Occurrence of Multiple Viruses in *Penaeus monodon* Shrimp Ponds and Their Effect on Shrimp Production

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ABSTRACT

The prevalence of multiple viruses (HPV, MBV and WSSV) in *Penaeus monodon* shrimp ponds along the west coast of Karnataka, India was studied by nested PCR. WSSV was found in all the 22 ponds studied (7 in non nested PCR and 15 in nested PCR). MBV and HPV were mostly found in dual infections with WSSV (19 ponds showed the presence of both MBV and WSSV and 9 ponds were positive for HPV and WSSV) or in triple infections (7 ponds were found positive for all the 3 viruses). Of these 22 ponds, 15 ponds that showed dual or triple infections by nested PCR resulted in successful crops with a total production ranging from 1.1 to 1.9 t/ha. However, mortality that resulted in emergency harvests occurred in 7 ponds where WSSV was found positive by non-nested PCR and other viruses by nested PCR. This result indicates that *P. monodon* can tolerate a low level of infection by all 3 of these viruses if environmental conditions are optimal.

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INTRODUCTION

The shrimp culture industry saw important growth during the 1980s that was mainly due to technological breakthroughs and high profitability. However, viral diseases have recently become an important limiting factor for the shrimp aquaculture industry throughout the world (Leung and Tran, 2000). To date, over 22 different viruses are known to infect shrimp, and several of them have been associated with mass mortalities in cultured shrimp (Hsu *et al.,* 2000).

Hepatopancreatic parvovirus (HPV) infects several penaeid shrimp species and was first reported in *Penaeus merguiensis* and *P. indicus* (Chang and Loh, 1984) and in *P. chinensis* (Lightner and Redman, 1985). Cultured as well as wild captured penaeid shrimp have been reported to act as hosts for HPV. For example, Manjanaik *et al.* (2005) reported the prevalence of HPV in wild penaeid shrimp in India. HPV-infected shrimp do not show specific gross signs of disease and those that do show signs of disease frequently tend to be infected by other pathogens that may mask the actual effect of HPV (Flegel *et al.,* 1992, 1999).

White spot syndrome virus (WSSV) continues to be the most serious cause of shrimp disease faced by shrimp growing countries. It affects most of the commercially important species of penaeid shrimp (Lightner, 1996), and many cultured and captured crustaceans have also been found to harbour it (Hossain *et al.,* 2001a; Chakraborty *et al.,* 2002). The presence of both WSSV and MBV in *P. monodon* postlarvae in India has been reported (Otta *et al.,* 2003). The presence of triple virus infections (WSSV, MBV and HPV) in postlarvae that were showing mass mortality has been reported by Manivannan *et al*. (2002).

Monodon baculovirus (MBV) was the first virus reported for *P. monodon* and the second virus reported for penaeid shrimp (Lighter and Redman, 1981). It has been implicated in mass mortalities in shrimp that are cultured at high densities (Fulks and Main, 1992). *Penaeus monodon* larvae showing 90% mortality due to MBV infection has been reported (Ramasamy *et al.,* 1995).

Penaeus monodon postlarvae showing dual infection of MBV and WSSV (Otta *et al.,* 2003) and triple infection of WSSV, MBV and HPV (Umesha *et al.,* 2003) has been reported in India. In this communication we report the occurrence of multiple viruses in culture ponds that achieved crop success.

MATERIALS AND METHODS

Sample collection

Samples of shrimp (*P. monodon*) were collected fortnightly from 22 ponds, each of approximately 1 ha area situated in Udupi and Kundapur along the southwest coast of Karnataka, India. Each sample comprised 4-6 shrimps. Soon after collection, the samples were brought to the laboratory on ice for immediate processing.

Extraction of DNA

From each sample of shrimp, gills, stomach, hepatopancreas and cuticle were removed, pooled and total DNA was extracted following the method described by Otta *et al.* (2003). DNA extracted from gills, stomach and cuticle were used for the detection of WSSV and the DNA extracted from hepatopancreas was used for the detection of both MBV and HPV.

