Asian Fisheries Science **20**(2007):227-239
ISSN: 0116-6514
https://doi.org/10.33997/j.afs.2007.20.3.001

Asian Fisheries Society, Manila, Philippines

A Study by PCR on the Prevalence of Whitespot Syndrome Virus, Monodon Baculovirus and Hepatopancreatic Parvovirus Infection in *Penaeus monodon* Post Larvae in Tamil Nadu, Southeast Coast of India

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Abstract

The present study reports the prevalence of Whitespot syndrome virus (WSSV), Monodon baculovirus (MBV) and Hepatopancreatic parvovirus (HPV) in the *Penaeus monodon* post larvae in Tamil Nadu, Southeast coast of India. The PCR diagnosis of postlarvae revealed that 9.2% of the samples were infected with WSSV as they were positive either in one step or nested PCR. The PCR diagnosis of samples also showed that 9.6 and 6.4% of the samples screened were positive for MBV and HPV, respectively. Dual infection with MBV and HPV was observed by PCR in 2.6% of the samples screened. Multiple infection with WSSV, MBV and HPV was observed at 1.6% of the samples by PCR. Wet mount squash preparation from the hepatopancreas of postlarvae

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revealed that 5.5, 5.2 and 1.8% of the samples tested were positive for MBV, HPV and dual infection with MBV and HPV, respectively.

Introduction

Disease outbreaks due to viral infection are the major constraints to the shrimp culture operation. Whitespot syndrome virus (WSSV) is highly virulent and it causes mass mortalities in shrimp (Lo et al. 1996; Lo and Kou 1998). Monodon baculovirus (MBV) causes disease and mortality in postlarvae and juveniles, predisposes shrimp to secondary infection and affects their growth (Ramasamy et al. 1995; Johnson and Lightner 1988). Hepatopancreatic parvovirus (HPV) has been reported to be associated with the mortalities in larval stages of shrimp and stunted growth in juveniles (Flegel et al. 1992; Lightner et al. 1993; Limsuwan 1999). Multiple infection in postlarvae with all the three above mentioned viruses have been reported in India (Manivannan et al. 2002). Various diagnostic methods have been developed for the detection of the viral pathogens in shrimp viz., histopathology (Lightner et al. 1993; Takahashi et al. 1994; Wang et al. 1995; Catap et al. 2003), immunological methods (Nadala et al. 1997; Shahul Hameed et al. 1998; Hsu et al. 2000), *in situ* hybridization method using DNA probes (Lu et al. 1993; Lu et al. 1995; Durand et al. 1996; Chang et al. 1996; Pantoja and Lightner 2001; Phromjai et al. 2002), and Polymerase chain reaction (PCR) technique (Nunan and Lightner 1997; Otta et al. 1999; Tapay et al. 1999; Hsu et al. 2000; Phromjai et al. 2002). Among the various diagnostic methods, polymerase chain reaction (PCR) method is considered to be the most sensitive method for detecting shrimp viruses (Lightner and Redman 1998; Phromjai et al. 2002). Data on the prevalence of various diseases will help the shrimp culturists follow the practice of screening the shrimp for carriers of pathogens and take up suitable management measures to avoid the entry of the pathogens into the rearing systems and subsequent production losses. The present study reports the prevalence of white spot syndrome virus (WSSV), monodon baculovirus (MBV) and hepatopancreactic parvovirus (HPV) by PCR.

Materials and Methods

Sample collection

Penaeus monodon postlarvae (PL 10-15) were collected from commercial shrimp hatcheries located in various parts of Tamil Nadu, Southeast coast of India, during the period October 2003 – December 2004. A total of 920 postlarvae samples were tested in this study. The postlarval samples were brought to the shrimp disease diagnosis laboratory, Tamil Nadu Veterinary and Animal Sciences University in live condition in oxygenated plastic bags. Each sample comprised of a minimum of 500 seeds collected from individual tanks. About 100 post larvae were randomly selected from the samples for screening of MBV and HPV infection by microscopic method and polymerase chain reaction (PCR). For PCR, the samples were fixed in 70% ethanol.

Diagnosis of MBV and HPV by wet mount method

The wet mount method as described by Lightner (1996) for rapid diagnosis of MBV was followed for the preliminary diagnosis of MBV and HPV infection in post larvae.

Diagnosis of WSSV, MBV and HPV infection by polymerase chain reaction (PCR).

