Prevalence of white spot syndrome virus infection detected by one-step and nested PCR in selected tiger shrimp (*Penaeus monodon*) hatcheries

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Abstract White spot syndrome (WSS) is considered as a great threat to commercial farming of the tiger shrimp (*Penaeus monodon*). The causal agent of WSS is a DNA virus called white spot syndrome virus (WSSV). The prevalence of this dreadful virus infection has been studied in five randomly selected hatcheries located in the Cox's Bazar district of Bangladesh. Both one-step and nested polymerase chain reaction (PCR) involving two pairs of primers, namely, 146F1/146R1 and 146F2/146R2, amplifying the 1447 bp and 941 bp fragments, respectively, were conducted to detect the WSSV. Out of 60 randomly collected shrimps, 12 (20%) were found to be positive by one-step PCR, while 18 (30%) were found to be positive by nested PCR. The nested PCR was found to be much more sensitive than the one-step PCR. The shrimp specimens showing clinical signs of WSS were positive for WSSV by both one-step and nested PCR. Some of the apparently healthy samples were also found to be positive for WSSV by nested PCR. Among the two primer-pairs, the inner pair amplifying the 941 bp fragment was more sensitive than the outer primer pair amplifying the 1447 bp fragment when used in one-step PCR.

Keywords Detection · Nested PCR · *Penaeus monodon* · Prevalence · White spot syndrome virus

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Abbreviations

ng	Nanogram
pg	Picogram
PCR	Polymerase chain reaction
PL	Post larvae
WSS	White spot syndrome
WSSV	White spot syndrome virus

Introduction

Viral diseases are threatening problems for the global shrimp culture industry. White spot syndrome (WSS) has resulted in heavy mortalities and consequent production losses to the shrimp culture industry in many countries in Asia and Latin America (Momoyama et al. 1994; Larkins 1995; Wongteerasupaya et al. 1995; Karunasagar et al. 1997) since 1992, when it was first reported in Taiwan (Chou et al. 1995). WSS is caused by an extremely virulent DNA virus popularly known as white spot syndrome virus (WSSV). It has a wide host range and targets various tissues (Chang et al. 1996; Lightner 1996; Lo et al. 1996a; Kou et al. 1998; Maeda et al. 1998; Hossain et al. 2001). The principal clinical sign of WSS is the appearance of white spots in the exoskeleton and epidermis of the diseased shrimp. Shrimps of all age groups and sizes, irrespective of sex (Peinado-Guevara and López-Meyer 2006), in all kinds of aquaculture systems, whether extensive, semi-intensive or intensive (Karunasagar et al. 1997), are affected by WSSV.

WSSV causes serious economic losses because massive mortality can result in total crop damage within 3–10 days under farming conditions (Lightner 1996). The best method of preventing loss has been to exclude the pathogen from the production system. Transfer from captured wild broodstocks to post larvae (PL) that are used to stock rearing ponds has been considered as one of the major routes of viral introduction. Therefore, the detection of viral infection either in the brooders before being used in the hatchery or PL before stocking could help avoid this problem. Polymerase chain reaction (PCR) is a powerful tool for the detection of viral pathogens in shrimp aquaculture. It has been used for WSSV detection in broodstock and PL for the investigation of WSSV transmission routes among different hosts and for epidemiological studies (Lo et al. 1996a, 1996b; Maeda et al. 1998; Sahul Hameed et al. 2005). One-step PCR (Takahashi et al. 1996) and nested (two-step) PCR methods have been described for WSSV and it is generally recognised that nested PCR amplification methods are more sensitive than one-step PCR methods (Lo et al. 1996b; Peinado-Guevara and López-Meyer 2006).

The Penaeid shrimp of Bangladesh is considered as a highly valued product and its production and export play a dominant role in the economy of Bangladesh. Among 36 marine shrimp species available in Bangladesh, the black tiger shrimp (*Penaeus monodon*) is cultured widely in the coastal ponds or ghers of the Cox's Bazar, Khulna, Bagherhat and Satkhira districts. The shrimp farming of Bangladesh had been considered as a lucrative industry until the occurrence of WSS in the country. WSS made its first appearance in southeastern Bangladesh in 1994 and an outbreak of the disease in cultured black tiger shrimp occurred in semi-intensive farms in Cox's Bazar, where it caused losses of 50–60%. Later, the disease occurred in southwestern Bangladesh, where it severely affected small-scale farms practising low stocking density, resulting in great economic loss. The loss due to WSS from 21 farms in Bangladesh in 1994 was estimated to be US$5 million (Larkins

1995). Heavy economic loss due to WSSV infection can be effectively reduced if timely diagnosis is carried out regarding the condition of WSSV infection.