PCR analysis

Two sets of primers were used for the detection of HPV. The PCR protocol and primer set described by Phromjai *et al.* (2002) was expected to yield a product of 441 bp (Figure 1). For nested PCR, primers internal to the 441 bp were designed in our laboratory to bind to nucleotides 156-176 and 398-420 in the GenBank sequence AF456476 and yield a 265 bp amplicon (Figure 1). The cycling conditions for the nested reaction consisted of an initial 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, with a final extension at 72°C for 5 min. The protocol and primer set described by Pantoja and Lightner (2000) that amplifies a 592 bp product was also employed.

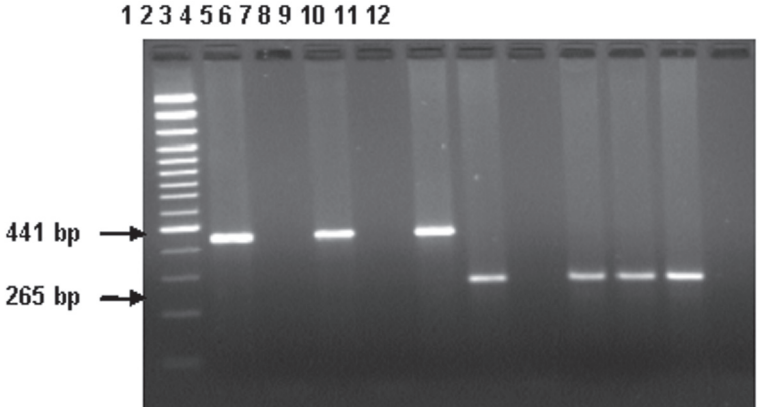

Figure.1. *Penaeus monodon*. Sample electrophoresis gel for detection of hepatopancreatic parvovirus (HPV) –specific PCR amplifications in adult *P. monodon* by 1-step. Lanes: (2 - 6) and nested (7 - 12) PCR. Lane 1: 100 bp DNA ladder Plus (Gene RulerTM genetix); Lane 2: 1-step PCR positive control; Lane 3: Negative control; Lanes 4 and 6: HPV detected by 1-step PCR; Lane 5: HPV 'negative' by 1-step PCR; Lane 7: Nested PCR positive control; Lane 8: Negative control; Lanes 9 to 11: HPV detected by nested PCR; Lane 12: HPV not detected by nested PCR

The nested primers and protocol described by Hossain *et al.* (2001a) were used for the detection of WSSV (486 bp and 310 bp respectively) (Fig. 2). For the detection of MBV, the PCR protocol and primers described by Belcher and Young (1998), yielding products of 533 and 361 bp were used (Figure 3).

PCR reactions were carried out in a 30 µl reaction mixture that consisted of 1X PCR reaction buffer, 10 pmol each primer, 200 µM dNTPs, 0.9 units of Taq DNA polymerase (Bangalore Genei, Bangalore), 2 µl of template DNA and sterile distilled water to adjust

the volume to 30 µl. All PCR reactions were carried out in an MJ Research thermocycler. The amplified products were analysed on 2% agarose gel containing ethidium bromide at a concentration of 0.5 µg/ ml and observed using a transilluminator (Gel doc system, Hero Lab).