About 50 post larvae randomly selected from a sample and fixed in 70% ethanol were used for viral DNA extraction. The preserved sample was rehydrated in distilled water for 1h and the DNA was extracted using a commercial DNA extraction kit (Bangalore Genei Pvt. Ltd., Bangalore). Aliquots of the extracted DNA were used for PCR diagnosis of WSSV, MBV, and HPV. Nested PCR assay of a commercial diagnostic kit (Bangalore Genie Pvt. Ltd.) was followed for the diagnosis of WSSV. The PCR program for the non-nested reaction consisted of an initial delay of 95^{o}C for 3 minutes followed by 28 cycles of 95^{o}C for 30 sec; 58^{o}C for 30 sec; 72^{o}C for 30 sec with a final extension at 72^{o}C for 5 min. For nested PCR reaction, 1µl of the non-nested PCR product was taken as DNA template. The components and the cycling conditions were similar to the non-nested reaction.

For MBV diagnosis, PCR primers (MBV 1.4F and MBV 1.4R, MBV 1.4NF and MBV 1.4 NR) of Belcher and Young (1998) were used. The PCR program followed was the same as the WSSV PCR program.

For HPV diagnosis, PCR primers and one step PCR protocol of Phromjai et al. (2002) were followed. The PCR program consisted of an initial delay of 95°C for 5 min followed by 40 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min with final extension of 72°C for 7 min.

Results

Microscopic observation of wet mounts of squashed hepatopancreatic tissue of postlarvae infected with MBV revealed the presence of multiple spherical occlusion bodies of MBV inside the enlarged nuclei of the hepatopancreatic cells (Fig. 1). The samples infected with HPV showed intranuclear inclusion bodies in hypertrophied nuclei with lateral displacement and compression of host cell nucleolus (Fig. 2). Multiple infections with MBV and HPV were also observed. Although some of the samples screened did not show MBV-specific occlusion bodies or HPV-specific inclusion bodies, they were positive for MBV and/or HPV by nested PCR and one step PCR, respectively.

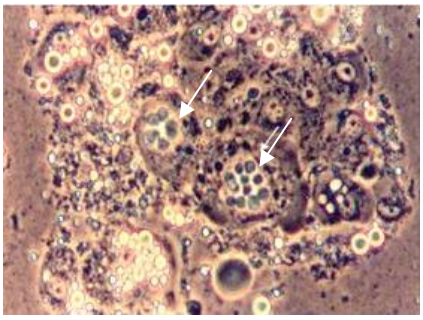


Fig. 1. MBV infected cells showing multiple spherical occlusion bodies in wet mount squashes of the hepatopancreas

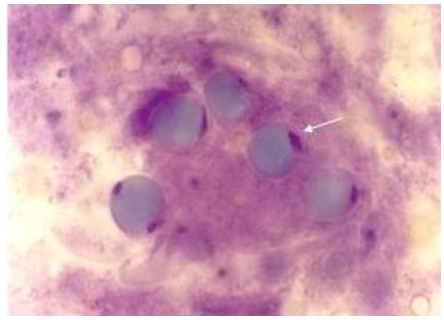


Fig. 2. HPV infected cells showing intranuclear inclusion bodies in wet mount squashes of the hepatopancreas

Post larval samples with severe WSSV infection yielded a band at 650bp in one step PCR and 300 bp in nested PCR. Samples with mild infection produced a band at 300 bp only by nested PCR (Fig. 3). In uninfected healthy post larvae, there was no viral DNA amplification and no band was seen on the gel. Similarly, post larvae with severe MBV infection yielded a band at 533 bp in one step PCR reaction. Mild infection with MBV in post larvae resulted in a band at 361 bp by nested PCR (Fig. 4). The PCR amplification of DNA from HPV infected post larvae resulted in a band of 441 bp size (Fig. 5) in one step PCR. In all the above

PCR reactions, positive controls, negative controls and distilled water were included to check for the false positive and false negative reactions.