Data on the prevalence of viruses in broodstock are important for developing strategies for health management. In the case of WSSV, virus-positive broodstock may yield either virus-positive or virus-negative larvae, depending on the degree of infection (Lo et al. 1997). It would be important to know the level of infection in the broodstock, so that the risk of producing infected larvae could be assessed. We report here the prevalence of WSSV infection in the broodstock of five selected hatcheries of the Cox's Bazar region. In this study, we have also compared the efficiency of one-step and nested PCR methods in detecting the virus.

Materials and methods

Collection of experimental animals

Two types of samples were collected for the present study: selected sample and random sample. As selected samples, seven tiger shrimps (*P. monodon* showing clinical symptoms of WSS were collected from the Niribili hatchery to test whether the PCR primers developed by Lo et al. (1996b) are also effective in detecting the Bangladesh strain of WSSV. As random samples, 60 shrimps were randomly collected from five different hatcheries (12 from each), namely, the S. Alam hatchery, Chingri Bangla hatchery, Saudia hatchery, Niribili hatchery and Bolaka hatchery of the Cox's Bazar district to study the prevalence of virus infection in this region.

A small piece of pleopod/uropod (approximately 500 mg) from each specimen was clipped aseptically using scissors and transferred to 1.5-ml microfuge tubes containing 95% ethanol and then brought to the laboratory of the Department of Fisheries Biology and Genetics, Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh, Bangladesh. Finally, the samples were stored at −20°C for future use.

Extraction of DNA from shrimp tissue

Approximately 30–40 mg of tissue (pleopod/uropod) from each collected sample was cut into small pieces with scissors, homogenised by a micro-tissue-grinder in a 1.5-ml microfuge tube and the total DNA was extracted by Proteinase-K digestion, phenol:chloroform:isoamyl alcohol extraction and the ethanol precipitation method, as described by Islam and Alam (2004). The DNA quality was checked by electrophoresis in 1% agarose gel and quantified using a spectrophotometer (Spectronic® Genesis™, Spectronic Instruments Inc., USA).

Polymerase chain reaction

The primer-pairs 146F1/146R1 and 146F2/146R2 developed from the sequence of a cloned WSSV *Sal*1 1,461 bp DNA fragment by Lo et al. (1996b) that amplifies 1447 bp and 941 bp fragments, respectively, were used in the present study to amplify the WSSV DNA fragments. Another primer pair, 143F/145R, yielding a PCR product of 848 bp, derived from a highly conserved region of the 18S rRNA sequence of decapods (Kim and Abele

Table 1 Primer sequences used for the amplification of white spot syndrome virus (WSSV) and tiger shrimp DNA

Primer name	Primer sequence (5′–3′)	Size (bp)	Reference
146F1	5′-ACT ACT AAC TTC AGC CTA TCT AG-3′	1447	(Lo et al. 1996b)
146R1	5′-TAA TGC GGG TGT AAT GTT CTT ACG A-3′		
146F2	5′-GTA ACT GCC CCT TCC ATC TCC A-3′	941	
146R2	5′-TAC GGC AGC TGC TGC ACC TTG T-3′		
143F	5′-TGC CTT ATC AGC TNT CGA TTG TAG-3′ (where N represents G, A, T or C)	848	(Kim and Abele 1990; Lo et al. 1996b)
145R	5′-TTC AGN TTT GCA ACC ATA CTT CCC-3′		

1990; Lo et al. 1996b) was used as a positive control to check the quality of the DNA extracted from shrimp samples and the PCR conditions used. The sequences of the three sets of primers are shown in Table 1. The primers were synthesised and supplied by 1st Base Company Ltd., Singapore.

