, displayed a slightly higher prevalence of WSSV infection (21.1%; 95% CI: 16.3-26.7%) than those from the south (15.7%; 95% CI: 9.17-25.3%), although this difference was not statistically significant. The prevalence of MBV infection in postlarvae from the central region was not significantly different the prevalence in samples from the south (44.3%, 95% CI: 37.9-50.8% and 46%, 95% CI: 33.5-58.9%, respectively). Differences in prevalence indicated that quality of postlarvae varies according to production areas and sources of supply. The implications of the findings for shrimp health management in Vietnam are discussed.

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## INTRODUCTION

The Mekong River Delta of Vietnam has greatest potential for shrimp farming in Vietnam and it is developing very rapidly. In the year 2000, the shrimp culture production area of the Mekong Delta accounted for 180,000 ha of total 226,000 ha area for the whole country. However, viral disease is a serious constraint for sustainable shrimp farming in the Delta. According to Hao (1999), reported that WSSV was responsible for mass mortalities of shrimp in the Duyen Hai District of Tra Vinh Province and that up to 58-62% of postlarval batches used for stocking were infected. Preston *et al.*, 2001 confirmed the presence of serious viral diseases, including white spot disease (WSD) in *Penaeus monodon* postlarvae, juveniles and adult shrimp from farms in Gia Rai District of Bac Lieu Province. However, the source(s) of these viral infections are yet to be determined. At the beginning of 2001, WSSV has had a serious impact on shrimp farming in 7 coastal provinces in the Mekong River Delta of Vietnam including Dong Nai, Ben Tre, Tra Vinh, Soc Trang, Bac Lieu, Ca Mau and Kien Giang (Vietnam Youth newspaper No 25 dated 20/02/2001). A survey of the epizootic of WSD in Taiwan showed that monodon baculovirus (MBV) was frequently present in co-infections with WSSV in *P. monodon* (Wang *et al.*, 1997). However, information on MBV infection in shrimp postlarvae in the Mekong River Delta is very limited.

As postlarvae supplied to shrimp farms in the Mekong Delta are mainly imported from the central provinces, they are in short supply and often in very poor condition after having been transported long distances by road. Information on the prevalence of WSSV and MBV infection in these postlarvae is lacking. This study has aimed to obtain information on the prevalence of these shrimp viral diseases in the region, in order to determine possible sources of infection of postlarvae and farm stocks, and to assist in developing management strategies to limit these infections.

## MATERIALS AND METHODS

### Source of shrimp postlarvae used for the study

Four hundred and seventy-one postlarval batches of *Penaeus monodon* were collected during the period between mid December 2001 to mid May 2002. Among these, 256 postlarval batches were collected from hatcheries in six provinces (Khanh Hoa, Ninh Thuan, Phan Thiet, Binh Thuan, Da Nang and Vung Tau) in the central region and 89 postlarval batches were collected from hatcheries in five (Soc Trang, Bac Lieu, Tra Vinh, Kien Giang and Ca Mau) provinces in the southern region of Vietnam. The remaining 126 postlarval batches were collected from shrimp nurseries located in Mekong River Delta provinces and had been transported from hatcheries either the central or southern regions of the country (Table 1). Each larval batch contained 200 postlarvae (PL<sub>12</sub>-PL<sub>15</sub>) sampled from the same tank. The postlarvae were transported live to the fish disease laboratory at Can Tho University in oxygenated nylon bags. Samples were analysed as soon as possible after arrival in the laboratory.

**Table 1.** Occurrence of infections and mixed-infection in *Penaeus monodon* postlarval (PL) batches collected from Vietnam in 2001-02.