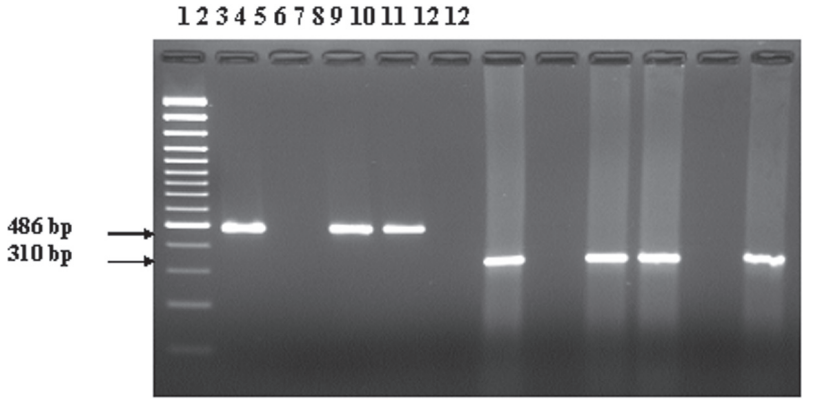

Figure 2. *Penaeus monodon.* Sample electrophoresis gel for detection of white spot syndrome virus (WSSV) –specific PCR amplifications in adult *Penaeus monodon* by 1-step (Lanes 2 to 6) and nested (Lanes 7 to 12) PCR. Lane 1: 100 bp DNA ladder Plus (Gene RulerTM genetix); Lane 2: 1-step PCR positive control; Lane 3: Negative control; Lanes 4 and 5: WSSV detected by 1-step PCR; Lane 6: WSSV 'negative' by 1-step PCR; Lane 7: Nested PCR positive control; Lane 8: Negative control; Lanes 9, 10 and 12: WSSV detected by nested PCR; Lane 11: WSSV not detected by nested PCR

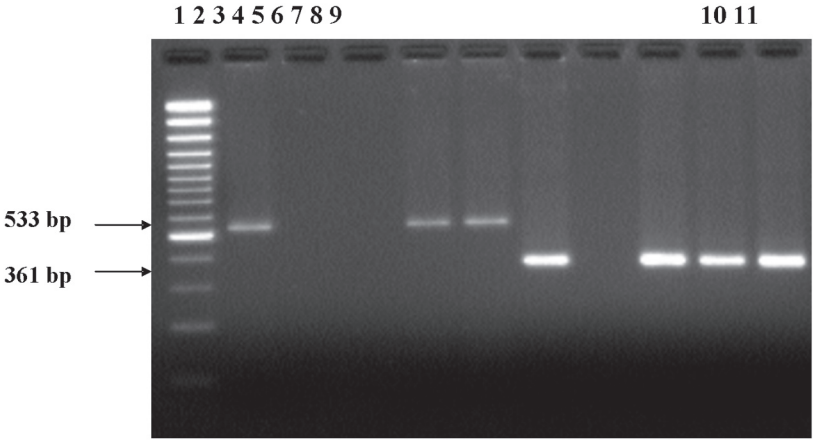

Figure 3. *Penaeus monodon.* Sample electrophoresis gel for detection of Monodon baculovirus (MBV) –specific PCR amplifications in adult *P. monodon* by 1-step (Lanes 2 to 6) and nested (Lanes 7 to 11) PCR. Lane 1: 100 bp DNA ladder Plus (Gene RulerTM genetix); Lane 2: 1-step PCR positive control; Lane 3: Negative control; Lane 4: MBV 'negative' by 1-step PCR; Lanes 5 and 6: MBV detected by 1-step PCR; Lane 7: Nested PCR positive control; Lane 8: Negative control; Lanes 9 to 11: MBV detected by nested PCR.

RESULTS AND DISCUSSION

Samples from one pond were found positive for HPV by the primers described by Phromjai *et al.* (2002) yielding a 441 bp product. Eight additional ponds were found positive for HPV by the nested PCR yielding a 265 bp product, bringing the total positive ponds to 9 (Table 1). It is interesting to note that, HPV alone was not found in any of the ponds studied, but was found in dual infections with WSSV (9/22) or in triple infections with WSSV and MBV (7/22) (Table 1). Shrimp samples from all the ponds were also analyzed for the presence of HPV by using the primers described by Pantoja and Lightner (2000), but none of the ponds were found positive. This result indicates that the strain of HPV present in India may be similar to HPV-mon reported from Thailand but different from HPV-chin. The DNA sequence of HPV in *P. monodon* from Thailand (HPV-mon) differs from that of HPV-chin in *P. chinensis* from Korea (HPV-chin) by approximately 30% (Phromjai *et al.,* 2002).

It has been reported that, HPV infection has the most significant impact in growout ponds (Flegel *et al.,* 1999; Lightner, 1996). However, in the present study, no significant difference in production ($p>0.05$) was observed between ponds with and without HPV infection.