One-step PCR for the diagnosis of WSSV

One-step PCR reactions were performed on each DNA sample in a 10-μl reaction mixture containing 2 μl of template DNA, 0.25 μM of each primer, 0.25 mM each of dATP, dCTP, dGTP and dTTP, 1 unit of Taq DNA polymerase (GENEI, Bengalore, India), 1× Taq polymerase buffer and 1.5 mM of $MgCl_2$. The PCR amplification was performed in a personal thermal cycler (OmmE, Hybaid, UK). The reaction mixture was preheated at 95°C for 3 min for initial denaturation, followed by 35 cycles comprising 1 min denaturation at 95°C, 1 min annealing at 55°C and 2 min elongation or extension at 72°C. A final step of 5 min at 72°C followed the last cycle to allow complete extension of all of the amplified fragments.

Nested PCR for the diagnosis of WSSV

In nested PCR, the amplified products of the first step of PCR served as the template DNA for the second step of amplification. In this case, the first-step amplification was carried out by the outer primer pair 146F1/146R1 with the following cycle parameters: initial denaturation at 95°C for 3 min, followed by 15 cycles of 95°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 2 min and a final extension at 72°C for 5 min. After the completion of the first step, 10 μl of the reaction mixture was added to another 10 μl of standard PCR cocktail containing the inner primer pair 146F2/146R2, dNTPs, buffer and Taq DNA polymerase. The thermal profile and the number of cycles were the same as in one-step PCR.

Sensitivity analysis of one-step and nested PCR

The sensitivities of one-step and nested PCR were determined by using different concentrations of template DNA. Two previously analysed samples showing strong positive signs of WSSV infection from two different hatcheries (Bolaka hatchery and Niribili

hatchery) were chosen to analyse the sensitivity of one-step and nested PCR. The DNA template of the chosen samples was first diluted from 50 ng/µl to 1.0 ng/µl, 0.5 ng/µl, 0.25 ng/µl, 0.125 ng/µl, 62.5 pg/µl and 31.3 pg/µl and 20.8 pg/µl. From each dilution, 2 µl was used as the template for one-step PCR and first step of the nested PCR. In the second step of the nested PCR, the amplified product of the first step served as the template DNA.

Agarose gel electrophoresis and visualisation of the PCR product

After the completion of PCR, 2.5 µl (5 µl in the case of 20 µl nested PCR) of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol) was added to each PCR tube, mixed well, centrifuged briefly and then loaded into the wells of 1.4% agarose gel. DNA molecular weight markers (λ DNA *Hin*dIII digest and/or 100 bp DNA ladder) were loaded on either side of the gel. The gel was then observed on a UV transilluminator and photographed by the BioDoc-It^{TM} system.

Results

Specificity analysis of WSSV primers

The specificity of both WSSV primer pairs was assessed using the extracted DNA template from a shrimp showing symptoms of WSS. The primer pairs 146F1/146R1 and 146F2/146R2 yielded the expected 1447 bp and 941 bp amplicons, respectively, which suggests that these primers can also amplify the Bangladesh strain of WSSV.

The prevalence of WSSV in samples collected from five different hatcheries of Cox's Bazar

The PCR profiles of selected and random shrimp specimens collected from five different hatcheries are presented in Figs. 1 and 2. All of the seven selected samples having clinical symptoms of WSS were found to be positive by both one-step and nested PCR. Among the 60 random samples 19.99 $\pm$ 13.3% (SE) were found to be positive by one-step PCR, while 29.99 $\pm$ 12.2% (SE) were found to be positive by nested PCR (Table 2). None of the samples of the S. Alam and Saudia hatcheries were found to be positive by one-step PCR. However, two shrimps from each of these hatcheries were proved to be positive by nested PCR (Fig. 1). Among 12 samples of the Chingri Bangla hatchery, none were positive by either one-step or nested PCR. Four of the 12 random samples of the Niribili hatchery were found to be positive by one-step PCR, while six were found to be positive by nested PCR. All of the positive samples again were positive with both outer (146F1/146R1) and inner (146F2/146R2) primer pairs in the one-step PCR (Fig. 1). Eight specimens of the Bolaka hatchery were found to be positive by both one-step PCR (Fig. 2) and nested PCR (Table 2).