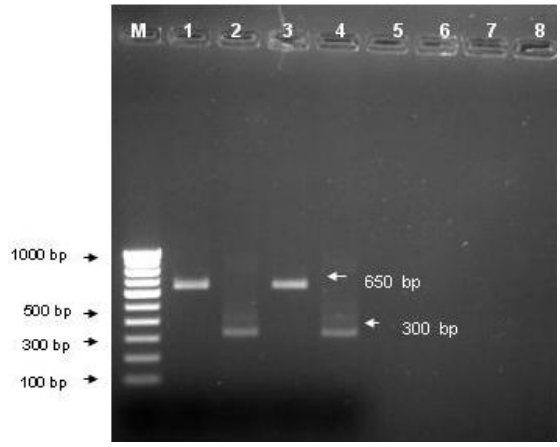
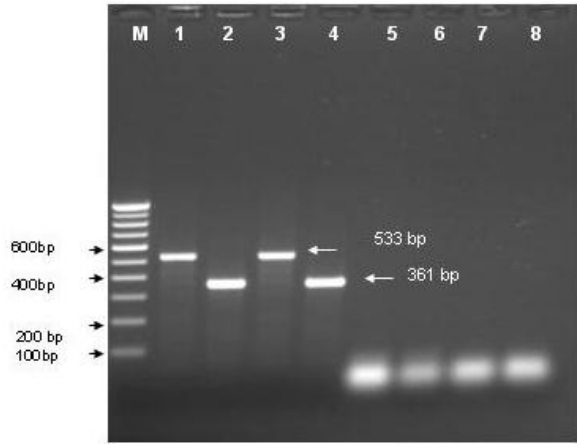


Fig. 3. Agarose gel electrophoresis of PCR amplicon from WSSV infected and uninfected postlarvae. Lane M: 100 bp molecular weight DNA ladder. Lanes 1 and 3: WSSV DNA from infected postlarvae and positive control yielding 650 bp amplicon by one step PCR. Lanes 2 and 4: WSSV DNA from infected postlarvae and positive control yielding 300 bp amplicon by nested PCR. Lanes 5 and 6: healthy uninfected postlarvae. Lanes 7 and 8: negative control

Fig. 4. Agarose gel electrophoresis of PCR amplicon from MBV infected and uninfected postlarvae. Lane M: 100 bp molecular weight DNA ladder. Lanes 1 and 3: MBV DNA from severely infected postlarvae and positive control yielding 533 bp amplicon by one step PCR. Lanes 2 and 4: MBV DNA from postlarvae with mild infection and positive control yielding 361 bp amplicon by nested PCR. Lanes 5 and 6: healthy uninfected postlarvae. Lanes 7 and 8: negative control



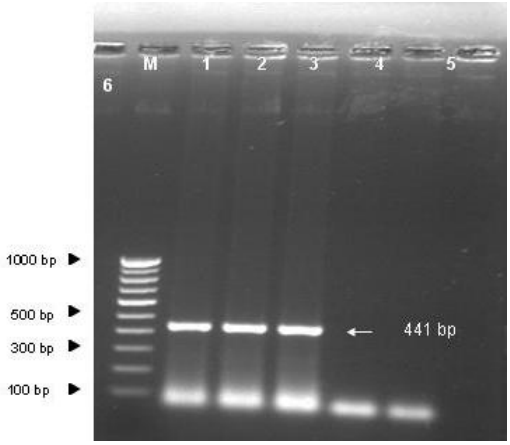


Fig. 5. Agarose gel electrophoresis of PCR amplicon from HPV infected and uninfected postlarvae. Lane M: 100 bp molecular weight DNA ladder. Lanes 1 and 2: HPV DNA from infected postlarvae yielding 441 bp amplicon. Lane 3: HPV positive control DNA yielding 441 bp amplicon. Lane 4: healthy uninfected postlarvae. Lane 5: negative control

The prevalence of WSSV in various post larval samples screened is presented in table 1. Out of 920 samples screened for WSSV by PCR, 109 samples were positive giving an overall prevalence of 11.8%. Forty six samples (5%) were positive in one step PCR indicating a high degree of infection. When the non-nested PCR negative samples were subjected to nested PCR, 63 (6.8%) of the total samples were found to be positive for WSSV. Monthly prevalence of WSSV varied from 0 - 24.1%.

Table 1. Prevalence of WSSV in shrimp post larvae from October 2003 to October 2004

Months	WSSV Positive		Prevalence % of WSSV in total
	One step PCR	Nested PCR*	
October 2003	11/75	2/75	17.3
November 2003	0/16	0/16	0
December 2003	0/14	0/14	0
January 2004	0/9	0/9	0
February 2004	1/39	4/39	12.8
March 2004	5/52	7/52	23
April 2004	4/125	9/125	10.4
May 2004	8/224	18/224	11.6
June 2004	8/262	15/262	8.8
July 2004	6/58	8/58	24.1
August 2004	1/26	0/26	3.8
September 2004	2/12	0/12	16.6
Total	46/920	63/920	11.8

* The one step PCR negative samples were subjected to nested PCR.