Collection location	WSSV(*)		MBV (#)		Mixed-infection
	PL batches analysed	No. positive batches and prevalence of infection (%)	PL batches analysed	No. of positive batches and prevalence of infection (%)	No. of positive batches and prevalence of infection (%)
<b>Central Provinces</b>					
Khanh Hoa	112	40	97	43	13
Ninh Thuan	44	2	40	19	0
Phan Thiet	53	6	53	25	9
Binh Thuan	29	4	29	10	2
Da Nang	8	0	8	5	0
Vung Tau	10	2	10	3	2
Total	256	54 (21%; 95% CI: 16.3-26.7%)	237	105 (44.3%; 95% CI: 37.9-50.8%)	26 (10.1%; 95% CI: 6.9-14.7%)
<b>Southern Provinces</b>					
Soc Trang	14	2	14	9	3
Bac Lieu	22	3	22	9	4
Tra Vinh	4	0	0	0	0
Kien Giang	41	8	22	11	2
Ca Mau	8	1	8	1	1
Total	89	14 (15.7%; 95% CI: 9.17-25.3%)	66	30 (46%; 95% CI: 5.8-20.2%)	10 (11.2%; 95% CI: 5.8-20.2%)
<b>Collected from nurseries in the Mekong Delta region</b>					
Total	126	29 (23%; 95% CI: 16.2- 31.5%)	85	45 (52.9%; 95% CI: 41.9-63.8%)	9 (7.1%; 95% CI: 3.5-13.5%)

(\*) WSSV infection as detected by PCR

(#) MBV infection as detected by histology

### Detection of monodon baculovirus

The rapid Malachite green staining method (Lightner *et al.* 1996) was used for detection of MBV occlusion bodies. Briefly, a squash preparation of hepatopancreas was stained with 0.05% aqueous Malachite green and examined directly under a light microscope. Infection was detected by observation of single or multiple spherical occlusion bodies which stained more intensely than similar sized normal host cell nuclei, secretory granules or lipid droplets. Twenty postlarvae from each batch were examined for MBV. Batches containing  $\leq 8$  infected postlarvae were recorded as light infections and higher numbers of infected postlarvae were recorded as medium infections.

### Detection of WSSV

**Extraction of total nucleic acid (TNA).** TNA was extracted from each postlarval batch using the DNA extraction kit IQ2000 WSSV (Farming IntelliGene Tech. Corp., Taiwan). About 25 postlarvae from each postlarval batch were first blot dried on tissue paper and emulsified with disposable grinding pestles in 2 ml Eppendorf tubes containing 0.6 ml of a premixed dodecyl-trimethylammonium bromide (DTAB) solution, incubated in a heating block at 75°C for 5 minutes and then cooled to room temperature. The mixture was then mixed briefly and centrifuged, 0.7 ml of chloroform was added, and then briefly vortexed

and centrifuged at 12,000 x g for 5 minutes. The upper aqueous phase was transferred to a new 2 ml Eppendorf tube, mixed briefly with 100 µl of a premixed N-cetyl N,N,N-trimethylammonium bromide (CTAB) solution and 900 µl double-distilled water, and incubated at 75°C for 5 minutes before cooling to room temperature. After centrifugation at 12,000 x g for 10 minutes and carefully decanting the supernatant, the pellet was resuspended with 150 µl Dissolving Solution (provided with the kit), incubated at 75°C for 5 minutes and then allowed to cool to room temperature. After centrifugation at 12,000 x g for 5 minutes, the supernatant was transferred to a fresh 0.5 ml Eppendorf tube mixed with 300 µl 95% ethanol, vortexed and centrifuged at 12,000 x g for 5 minutes. The pellet was then washed with 200 µl 70% ethanol, dried at room temperature and resuspended in 200 µl ET buffer (0.1 mM EDTA, 0.1 mM Tris-HCl, pH 8.0).