Sensitivity analysis of one-step and nested PCR

A relationship was observed between the concentration of the template DNA and the PCR signal in the one-step PCR, where the PCR signals became fainter with increased dilution
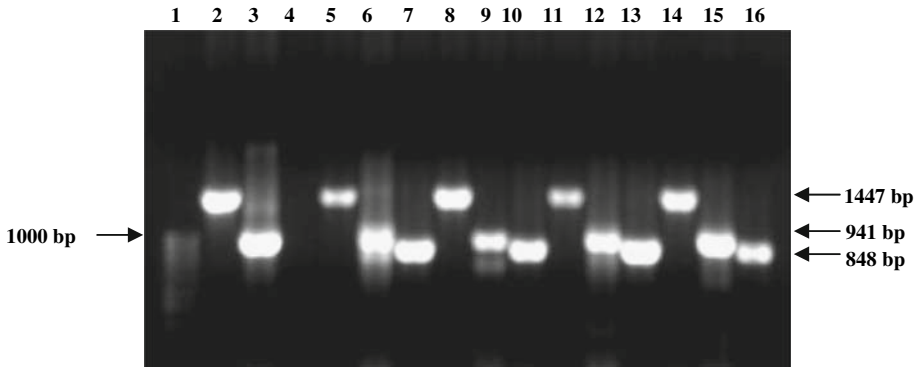
Fig. 1 Detection of white spot syndrome virus (WSSV) in *Penaeus monodon* collected from the Bolaka and Niribili hatcheries by one-step polymerase chain reaction (PCR) with primer pairs 146F1/146R1 (lanes: 2, 5, 8, 11, 14) and 146F2/145R2 (lanes: 3, 6, 9, 12, 15). The 143F/145R primer pair was used to check the quality of the DNA and the reproducibility of the PCR technique (lanes: 7, 10, 13, 16). Lanes 2–13 represent the DNA template of WSSV-infected shrimp of the Bolaka hatchery (lane 4 contained no DNA template); lanes 14–16 represent the DNA template of WSSV-infected shrimp of the Niribili hatchery. Lane 1: molecular weight marker (100 bp DNA ladder)

Fig. 2 Detection of WSSV in *P. monodon* collected from the S. Alam hatchery, Saudia hatchery, Chingri Bangla hatchery and Niribili hatchery by nested PCR. Lanes 2–7 represent the DNA templates of WSSV-infected shrimp of the S. Alam hatchery; lanes 8–14 represent the DNA template of WSSV-infected shrimps of the Saudia hatchery; lanes 15–18 represent the DNA templates of WSSV-infected shrimps of the Chingri Bangla hatchery; lanes 19–22 represent the DNA templates of WSSV-infected shrimps of the Niribili hatchery; lanes 23–24 represent the DNA templates of WSSV-infected shrimps of the Bolaka hatchery. Lane 1: molecular weight marker (λ DNA *Hin*dIII digest). Lane 25: molecular weight marker (100 bp DNA ladder)

of the template DNA and vice-versa. In the case of nested PCR however, the PCR signals were more or less similar up to certain dilutions of the template DNA (Fig. 3). In the case of the Bolaka hatchery sample, 41.6 pg templates DNA showed very weak positive signal and the sample of the Niribili hatchery showed negative sign at a template content of 0.50 ng in one-step PCR. In the case of nested PCR, both samples showed a clear positive signal of WSSV infection, even at a 41.6 pg template content (Fig. 3). Then, the sample of the Bolaka hatchery was chosen to analyse the sensitivity of non-nested PCR with outer and inner primer pairs and, for this purpose, 10 ng to 31.2 pg template DNA was used and

Table 2 Prevalence of WSSV infection in random samples collected from five different tiger shrimp (*P. monodon*) hatcheries located in the Cox's Bazar region, Bangladesh