The prevalence of MBV in various post larval samples screened by PCR is presented in table 2. Out of 920 samples screened, 62 (6.7%) were positive for MBV by wet mount method and 121 (13.1%) samples were positive for MBV by PCR. The prevalence during various months ranged from 0 to 15.4% and 7.7 to 42.0% by wet mount method and PCR, respectively.

The prevalence of HPV in various post larval samples screened by PCR is presented in table 3. Out of 920 samples screened, 48 (5.2%) were positive for MBV by wet mount method and 73 (7.9%) were positive for HPV by PCR. The prevalence of HPV during various months ranged from 0 to 34.6% by PCR.

The prevalence of multiple viral infections due to MBV-HPV and WSSV-MBV-HPV in various post larval samples screened by PCR is presented in table 4. Out of the 920 screened by PCR, 30 (3.3%) were observed to have dual infection with MBV and HPV. Twelve (1.3%) samples had multiple infections with WSSV, MBV and HPV. The preva-

lence of MBV-HPV and WSSV-MBV-HPV infections in the samples in the various months ranged from 0 to 19.2% and 0 to 3.8%, respectively.

Table 2. Prevalence of MBV in shrimp post larvae from October 2003 to October 2004

Months	MBV Positive			Prevalence % of MBV	
	Wet mount	One step PCR	Nested PCR*	Wet mount	PCR in total
October 2003	0/75	0/75	7/75	0	9.3
November 2003	2/16	2/16	0/16	12.5	12.5
December 2003	0/4	0/4	1/4	0	25.0
January 2004	0/9	0/9	2/9	0	22.2
February 2004	1/39	3/39	0/39	2.5	7.7
March 2004	3/52	4/52	3/52	5.8	13.5
April 2004	8/125	10/125	5/125	6.4	12.0
May 2004	14/224	19/224	4/224	6.25	10.3
June 2004	22/262	28/262	7/262	8.4	13.4
July 2004	8/58	10/58	6/58	13.8	42.0
August 2004	4/26	7/26	2/26	15.4	34.6
September 2004	0/12	0/12	1/12	0	8.3
October 2004	0/18	0/18	2/18	0	
Total	62/920	83/920	38/920		

* The one step PCR negative samples were subjected to nested PCR.

Table 3. Prevalence of HPV in shrimp post larvae from October 2003 to September 2004

Months	HPV Positive		Prevalence % of HPV	
	Wet mount	PCR	Wet mount	PCR
October 2003	4/75	7/75	5.3	9.3
November 2003	0/16	0/16	0	0
December 2003	0/4	1/4	0	25
January 2004	1/9	2/9	11.1	22.2
February 2004	0/39	2/39	0	5.1
March 2004	0/52	3/52	0	5.8
April 2004	6/125	10/125	4.8	8.0
May 2004	15/224	17/224	6.7	7.6
June 2004	12/262	16/262	4.6	6.1
July 2004	4/58	6/58	6.9	10.3
August 2004	6/26	9/26	23.1	34.6
September 2004	0/12	0/12	0	0
Total	48/920	73/920	5.2	7.9

Discussion

The above results show that the prevalence of diseases due to WSSV, MBV and HPV is a problem for shrimp hatchery and farm operations in India. Prevalence of viral infections due to WSSV, MBV and HPV have been reported from various countries (Fegan et al. 1991; Light-

ner et al. 1992; Lo and Kou 1998; Ramasamy et al. 1995; Lu et al. 1993; Magbanua et al 2000; Umesha et al. 2003; Corsin et al. 2003; Withyachumnarnkul et al. 2003). Among the various diagnostic methods, PCR is considered to be the most suitable method for accurate and rapid detection of shrimp viral pathogens. The PCR assays for the detection of shrimp viruses have been reported by various researchers (Sukhumsirichart et al. 1999; Pantoja and Lightner 2000; Phromjai et al. 2002). It has been reported that WSSV infection cause heavy mortalities in shrimp and subsequent production losses in shrimp aquaculture (Chang et al. 1996; Chou et al. 1998). Detection of WSSV by PCR in the brooder and wild shrimp populations has been reported in India (Hossain et al., 2001; Uma et al. 2002). WSSV can invade the follicle cells, oogonia, developing oocytes and connective tissue in the ovary and WSSV positive brooders produce post larvae which are either positive or negative for WSSV by PCR (Lo et al. 1997). The prevalence of WSSV in post larvae (11.8%) reported in this study suggests the possibility of vertical transmission of the virus. The prevalence of WSSV observed in this study is lower when compared to our earlier observation (13%) during the period from March 2000 to May 2002 in the southeast coast of India (Uma et al. 2002) and the prevalence (75%) reported by Otta et al. (2003) from the west coast of India.