**Amplification of WSSV DNA.** The IQ 2000 WSSV detection kit is a two-step sequential semi-quantitative assay targeting a conserved region of WSSV DNA sequence. In the first step, a 910 bp fragment was amplified. 2 µl of TNA template was added to 8 µl PCR Pre-Mix reagent and 0.5 µl of IQzyme DNA polymerase (2U/µl). Paraffin oil was used to cover the mixture and PCR was conducted as follows: 5 cycles at 94°C for 20 seconds, 62°C for 20 seconds, and 72°C for 20 seconds; 15 cycles at 94°C for 10 seconds, 62°C for 10 seconds, and 72°C for 15 seconds; and a final extension cycle at 72°C for 30 seconds. In the second step, PCR Pre-Mix reagent including nested primers and IQzyme DNA polymerase (2U/µl) were added to amplify three fragments (910, 550, 296 bp) from the initial amplification product. PCR was conducted as follows: 25 cycles of 94°C for 20 seconds, 62°C for 20 seconds, 72°C for 30 seconds, followed by 72°C for 30 seconds. A series of dilutions of WSSV plasmid DNA containing 10<sup>3</sup>, 10<sup>2</sup> and 10<sup>1</sup> copies (supplied with the kit) were used as positive standards, and yeast tRNA (also supplied with the kit) was used as a negative control.

After amplification, 10 µl of the PCR product was mixed with 2 µl of 6 × loading dye (provided with the kit) and loaded onto a 2% agarose gel containing 0.5 µg/ml ethidium bromide. Three serial dilutions 10<sup>1</sup>, 10<sup>2</sup> and 10<sup>3</sup> (supplied with the kit) of WSSV plasmid DNA were used as positive controls to interpret as light, medium or severe infections, respectively. Electrophoresis was performed in 1 × TAE buffer (20 mM Tris-HCl pH 7.5, 20 mM glacial acetic acid, 0.5 mM EDTA, pH 8.0) at 100 V for ~30 minutes. Amplified fragments were visualised under ultraviolet transillumination and photographed.

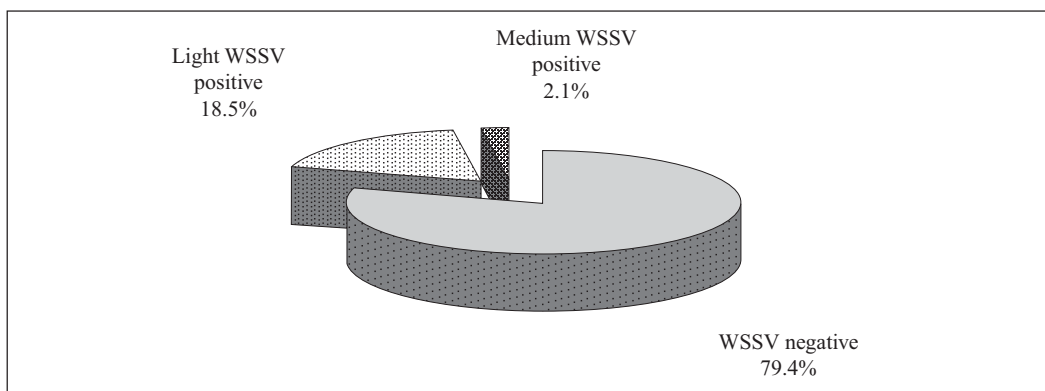
### Statistical analysis

Prevalence data were analysed using the EpiCalc 2000 program at 95% confidence interval.