| Name of hatchery	Sample size	Prevalence of infection detected			
		One-step		Nested	
		146F1/146R1	146F2/146R2	143F/145R	146F1/146R1–146F2/146R2
S. Alam hatchery	12	0 (0.00)	0 (0.00)	12	2 (16.66)
Chingri Bangla hatchery	12	0 (0.00)	0 (0.00)	12	0 (0.00)
Saudia hatchery	12	0 (0.00)	0 (0.00)	12	2 (16.66)
Niribili hatchery	12	4 (33.33)	4 (33.33)	12	6 (50.00)
Bolaka hatchery	12	8 (66.66)	8 (66.66)	12	8 (66.66)
Total no. (%) (±SE)	60	12 (19.99 ± 13.3)	12 (19.99 ± 13.3)	60 (100%)	18 (29.99 ± 12.25)
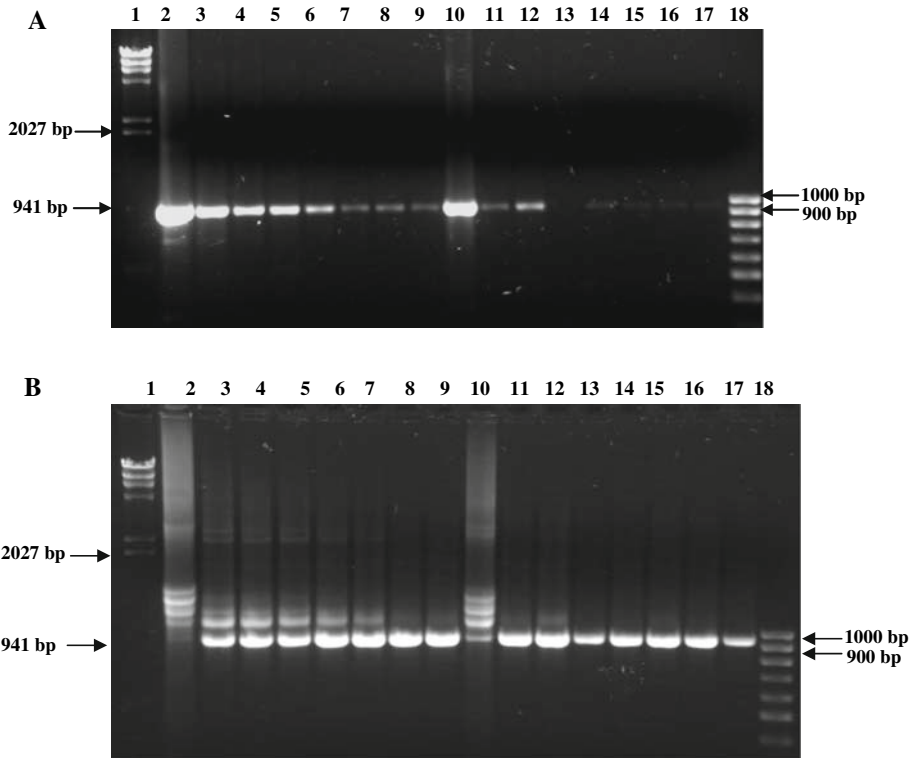
Fig. 3 Sensitivity analysis of PCR. (**a**) One-step PCR reaction. (**b**) Nested PCR. Lanes 2–9 represent serial dilutions of a positive sample of the Bolaka hatchery and lanes 10–17 represent serial dilutions of a positive sample of the Niribili hatchery. Template DNA concentrations: lanes 2 and 10, 100 ng; 3 and 11, 2 ng; 4 and 12, 1.0 ng; 5 and 13, 0.5 ng; 6 and 14, 0.25 ng; 7 and 15, 0.125 ng; 8 and 16, 62.5 pg; and 9 and 17, 42 pg. Lane 1: molecular weight marker (λ DNA *Hin*dIII digest). Lane 18: molecular weight marker (100 bp DNA ladder)
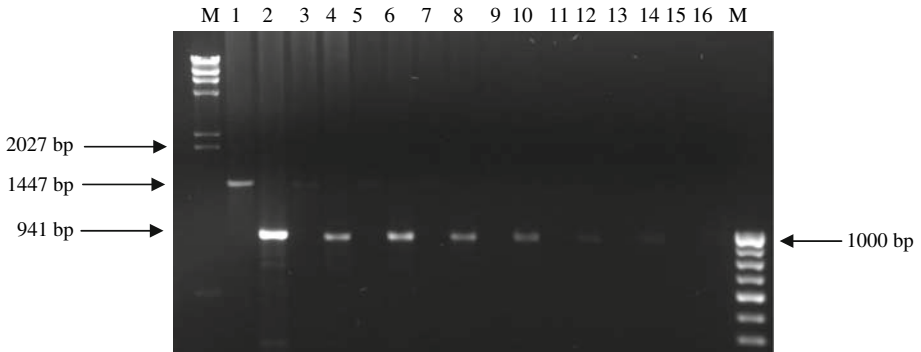
Fig. 4 Sensitivity analysis of one-step PCR with outer (1447 bp) and inner (941 bp) primer pairs using the Bolaka hatchery sample DNA. Template DNA concentrations: lanes 2 and 3, 10 ng; 4 and 5, 2.00 ng; 6 and 7, 1.00 ng; 8 and 9, 0.50 ng; 10 and 11, 0.25 ng; 12 and 13, 0.125 ng; 14 and 15, 62.5 pg; 16 and 17, 31.3 pg. Lane 1: molecular weight marker (λ DNA *Hin*dIII digest). Lane 9: molecular weight marker (100 bp DNA ladder)
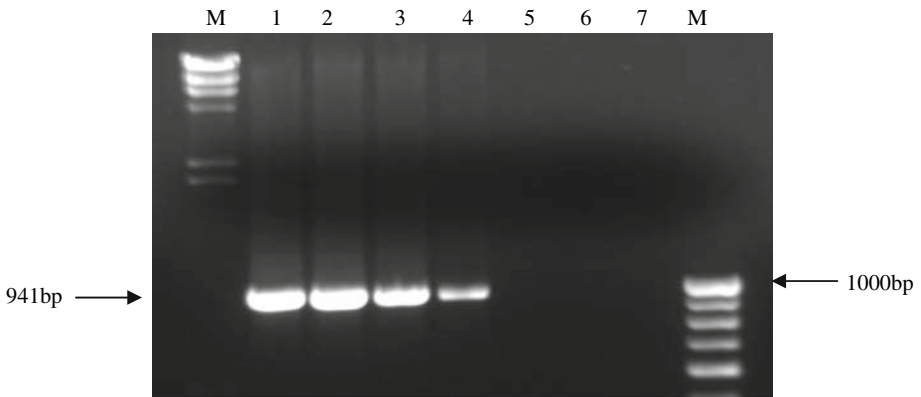