Table 4. Prevalence of multiple viral infections due to WSSV, MBV and HPV in shrimp post larvae from October 2003 to September 2004

Months	MBV-HPV	WSSV-MBV-HPV	Prevalence % of multiple infection	
			MBV-HPV	WSSV-MBV-HPV
October 2003	1/75	0/75	1.3	0
November 2003	0/16	0/16	0	0
December 2003	0/4	0/4	0	0
January 2004	1/9	0/9	11.1	0
February 2004	0/39	0/39	0	0
March 2004	1/52	0/52	1.9	0
April 2004	2/125	1/125	1.6	0.8
May 2004	8/224	5/224	3.6	2.2
June 2004	9/262	4/262	3.4	1.5
July 2004	3/58	1/58	5.2	1.7
August 2004	5/26	1/26	19.2	3.8
September 2004	0/12	0/12	0	0
Total	30/920	12/920	3.3	1.3

MBV is considered to be a potentially serious pathogen in the larval stages of shrimp (Baticados et al. 1991; Natividad and Lightner 1992). A mortality of up to 90% has been recorded in India in the postlarvae of

P. monodon due to MBV infection (Ramasamy et al. 1995). The MBV prevalence in post larvae has been reported in India by various researchers (Ramasamy et al. 1995; Karunasagar et al. 1998; Otta et al. 2003). An MBV prevalence of 81% by wet mount method and 54% by PCR has been recorded in the hatcheries located in the southeast coast (Ramasamy et al. 1995) and west coast of India respectively (Otta et al. 2003). The MBV prevalence reported in this study by wet mount method and PCR were 6.7% and 13.1%, respectively. The variations in the sampling method and the number of samples screened may contribute for the difference in the prevalence rate. The MBV is prevalent in *Penaeus monodon* broodstocks in many countries including India (Fegan et al. 1991; Lightner et al. 1992; Ramasamy et al. 2000). Transmission of MBV occurs from broodstocks to larvae by faecal contamination of the spawned eggs (Chen et al. 1990). Use of infected broodstock would be the major source of MBV infection in the post larvae. However, avoiding faecal contamination of spawned eggs and larvae by thoroughly washing nauplii or eggs with formalin, iodophore and clean seawater would help to prevent MBV infection (Chen et al. 1990). The lower prevalence of MBV observed in this study could be due to such preventive measures followed in the hatcheries.

The HPV infection in shrimp has been reported to be associated with mortalities in larval stages (Lightner et al. 1993), stunted growth in juvenile stages (Flegel et al. 1992; Limsuwan 1999; Phromjai et al. 2002). The HPV infection has been reported in postlarvae by various researchers (Manivannan et al. 2002; Phromjai et al. 2002; Catap et al. 2003), and HPV transmission is likely to occur from broodstocks to offspring through feacal contamination. Screening of broodstocks for HPV and use of uninfected healthy broodstocks for seed production would help to avoid the infection due to HPV.

Earlier reports (Manivanan et al. 2002; Umesha et al. 2003) and the results of the present study on multiple infections of post larvae with HPV, WSSV and MBV in Indian shrimp hatcheries stress the need for rigorous screening of the brooders and post larvae for viral infections.

Conclusions

The results of the above study show that WSSV, MBV and HPV are prevalent in the *P. monodon* post larvae produced from the shrimp hatcheries of Tamil Nadu, Southeast coast of India. It is evident from the results that although wet mount method will be useful for preliminary screening of post larvae for MBV and HPV, screening of post larvae for WSSV, MBV and HPV by highly specific and sensitive method like PCR is necessary to avoid the entry of the virus into the grow out systems.

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Received January 27, 2005; Accepted May 10, 2006