## RESULTS

### Overall prevalence of WSSV infection

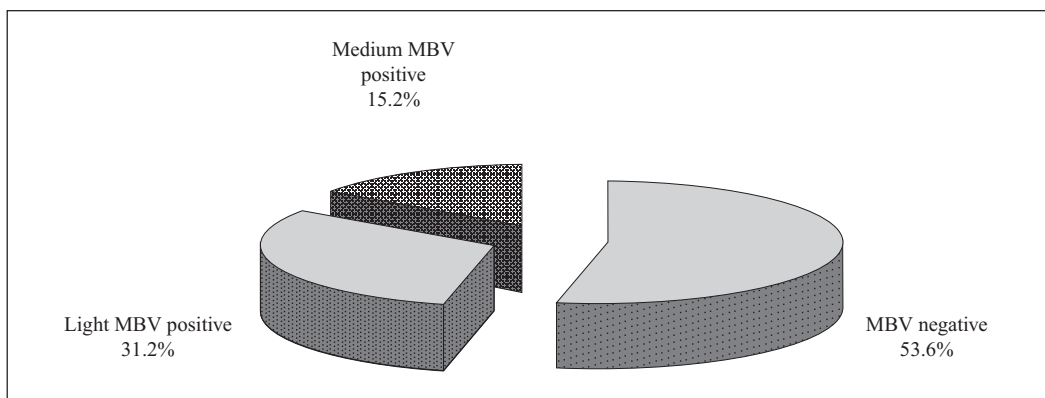
Nested PCR was used to determine the prevalence of WSSV infection in 471 postlarval batch samples originating from hatcheries in the central and southern regions of Vietnam and collected from hatcheries or nurseries between December 2001 and May 2002. The overall prevalence of WSSV infection in all postlarvae samples collected was 20.6% (95% CI: 17.1-24.6%) (Table 1). A total of 87 samples (18.5%; 95% CI: 15.2-22.4%) were graded as medium level infections and 10 samples (2.1%; 95% CI: 1.1-4%) were graded as light infections (Figure 1).



**Figure 1.** WSSV detection in *P. monodon* postlarval batches

### Overall prevalence of MBV infection

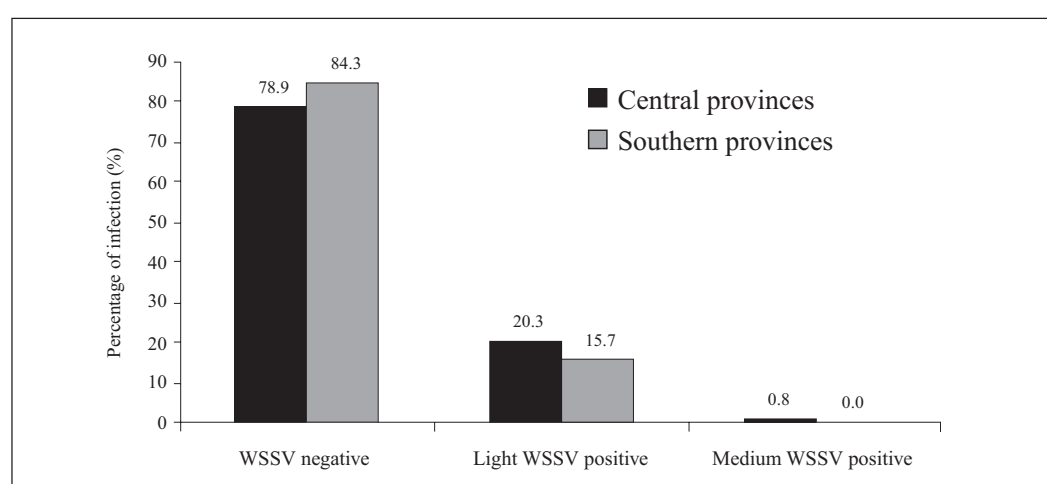
The Malachite green staining method for inclusion bodies was used to determine the prevalence of MBV infection in 388 of the 471 samples tested for WSSV. The overall prevalence of MBV infection in all postlarval batch samples tested was 46.4% (95% CI: 41.4-51.5%) (Table 1). A total of 59 samples (15.2%; (95% CI: 11.9-19.3%)) were graded as medium level MBV infections and 121 samples (31.2% (95% CI: 26.7-36.1%)) were graded as light infections (Figure 2).