Fig. 5 Sensitivity analysis of nested PCR using the Niribili hatchery sample DNA. Template DNA concentrations: lanes 2, 0.25 ng; 3, 0.125 ng; 4, 62.5 pg; 5, 31.3 pg; 6, 15.6 pg; 7, 7.8 pg; 8, 3.9 pg template DNA. Lane 1: molecular weight marker (λ DNA *Hin*dIII digest). Lane 9: molecular weight marker (100 bp DNA ladder)

we found that the outer primer pair (1447 bp) showed a negative signal at 1.00 ng, whereas the inner primer pair (941 bp) showed a negative signal at a 31.2 pg concentration (Fig. 4). The other sample (Niribili hatchery) was further diluted up to 2.0 pg/µl and we found a negative signal at 15.6 pg DNA content in the nested PCR reaction (Fig. 5).

Discussion

P. monodon is considered as one of the most important commercially cultured species in Bangladesh. *P. monodon* is the most susceptible host of WSSV (Wongteerasupaya et al. 1995). This species has been badly affected by WSSV in all of the shrimp-producing countries in Asia, including Bangladesh (Larkins 1995; Wongteerasupaya et al. 1995;

Karunasagar et al. 1997). For definitive diagnosis and certification of the WSSV infection status of broodstock and fry, PCR technology has been recommended (OIE 2003). Molecular-based detection methods for shrimp viruses include normal PCR using degenerate primers (Chang et al. 1993) and nested PCR (Lo et al. 1996b; Belcher and Young 1998), among others. Due to higher sensitivity limits than most classical diagnostic methods, PCR has become the preferred method for the diagnosis of most shrimp viruses (Tang and Lightner 2000).

Natividad et al. (2006) tested both Philippine and Japanese WSSV isolates with primer pair 146F1/146R1 and found that the primer pair was able to amplify only the isolates from Japan but not the isolates from the Philippines. So, one of the objectives of the present study was to reveal whether the effectiveness of the primer sets 146F1/146R1 and 146F2/146R2 developed by Lo et al. (1996b) in Taiwan can also detect WSSV samples from Bangladesh. We found that both primer pairs were able to amplify expected size fragments from Bangladesh isolates of WSSV successfully.