WSSV is the most serious cause of shrimp disease in most countries where shrimp are cultivated. In the present study all the ponds studied (22/22) (Table 1) were found positive for this virus (7 ponds by non-nested PCR and 15 by nested PCR). In the initial stage of culture (2-5 weeks), WSSV alone was detected by nested PCR in 15 ponds. However, 19/22 ponds eventually showed dual infections with MBV and 9/22 showed dual infections with HPV (Table 1). Although all the ponds were positive for WSSV infection, 15 ponds were positive only by nested PCR and these ponds went through to successful harvests at between 15-17 weeks after stocking with a total production ranging from 1.1 to 1.9 t/ha (Table 1). This is normal production for India where stocking levels (6-18 postlarvae per square meter) are relatively low when compared to countries were more intensive culture (i.e., 50 or more PL per square meter) is practiced. In the 7 ponds (P15, P16, P18-P22) that were positive for WSSV by single step PCR and for other viruses by nested PCR between 5-10 weeks, mass mortality occurred and led to emergency harvests that gave production yields ranging from 0.4 – 1.4 t / ha. These results indicate that shrimp (*P. monodon*) can tolerate a low level of WSSV, WSSV/MBV, WSSV/HPV and WSSV/MBV/HPV infection. Long term presence of WSSV in shrimp culture ponds has been reported previously (Tsai *et al.,* 1999). However, stress induced by environmental factors such as pH, salinity, temperature (Hossain *et al.,* 2001b), poor pond management (Flegel *et al.,* 1995a, b) and high stocking density may promote conversion to the disease state.

Out of 22 ponds studied, MBV alone was found in only one pond (P4) in the initial stage of culture. However, 19 ponds showed dual infections with WSSV and 7 showed triple infections (Table 1). This result indicates a high prevalence of MBV in culture ponds in India. It has been reported that MBV is relatively well tolerated by *P. monodon* if other conditions are optimal (Fegan *et al.,* 1991). During the period of study, none of the shrimp from any of the ponds showed mortality that could be ascribed to MBV. We also found that MBV and HPV occurred mostly in dual or multiple viral infections, while WSSV was more frequently found alone.

Table 1. Detection of multiple viral infections in *Penaeus monodon* culture ponds by nested polymerase chain reaction.

Pond No.	Number of shrimp positive for various viruses by PCR								Pond parameters		
	WSSV	MBV	WSSV/ MBV	WSSV/ HPV	Triple	Any WSSV	Any MBV	Any HPV	Stocking density	Week of harvest	Total production
P1	3	0	4	0	0	7	4	0	7-8	16	1.4
P2	2	0	4	2	2	6	4	2	7	16	1.4
P3	3	0	3	2	2	6	3	2	9	16	1.8
P4	2	1	4	0	0	6	5	0	7	17	1.5
P5	1	0	5	3	3	6	5	3	8	17	1.5
P6	7	0	0	0	0	7	0	0	8	16	1.6
P7	4	0	3	0	0	7	3	0	9	17	1.9
P8	1	0	7	0	0	7	7	0	8	16	1.5
P9	1	0	2	0	0	3	2	0	11	15	1.4
P10	3	0	4	0	0	7	4	0	6	15	1.3
P11	2	0	3	0	0	5	3	0	8-10	15	1.6
P12	3	0	0	2	0	5	0	2	6	15	1.3
P13	0	0	3	0	0	3	3	0	7	15	1.3
P14	4	0	2	0	0	6	2	0	8-9	15	1.5
P15	0	0	4	5	4	5	4	5	16	12	1.4
P16	1	0	3	0	0	4	3	0	14	10	1.3
P17	1	0	3	2	2	4	3	2	6-7	15	1.1
P18	0	0	2	3	2	3	2	3	9	10	1.2
P19	0	0	5	1	1	5	5	1	16	7	0.8
P20	0	0	0	3	0	3	0	3	14-15	8	0.8
P21	0	0	3	0	0	3	3	0	10-11	6	0.4
P22	0	0	3	0	0	3	3	0	18	6	0.5

CONCLUSION

From this study, it can be concluded that cultured populations of P. *monodon* may serve as hosts for many viruses. While WSSV was found in single infections, MBV and HPV were mostly found as components in dual or multiple viral infections. Though *Penaeus monodon* can tolerate low-level single (WSSV), dual (WSSV/MBV, WSSV/HPV) or triple (WSSV/MBV/HPV) viral infections, it is necessary to screen the larvae for all these viruses before stocking in grow out ponds. This, together with stocking at low density can help to avoid disease outbreaks when combined with proper management practices.

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