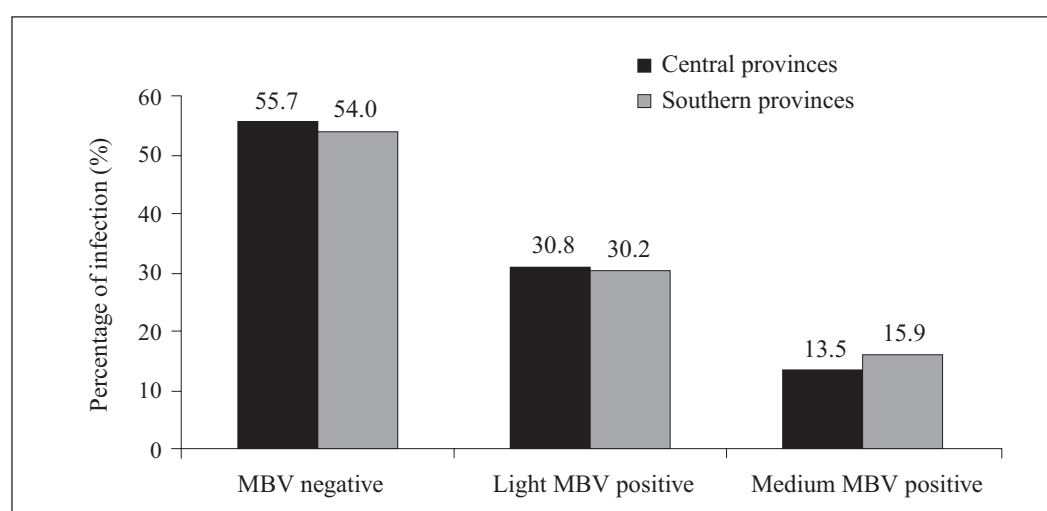
**Figure 2.** MBV detection in *P. monodon* postlarval batches

### Prevalence of WSSV and MBV infections by production areas

There was a slightly higher prevalence of WSSV infection in samples from the central region (21.1%; 95% CI: 16.3-26.7%), where hatcheries supply most of postlarvae used for grow-out ponds in southern Provinces, than those from the southern region (15.7%; 95% CI: 9.17-25.3%) (Figure 3). However, the difference in prevalence was not statistically significant (Chi-squared = 1.2,  $P = 0.27$  for 1 degree of freedom). The prevalence of MBV infection in postlarvae from the central Provinces (44.3%, 95% CI: 37.9-50.8%) also did not vary significantly compared with samples from the southern Provinces (46%, 95% CI: 33.5-58.9%) (Chi-squared = 0.03,  $P = 0.86$  for 1 degree of freedom) (Figure 4).



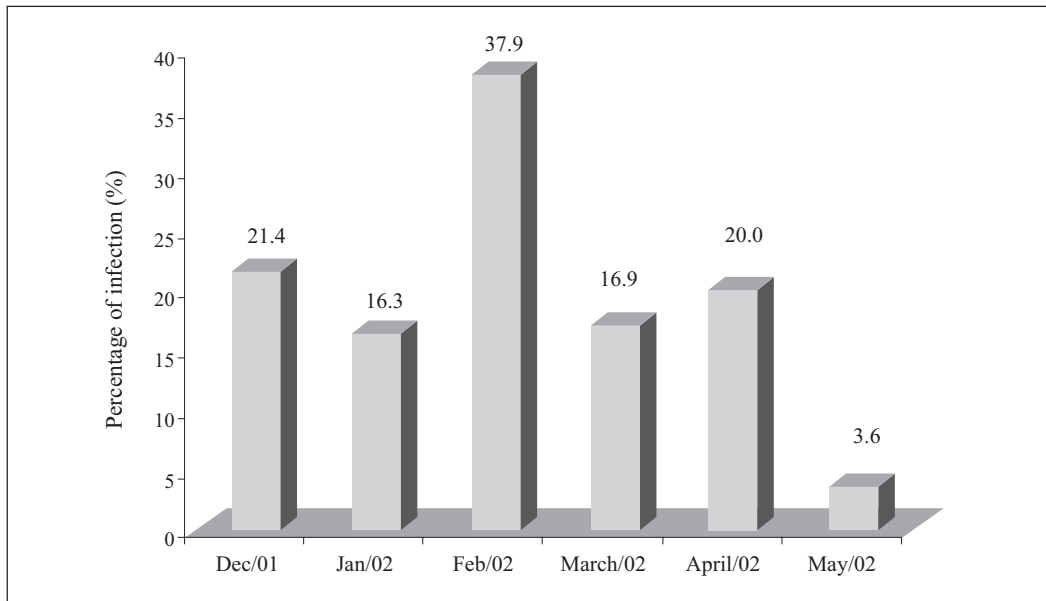
**Figure 3.** Prevalence infection of WSSV by production areas.