We found that apparently healthy hatchery samples were positive for WSSV only by nested PCR, while the samples showing clinical signs of WSS were positive for WSSV by both one-step and nested PCR. However, apparently healthy samples of one hatchery were also found to be positive by one-step PCR. Those samples that were positive by one-step PCR were highly infected by WSSV and those that were positive only by nested PCR indicated a lower level of infection. A similar type of finding was also reported by Lo et al. (1996b). They revealed that the nested PCR technique allowed the gradation of viral infection, with highly infected animals being positive in non-nested PCR and lightly infected animals being positive only in nested PCR. The presence of WSSV can often be detected in apparently healthy PL by PCR (Lo et al. 1996b; Magbanua et al. 2000; Otta et al. 2003). Manivannan et al. (2002) reported that the PL samples from the affected tanks were positive for WSSV by one-step PCR, while those from the healthy tanks (not showing mortality) were positive only by nested PCR.

In our study, we used two pairs of primers designed by Lo et al. (1996b) containing 1447 bp and 941 bp amplicons specific for WSSV. It can be mentioned that the samples which were positive in one-step and nested PCR with primer pairs 146F1/146R1 and 146F2/146R2 were highly infected by WSSV. Again, we compared the sensitivity between two primer pairs and found that the primer pair amplifying 941 bp was much more sensitive than the primer pair amplifying 1447 bp (Fig. 4). Our result supports the views of Hossain et al. (2004), who reported that primers containing smaller amplicon size were much more sensitive than the primers containing larger amplicon size.

We also compared the sensitivity between one-step and nested PCR and, for that purpose, the same samples with the same dilutions were used and we found that nested PCR was much more sensitive than one-step PCR. Through a sensitivity analysis, we could optimise the PCR technique and the optimisation of PCR is necessary to assess the quality of a brood or PL stock. It would be important to know the level of infection in the brood stock, so that the risk of producing infected larvae could be assessed (Uma et al. 2005).

As mentioned earlier, we originally planned to validate whether the primers developed by Lo et al. (1996b) can also detect the WSSV strain infecting shrimps in Bangladesh hatcheries. Therefore, we collected seven samples with clinical signs of WSS from the Niribili hatchery. When we succeeded in detecting the virus in the infected shrimps by both one-step and nested PCR, we then collected some random samples (apparently healthy) from five hatcheries, including the Nirbili hatchery (Table 2). Our results of the initial detection of WSSV were validated in the PCR laboratory (Cox's Bazar) originally established by the ATDP (Agro-based Industries and Technology Development Project)

funded by United States Agency for International Development (USAID), at present taken over by the Bangladesh Office of the WorldFish Center. They use a commercial WSSV detection kit manufactured by Farming IntelliGene Tech Corp., Taiwan, ROC. It is the only laboratory in Bangladesh where WSSV can be detected. Therefore, apart from this laboratory, there was no scope for inter-lab collaboration to validate the detection results. In the random samples, we found 30% infection, which is lower compared to the 75% prevalence reported by Otta et al. (1999) from the west coast of India and also lower compared to the 39.4% prevalence reported by Uma et al. (2005) from the south coast of India.

We extracted DNA from infected/non-infected shrimp tissue for this study so that the templates contained DNA from shrimp as well as from the virus if the shrimp was infected. Therefore, it was not possible for us to obtain an estimate on the concentration of viral DNA. However, because of the high sensitivity of the nested PCR, it may be useful in confirming the early stages of WSSV infection when the virus concentration is relatively low or before the manifestation of infection. The determination of the exact viral load in shrimps at certain times of culture may be useful to predict either an outbreak of the WSSV disease or the completion of the culture (Peinado-Guevara and López-Meyer 2006). Apparently healthy samples may also carry the WSSV, which has been found to be positive mainly by the nested PCR method. Therefore, the samples that would be proved as negative by nested PCR may only be certified as non-infected. It is recommended to test the mother shrimps before use in the hatchery and representative samples of PLs by PCR before release into the ponds to avoid serious losses from WSS.

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