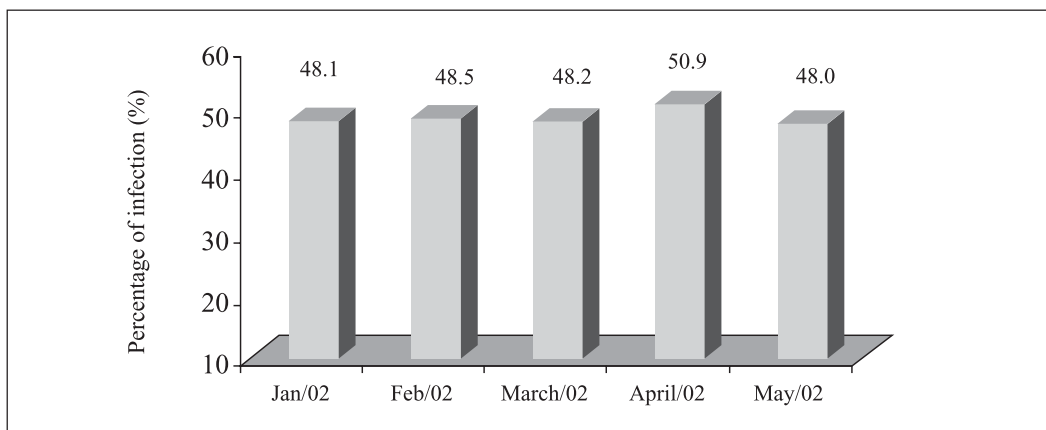
**Figure 4.** Prevalence infection of MBV by production areas.

### Prevalence of WSSV and MBV infections by sampling months

Prevalence of WSSV infection fluctuated according to sampling month (Figure 5). The difference was statistically significant (Chi-squared = 30.73,  $P = 0.000011$  for 5 degree of freedom) and the highest prevalence occurred in February (37.7%; 95% CI: 28.7-48%) (Table 2). However, there was no statistically significant difference in the prevalence of MBV infection (Figure 6) in different months of the sampling period (Chi-squared = 0.14,  $P = 0.99$  for 4 degree of freedom).



**Figure 5.** Prevalence infection of WSSV by sampling month.



**Figure 6.** Prevalence of MBV positive by sampling month.



**Table 2.** Occurrence of infections in *Penaeus monodon* postlarval (PL) batches collected from Vietnam by sampling months in 2001-02.

Collection period (2001-2002)	WSSV(*)		MBV#	
	PL batches analysed	No. positive batches and infection prevalence (%)	PL batches analysed	No. of positive batches and infection prevalence (%)
December	42	9 (21.4%; 95% CI: 10.8 - 37.2%)	-	-
January	86	14 (16.3%; 95% CI: 9.5-26.2%)	54	26 (48.1%; 95% CI: 34.5-62%)
February	103	39 (37.9%; 95% CI: 28.7- 48%)	101	49 (48.5%; 95% CI: 38.5-58.6%)
March	124	21 (16.9%; 95% CI: 11- 25%)	110	53 (48.2%; 95% CI: 38.6-57.9%)
April	60	12 (20.0%; 95% CI: 11.2-32.7%)	55	28 (50.9%; 95% CI: 37.2-64.5%)
May	56	2 (3.6%; 95% CI: 0.6-13.4%)	50	24 (48.0%; 95% CI: 33.9-62.4%)
Total	471	97 (20.6%; 95% CI: 17.1-24.6%)	388	180 (46.4%; 95% CI: 41.4-51.5%)

\*WSSV infection as detected by PCR

#MBV infection as detected by histology

### Prevalence of mixed infection in postlarval batches

Postlarval batches scoring as mixed WSSV and MBV infections are shown in Table 1. The overall prevalence of mixed infection in all postlarval batches tested was 9.6 % (95 % CI: 7.2-12.7%). The prevalence of mixed-infection in tested poslarval batches from the central region was 10.1% (95% CI: 6.9-14.7%) and the prevalence in batches from the southern region was 11.2% (95% CI: 5.8-20.2%).

## DISCUSSION

The total prevalence of WSSV infection in all tested poslarval batches in the present study in Vietnam was 20.6% which is relatively low compared with data from other reports. In Taiwan, a prevalence of WSSV infection in a survey of wild *P. monodon* has been reported to be 75.8% (Lo *et al.*, 1996). In a survey in India, 61% of healthy *P. monodon* poslarvae in collected from hatcheries were reported to be positive for WSSV by using nested PCR test (Otta *et al.*, 1999). In Vietnam, Hao (1999) reported 58-62% prevalence of WSSV infection in stocked *P. monodon* postlarval batches tested in the Duyen Hai District of Tra Vinh Province. The lower prevalence of WSSV infection detected in samples collected in our study may have been due to the different geographical origin of the samples, reflecting real special or temporal differences in the prevalence of infection in wild broodstock populations, or it may be due be different or improved management practices that have led to improved postlarval quality in hatcheries. Prevalence of MBV infection in the 388 samples tested was 46.4%. Unfortunately, other information on prevalence of MBV infection in stocked shrimp in Vietnam is very limited. According to Flegel 1999, MBV is not considered to be a serious pathogen for the black tiger shrimp but high prevalence infection in seed stock is still a threat with potential to cause slow growth in intensive culture.



Postlarval samples from the central region displayed a slightly higher prevalence of WSSV infection than those from the south, although this difference was not statistically significant. Thuy (2001) also found no difference in prevalence of WSSV infection from tested postlarvae from Khanh Hoa Province in the central region and Ca Mau Province in the south. In our study, batches of postlarvae collected in the central Provinces were transported for about 7 hours to the laboratory prior to testing and this represents similar transportation conditions to those batches supplied to nurseries and farms in the Mekong Delta.

The prevalence of MBV infection did not vary significantly during the sampling period and ranged from 48.0% to 50.9%. Chanratchakool *et al.* (1998) has observed that MBV may be very widespread in Southeast Asia, making it difficult to find postlarvae larvae that are free from MBV infection. Unlike MBV, the prevalence of WSSV infection varied significantly by sampling months during our study with the lowest prevalence (2.4%) observed in samples collected in October 2001, increased gradually until January 2002 (16.3%) and peaked in February (37.7%), after which the prevalence dropped to 3.6% in May. The maximum prevalence of WSSV infection in postlarval batches collected in February coincided with the season when weather conditions are variable causing changes such as high fluctuation of water temperatures between day and night. This season also corresponded to the most common stocking period in the Mekong Delta when there was a high demand for shrimp seed, potentially resulting in the common use of poor quality postlarvae.

The overall prevalence of mixed WSSV and MBV infections in tested postlarval batches was 9.5%. In a survey of white spot disease in Taiwan, Wang *et al.* (1997) reported that MBV and WSSV co-infections occur commonly with WSSV in *P. monodon*. The study described WSSV as the main cause of mass mortality and suggested MBV or *Vibrio* spp., together with the effect of various environmental stressors, may also play a role in the mass mortality of infected shrimp.

There were difficulties to obtain a truly representative sample of postlarval batches under the management and conditions in place in Vietnam. Therefore, the collection of postlarval batches used in this study was done to ensure that these samples were as representative as possible under the circumstances. However, we recognise that the observations reported here apply only to the batches of postlarvae collected from these sources at these times and may not be entirely representative.

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